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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 maximun 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 dm^3 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 ± 3/15 ± 2 °C and irradiance, of 250 - 280 μmol m^{-2} 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 μmol m^{-2} s^{-1}, CO_2 concentration of 370 to 380 μmol 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.C1.11.1.7) 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 ultramicrotone (*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 throught 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 ± 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 ± 0.08a	5.49 ± 0.04a	5.28 ± 0.1b	4.90 ± 0.09c	4.58 ± 0.09d
Dry mass [g $plant^{-1}$]	1.72 ± 0.03a	1.67 ± 0.03a	1.57 ± 0.04b	1.45 ± 0.02c	1.33 ± 0.04d
F content [mg kg^{-1}(d.m.)]	1283 ± 16a	1525 ± 6b	1702 ± 15c	1763 ± 20d	1876 ± 14e
Chl content [mg g^{-1}(f.m.)]	2.21 ± 0.02a	2.26 ± 0.01a	2.15 ± 0.03b	1.99 ± 0.04c	1.86 ± 0.03d
P_N [μmol m^{-2} s^{-1}]	12.70 ± 0.16a	13.30 ± 0.26a	10.70 ± 0.36b	8.60 ± 0.70c	3.90 ± 0.16d
Total SOD [U g^{-1}(f.m.)]	3191 ± 46a	3084 ± 22b	2899 ± 25c	2818 ± 52c	2639 ± 64d
Cu/Zn-SOD [U g^{-1}(f.m.)]	2314 ± 34a	2239 ± 113a	2109 ± 67b	2091 ± 46b	1934 ± 45c
Mn-SOD [U g^{-1}(f.m.)]	918 ± 66a	921 ± 32a	806 ± 47b	711 ± 12c	705 ± 58c
CAT [U g^{-1}(f.m.)]	380 ± 13c	430 ± 11b	447 ± 7a	395 ± 11c	358 ± 22d
GPX [U g^{-1}·min^{-1}]	1143 ± 28c	1149 ± 70c	1274 ± 34b	1514 ± 5a	1206 ± 50c
Proline [μg g^{-1}(f.m.)]	22.66 ± 1.54c	30.68 ± 1.72b	32.91 ± 1.37b	48.39 ± 1.03a	49.73 ± 0.74a
H_2O_2 [μmol g^{-1}(f.m.)]	29.08 ± 1.60c	30.18 ± 0.63bc	33.20 ± 0.81ab	36.08 ± 2.39a	36.65 ± 2.22a
MDA [nmol g^{-1}(f.m.)]	6.56 ± 0.51c	6.69 ± 0.57c	8.15 ± 0.02b	10.35 ± 0.49a	10.30 ± 0.26a

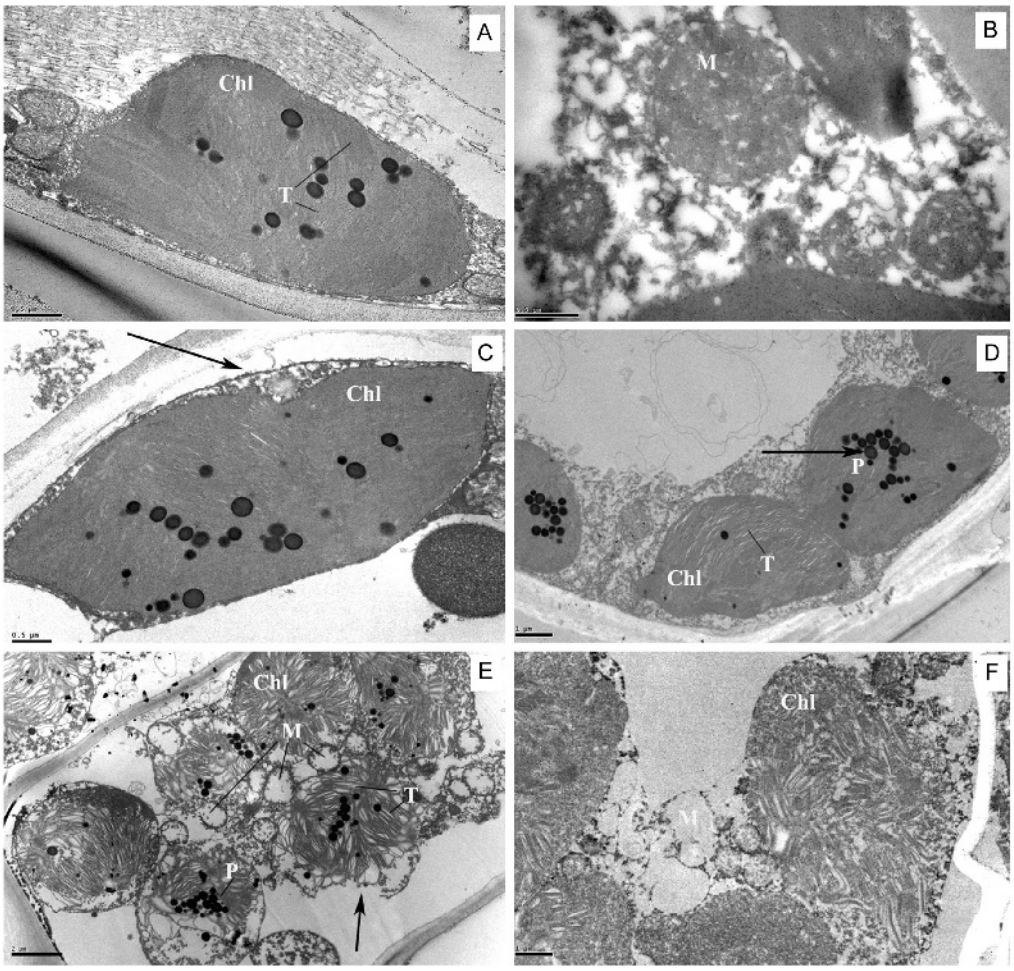

Fig. 1. Leaf ultrastructure of tea seedlings grown under 0 mM (control, *A*,*B*), 0.11 mM (*C*), 0.21 mM (*D*), 0.32 mM (*E*) and 0.53 mM(*F*) fluorine for 30 d, respectively. *Bars* = 0.5 μm (*A*,*B*,*C*), 1 μm (*D*,*F*) and 2 μm (*E*), respectively. *A* - chloroplast was elliptical and thylakoids were 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, Tasgín *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. 1*E*). 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 expantion (Fig 1*E*) and even disintegration (Fig 1*F*) 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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