

BRIEF COMMUNICATION

Changes of leaf antioxidant system, photosynthesis and ultrastructure in tea plant under the stress of fluorine

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Abstract

Seedlings of *Camellia sinensis* were grown hydroponically for 30 d in order to study the effect of fluorine (F) on growth parameters, antioxidant defence system, photosynthesis and leaf ultrastructure. Fresh and dry mass, chlorophyll (Chl) content and net photosynthetic rate (P_N) decreased with increasing F concentration. Superoxide dismutase (SOD) activity decreased significantly, catalase (CAT) and guaiacol peroxidase (GPX) activities reached maximum under 0.21 and 0.32 mM F, respectively. Proline, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents increased significantly. These results suggested, that antioxidant defence system of leaves did not sufficiently scavenge excessive reactive oxygen species. The cell ultrastructure was not changed under 0.11 - 0.21 mM F, however, it was destroyed at 0.32 - 0.53 mM F. So tea plants tolerated F in concentration less than 0.32 mM.

Additional key words: catalase, *Camellia sinensis*, chloroplasts, guaiacol peroxidase, H_2O_2 , malondialdehyde, net photosynthetic rate, proline, superoxide dismutase.

Fluorine, a phytotoxin in air, water, soil, and vegetation, is released into the environment from a number of industrial sources (Mackowiak *et al.* 2003), application of phosphate fertilizers in agriculture (Loganathan *et al.* 2001), and weathering of volcanic ashes (Cronin *et al.* 2003). F is transferred from soil to roots, and then to above ground parts, or absorbed by leaves from the air. The F content was reported to reach 871 - 1337 mg kg⁻¹(f.m.) in mature tea leaves, and even more than 2000 mg kg⁻¹(f.m.) in leaves of old tea plants (Ruan and Wong 2001, Shu *et al.* 2003). To our knowledge, little information is available regarding the effects of excessive F on physiological functions in tea plant. In the present study, the changes of antioxidant defence system, photosynthesis and cell ultrastructure of tea leaves under F stress were investigated, in order to study the mechanisms of tea plant F tolerance.

Camellia sinensis (L.) O. Kuntze cv. Fu Ding da Bai 1-year-old cuttings, provided by the Fruit and Tea Research Institute of Hubei Agricultural Academy, China, were planted in plastic pots containing 1.5 dm³ of 1/2 strength Hoagland nutrient solution (Hoagland and Arnon 1950). F (as NH_4F) was supplied at five concentrations: 0 (control), 0.11, 0.21, 0.32, 0.53 mM. For each treatment, 5 pots (with 5 seedlings each) were used, and the pots were arranged in the glasshouse in random design. The liquid solutions (pH 5.5) were ventilated with air pumps and replaced completely every 5 d. The seedlings were cultivated for 30 d in a glasshouse under day/night temperature of $25 \pm 3/15 \pm 2$ °C and irradiance, of 250 - 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during 16-h photoperiod.

Fresh mass (f.m.) of the whole plant was determined immediately after harvesting. Dry mass (d.m.) of the whole plant was determined after drying at 80°C till

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Abbreviations: CAT - catalase; Chl - chlorophyll; F - fluorine; GPX - guaiacol peroxidase; MDA - malondialdehyde; P_N - net photosynthetic rate; SOD - superoxide dismutase; TEM - transmission electron microscope.

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constant mass. Chlorophyll (Chl) content was measured in an 80 % acetone extract spectrophotometrically at 663 and 645 nm as described by Jiang *et al.* (2007). Net photosynthetic rate (P_N) was measured by *TPS-1* photosynthesis system (*PP Systems*, Amesbury, MA, USA). The seedlings were put outdoors in the morning from 9:00 to 11:00. The photosynthesis of the 3rd or 4th leaf from the top was detected at irradiance of 800 to 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$, CO_2 concentration of 370 to 380 $\mu\text{mol mol}^{-1}$, air temperature of 28 ± 0.5 °C and relative humidity of 60 ± 0.8 %. Three seedlings were measured in each treatment and repeated three times. Total F was tested with an ion selective electrode (*Orion 9609BNWP* with *Orion pH/ISE meter 710*, both *Thermo Scientific*, Waltham, MA, USA). The leaves were oven-dried at 80 °C for 24 h, and then ground to pass through a 2-mm sieve. The grounded sample (0.2 g) was extracted with 10 cm^3 of 0.2 M HCl at room temperature for 1 h. After filtration, 25 cm^3 total ionic strength adjustment buffer (TISAB) was added. Finally, the volume was fixed to 100 cm^3 with super-pure water produced by a purification system (*Millipore*, Bedford, MA, USA).

For enzyme extraction, fresh leaves (0.5 g) were ground on ice with 0.5 g quartz sands and 5 cm^3 of 50 mM precooled phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetracetic acid (EDTA) and 5 % (m/v) polyvinylpyrrolidone (PVP). The homogenate was centrifugated (16 000 g) at 4 °C for 15 min. Then the supernatant was used for superoxide dismutase (SOD; E.C.1.15.1.1), catalase (CAT; E.C.1.11.1.6) and guaiacol peroxidase (GPX; E.C.1.11.1.7) analysis (Pereira *et al.* 2002). The activity of total SOD, Cu/Zn-SOD, Mn-SOD and CAT were determined by using the reagent kit (Nanjing Jiancheng Bioengineering institute, Nanjing, China). The GPX was determined by modified guaiacol method. 0.1 cm^3 of enzyme solution, 0.9 cm^3 of 2 %

guaiacol and 1.0 cm^3 of 0.3 % H_2O_2 were added into 1.0 cm^3 of potassium phosphate buffer (pH 5.7), following absorbance variation at 470 nm within 5 min (Ramiro *et al.* 2006).

Proline content in the leaves was measured by acidic ninhydrin method according to Khedr *et al.* (2003). The content of malondialdehyde (MDA) in the leaves was measured by thiobarbituric acid (TBA) method according to Dhindsa *et al.* (1981). The H_2O_2 content in the leaves was measured according to Patterson *et al.* (1984).

For transmission electron microscope (TEM) analysis, the middle sections of the third leaves were cut (1×1 mm), and then put into the fixation solution composed of 2.5 % glutaraldehyde at pH 7.4 immediately to make the samples sunk. The samples were fixed in 1 % OsO_4 for 2 h, dehydrated by acetone, embedded with epoxy resin (*SPI-812*) for 24 h, and sliced up by ultramicrotome (*UC6*, *Leica Microsystems*, Wetzlar, Germany). The sections were stained with a mixture of lead citrate and uranyl acetate. Finally the configuration of cells was checked using TEM (*H-7650*, *Hitachi*, Tokyo, Japan) and photographed with *Gatan832* digital imaging system (*Gatan*, Pleasanton, USA).

All statistical analyses were done using the statistical package of the SAS software computer program. *ANOVA* followed by LSD test were carried out to test the significance.

SODs are ubiquitous metalloenzymes that play a role in defense against toxic reduced oxygen species. Cu/Zn-SODs are found throughout the plant cell, existing in both chloroplastic and cytosolic forms; Mn-SODs are located in mitochondria and also in peroxisomes (Attia *et al.* 2008). Our data showed that the activity of total SOD, Cu/Zn-SOD and Mn-SOD all decreased significantly ($P < 0.01$) with increasing F concentration (Table 1). The result is consistent with the findings of Wilde and Yu

Table 1. The whole plant fresh and dry masses, F, Chl, proline, H_2O_2 and MDA contents, P_N , and activities of antioxidative enzymes in leaves of tea cultivated in a half strength Hoagland nutrient solution with 0, 0.11, 0.21, 0.32 or 0.53 mM F for 30 d. Means \pm SD ($n = 3$). Different letter in each row represents significant differences at $P < 0.05$, based on LSD's multiple range test.

Parameters	0	0.11	0.21	0.32	0.53
Fresh mass [g plant^{-1}]	$5.51 \pm 0.08\text{a}$	$5.49 \pm 0.04\text{a}$	$5.28 \pm 0.1\text{b}$	$4.90 \pm 0.09\text{c}$	$4.58 \pm 0.09\text{d}$
Dry mass [g plant^{-1}]	$1.72 \pm 0.03\text{a}$	$1.67 \pm 0.03\text{a}$	$1.57 \pm 0.04\text{b}$	$1.45 \pm 0.02\text{c}$	$1.33 \pm 0.04\text{d}$
F content [$\text{mg kg}^{-1}(\text{d.m.})$]	$1283 \pm 16\text{a}$	$1525 \pm 6\text{b}$	$1702 \pm 15\text{c}$	$1763 \pm 20\text{d}$	$1876 \pm 14\text{e}$
Chl content [$\text{mg g}^{-1}(\text{f.m.})$]	$2.21 \pm 0.02\text{a}$	$2.26 \pm 0.01\text{a}$	$2.15 \pm 0.03\text{b}$	$1.99 \pm 0.04\text{c}$	$1.86 \pm 0.03\text{d}$
P_N [$\mu\text{mol m}^{-2} \text{s}^{-1}$]	$12.70 \pm 0.16\text{a}$	$13.30 \pm 0.26\text{a}$	$10.70 \pm 0.36\text{b}$	$8.60 \pm 0.70\text{c}$	$3.90 \pm 0.16\text{d}$
Total SOD [$\text{U g}^{-1}(\text{f.m.})$]	$3191 \pm 46\text{a}$	$3084 \pm 22\text{b}$	$2899 \pm 25\text{c}$	$2818 \pm 52\text{c}$	$2639 \pm 64\text{d}$
Cu/Zn-SOD [$\text{U g}^{-1}(\text{f.m.})$]	$2314 \pm 34\text{a}$	$2239 \pm 113\text{a}$	$2109 \pm 67\text{b}$	$2091 \pm 46\text{b}$	$1934 \pm 45\text{c}$
Mn-SOD [$\text{U g}^{-1}(\text{f.m.})$]	$918 \pm 66\text{a}$	$921 \pm 32\text{a}$	$806 \pm 47\text{b}$	$711 \pm 12\text{c}$	$705 \pm 58\text{c}$
CAT [$\text{U g}^{-1}(\text{f.m.})$]	$380 \pm 13\text{c}$	$430 \pm 11\text{b}$	$447 \pm 7\text{a}$	$395 \pm 11\text{c}$	$358 \pm 22\text{d}$
GPX [$\text{U g}^{-1} \cdot \text{min}^{-1}$]	$1143 \pm 28\text{c}$	$1149 \pm 70\text{c}$	$1274 \pm 34\text{b}$	$1514 \pm 5\text{a}$	$1206 \pm 50\text{c}$
Proline [$\mu\text{g g}^{-1}(\text{f.m.})$]	$22.66 \pm 1.54\text{c}$	$30.68 \pm 1.72\text{b}$	$32.91 \pm 1.37\text{b}$	$48.39 \pm 1.03\text{a}$	$49.73 \pm 0.74\text{a}$
H_2O_2 [$\mu\text{mol g}^{-1}(\text{f.m.})$]	$29.08 \pm 1.60\text{c}$	$30.18 \pm 0.63\text{bc}$	$33.20 \pm 0.81\text{ab}$	$36.08 \pm 2.39\text{a}$	$36.65 \pm 2.22\text{a}$
MDA [$\text{nmol g}^{-1}(\text{f.m.})$]	$6.56 \pm 0.51\text{c}$	$6.69 \pm 0.57\text{c}$	$8.15 \pm 0.02\text{b}$	$10.35 \pm 0.49\text{a}$	$10.30 \pm 0.26\text{a}$

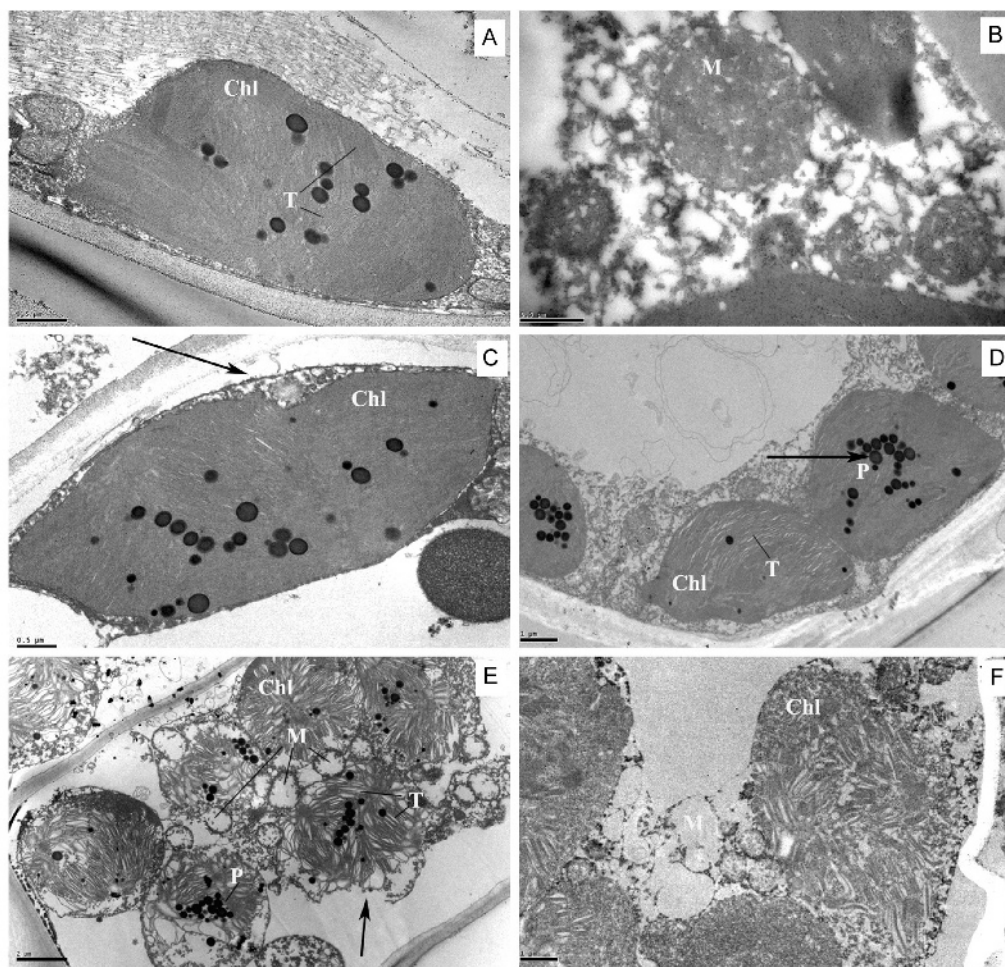


Fig. 1. Leaf ultrastructure of tea seedlings grown under 0 mM (control, A,B), 0.11 mM (C), 0.21 mM (D), 0.32 mM (E) and 0.53 mM (F) fluorine for 30 d, respectively. Bars = 0.5 μ m (A,B,C), 1 μ m (D,F) and 2 μ m (E), respectively. A - chloroplast was elliptical and thylakoids were arranged closely and aligned in an orderly manner; B - mitochondria were intact, with complete membrane structure and clear cristae; C - the plasmolysis observed at 0.11 mM F treatment; D - the plastoglobulus increased (arrow) and thylakoids expanded slightly at 0.21 mM F treatment; E - the shape of chloroplast changed, membrane was ruptured (arrow), the system of the membranes was obliterated, mitochondria were cavitated completely; F - chloroplasts and thylakoids were disintegrated, and mitochondria degraded. Chl - chloroplast, T - thylakoid, M - mitochondria, P - plastoglobulus.

(1998). CAT and GPX activities increased at low F concentrations, reached their peaks when the F concentrations were 0.21 and 0.32 mM, respectively, and decreased at higher concentrations (Table 1). Generally, antioxidant enzymes and non-enzymatic antioxidants including proline have synergistic effects on free radical scavenging, thus the generation and removal of free radical is balanced (Mascher *et al.* 2002, Tasg n *et al.* 2003). However, the reduced activity of antioxidant enzymes under stressed condition can lead to lipid peroxidation and membrane damage (Scandalios 2005). The content of MDA, a product of peroxidation of membrane lipids, is often used as an indicator of oxidative damage (Gunes *et al.* 2007). In the present study, MDA and H_2O_2 contents increased significantly (Table 1). Accumulation of H_2O_2 and lipid peroxidation resulted in a significant decrease in cell membrane stability. TEM also

proved it, *e.g.* membranes of chloroplasts were ruptured (Fig. 1E). These results suggested that antioxidants did not sufficiently scavenge excessive reactive oxygen species to protect the tissue from free radical injury under the F stress.

The related research had proved that the increase of proline was a common response of most plants to environmental stress (Almansouri *et al.* 1999, Meloni *et al.* 2001). In addition to osmotic adjustment (Tripathi and Gaur 2004), proline also stabilizes cellular structures and acts as a free radical scavenger (Alia and Matysik 2001, Siripornadulsil *et al.* 2002). In this trial, proline content increased remarkably ($P < 0.01$). Significant increase in proline content has been reported earlier in tea under Cu^{2+} and Al^{3+} stress (Yadav and Mohanpuria 2009).

Based on the results of TEM, it was discovered that cell ultrastructure changed little under 0.11 - 0.21 mM F, but an irreversible destruction under the concentration of F

higher than 0.32 mM was observed. Under high F stress, chloroplast damage, such as membrane rupture, thylakoid expansion (Fig 1E) and even disintegration (Fig 1F) was found, which would strongly influence the photosynthesis. In present study, both Chl content and P_N increased slightly at low F concentration (0.11 mM) but not significantly. However, these two parameters decreased significantly ($P < 0.05$) with increasing F concentration. The high electronegativity of F destroyed the Chl molecule and accelerated disintegration of chloroplasts

(Fornasiero 2001, Aboal *et al.* 2008).

In conclusion, exposure of tea seedlings to F decreased fresh mass, dry mass, Chl content and P_N , increased MDA, proline and H_2O_2 contents and changed SOD, POX, CAT activities. It is suggested that antioxidant defence system did not sufficiently protect the tissue under severe F stress. This was confirmed by the damage of cell ultrastructure. Tea plants are able tolerate F in concentration less than 0.32 mM.

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