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A Simple Method for Testing Freezing Resistance Based on Chlorophyll Fluorescence in Tea (*Camellia sinensis* L.)

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ABSTRACT: For the stable production of high quality tea, the freezing resistance is a very important character. Most of the farmers have planted out-pollinated seeds that are not genetically pure. So, with small sample, a quick and simple method is required to test freezing resistance of lots of germplasm and early generation of hybrids. The absorbances (A530 nm) of TTC reduction solution at -5°C were positively correlated with resistance to photoinhibition of PSII in 6 hour photoinhibitory treatments, being significantly fitted by simple linear regression (R^2=0.64). Chlorophyll fluorescence measured by Fv/Fm was found to be very useful in evaluating the relative levels of freezing resistance in tea.**

Keywords **:** freezing resistance, chlorophyll fluorescence, Fv/Fm, TTC, tea.

Yabukita (cultivated about 30%) with less freezing resistance and native land races have been cultivated or grown naturally in the southern areas of Korea. *Camellia sinensis* is a shrublike evergreen tree related to the flowering *Camellia*. The plant has a strong self-incompatibility controlled by multi-alleles (de Nettancourt, 1977). Thus, native land races might have acquired some genes related to freezing resistance through natural selection for a long time. There are no formally registered tea varieties, and tea plant breeders have tried to breed a new variety with high yield, good quality, and resistance to environmental stresses, especially, freezing resistance with introduced or domestic native land races. The freezing resistance is controlled with genetic and environmental factors such as amounts of sucrose, protein, moisture of leaf, phosphorylase activity and others. Growth and development of winter cultivars of cereals such as rye and wheat at low nonfreezing temperatures induce freezing resistance (Levitt, 1980). However, the growth at low temperature not only induces freezing resistance but also induces an increased resistance to low temperature-induced photoinhibition of photosynthesis (Ort & Baker, 1988; Powles, 1984).

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Photoinhibition of photosynthesis occurs at the reaction centre of PSII (Powles, 1984). It typically results in reduction of the quantum yield of PSII photochemistry, coupled to increased thermal de-excitation of excited chlorophyll. Photoinhibition reduces the photochemical efficiency of PSII and it is typically detected as a decrease in Fv, Fv/Fm, or a decrease in the quantum yield of O_2 evolution (Hurry and Huner, 1991; Krause and Weis, 1991). Winter rye (Öquist and Huner. 1991) and spinach (Boese and Huner, 1990; Somersalo and Krause, 1990) exhibit reduced sensitivity to low temperature-induced photoinhibition when acclimated at cold-hardening conditions. The growth and development of plants at low temperature (5°C) are prerequisites for exhibiting this increased resistance to photoinhibition (Boese and Huner, 1990, Öquist and Huner. 1991). During fall and winter in the field, plants are exposed to a combination of low temperatures and high irradiance. The relative susceptibility of plants to photoinhibition may contribute to their ability to cold hardening and survive the winter. Chun *et al.* (1997) suggested that chlorophyll fluorescence attribute, Fv/Fm ratio has been very useful in evaluating freezing tolerance in wheat.

Our study examined the potential utility of Fv/Fm ratio as a tool to test freezing resistance in native land races of tea plants and to establish a simple method for testing the character.

MATERIALS AND METHODS

Plant material

The secondary leaves of native tea land races were sampled from different sites, placed into a vinyl bag and exposed to each temperature treatment; a control (+3°C), -3~-6°C. All samples except control were placed in a programmable freezing chamber at +3°C. Chamber temperature was lowered by 3 h^{-1} and held at the set point (treatment temperature) for 2h. After thawing at 4°C overnight, TTC (2,3,5-triphenyl tetrazolium chloride) test was performed (Towill & Mazur, 1974).

Photoinhibition

Leaf discs (diam. 15 mm) were floated on water in a Petri

dish with their upper surface exposed to air. A tray with Petri dishes was placed in a 5°C cold room. Photoinhibition was imposed on floating leaf discs by exposing them to 1,500 μmol $m^{-2}s^{-1}$ of PAR with high-pressure sodium lamps (AGRO 400, Phillips) for 1~7h. Following the period of irradiation, photoinhibitions of the leaf samples were measured by the decrease in the ratio of the induced to the maximum Chl fluorescence, Fv/Fm measured at room temperature. The change in the Fv/Fm ratio relative to control was used to quantify photoinhibition. Prior to measurements, the leaf discs were dark-adapted at room temperature for at least 30 min. Modulated fluorescence was measured with Chlorophyll Fluorometer (PAM-2000, H. Walz Effeltrich, Germany). Fv and Fm are variable fluorescence after dark adaptation (Fm-Fo), and maximal fluorescence after dark adaptation, respectively. Fv/Fm was used as a measure of the maximal photochemical efficiency of PSII (Genty *et al.*, 1989).

RESULTS AND DISCUSSION

Change in Fv/Fm of tea leaves according to freezing temperatures and durations of photoinhibition

Chlorophyll fluorescence (CF) is used to indicate the efficiency of the light-absorbing portion (PSII) of a plant photosynthetic system. Photosystem II captures light energy and converts it to chemical energy. It can be used to estimate the effects of environmental stresses, including freezing, on photosynthesis (Binder and Fielder, 1996). The variable fluorescence attribute, Fv/Fm has been found to be very useful in detecting freezing damage. Compared with measurement of CO_2 uptake, the CF measurement can be done easily with a portable fluorometer and does not require calculation of leaf area.

The changes of Fv/Fm in nonhardened tea leaves with different photoinhibitory hours at 5°C were shown in Fig. 1. The means were 0.790~0.801 at the control, 0.675~0.716 at the 2h photoinhibitory plot, 0.474~0.506 at the 3h plot, 0.397~0.426 at the 4h plot, 0.274~0.283 at the 5h plot, 0.266~0.269 at the 6h plot, and 0.139~0.178 at the 7h plot. As the photoinhibitory duration was lengthened, the values of Fv/Fm were reduced linearly. The Fv/Fm values were not different between the first and second leaves.

Changes in Fv/Fm of whole or disc tea leaves treated with different temperatures for 2h were shown in Fig. 2 and 3. The Fv/Fm values treated with low temperatures, and thawed at 3°C overnight (A) were a little greater than those exposed to low radiation at room temperature for 4h (B, C) for recovery, the difference being greatest at -10°C~-12°C plots. The Fv/Fm values in the tea leaves which were treated at -5°C~-8°C were decreased slightly with lowering temperature. While the temperature lowered down, below -9°C, the values were decreased very sharply, indicating that the tea tissue had lost photosynthetic potentiality.

The Fv/Fm values were 0.775~0.803 at the control, 0.648~0.710 at the -8°C plot, 0.244~0.466 (30~58% of the control) at the -9°C plot, showing that LT_{50} was between -9°C and -10°C at nonhardened tea leaves. Compared to the treatments with low temperatures for 2h and thawed at 3°C overnight, the tea samples treated with low temperatures and

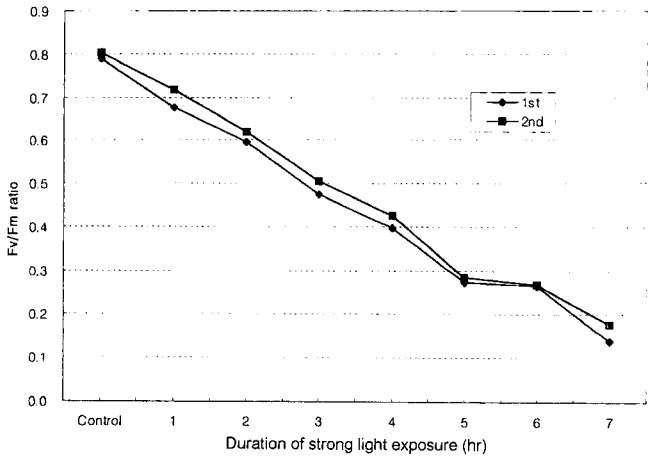

Fig. 1. Changes in chlorophyll fluorescence (Fv/Fm) of the first and second tea leaves photoinhibited for different durations. They were sampled on 12 October in 1999 at Hyanglymsa, Sunchon.

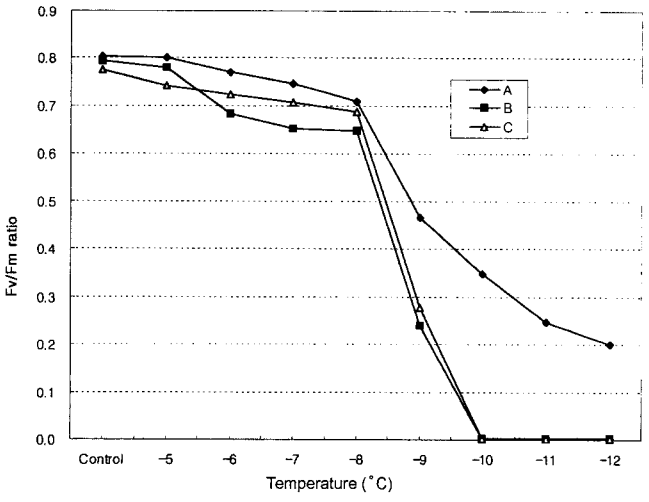

Fig. 2. Changes in chlorophyll fluorescence (Fv/Fm) of whole tea leaves treated with different low temperatures for 2 hours. They were sampled on 29 October in 1999.
A: low temp. (2h) & 3°C overnight in dark,
B: low temp. (2h) & 3°C overnight & recovery (4h) in light.
C: low temp. (2h) & 3°C overnight & recovery (4h) in light & dark adaptation (30 min.)

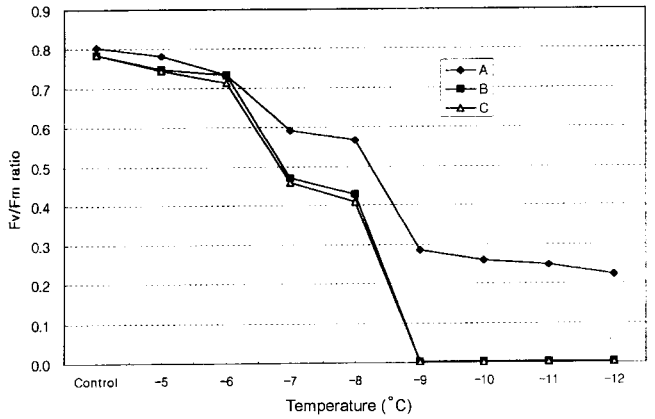

Fig. 3. Changes in chlorophyll fluorescence (Fv/Fm) of leaf discs treated with different low temperatures for 2 hours. They were sampled on 29 October in 1999.
A: low temp. (2h) & 3°C overnight in dark,
B: low temp. (2h) & 3°C overnight & recovery (4h) in light.
C: low temp. (2h) & 3°C overnight & recovery (4h) in light & dark adaptation (30 min.)

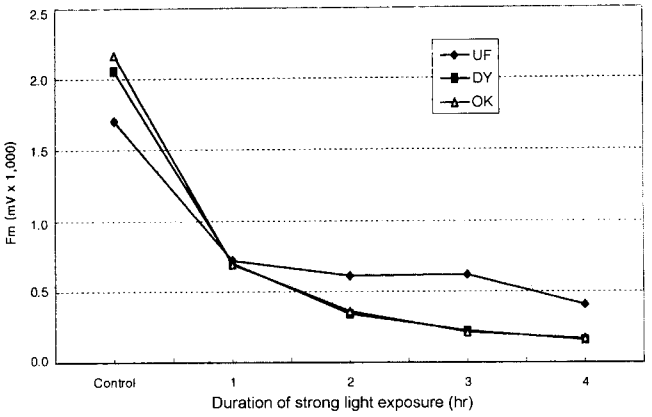

Fig. 4. Comparison of maximal fluorescence yield (Fm) in tea leaves sampled from different places with different growth conditions on 22 June in 2000. Tea leaves were photoinhibited with different durations under strong light intensity (1,500 μmol $m^{-2}s^{-1}$). UF: University farm, nonshading, DY: Damyang, 70~80% shading. OK: Okkwa 70~80% shading.

recovered at room temperature for 4h, had lower Fv/Fm values, the difference being greatest at -9°C plot.

Those results suggested that the frozen and thawed tea tissues were already dead even during recovery at room temperature and 200 μmole $m^{-2}s^{-1}$. Fig. 3 showed that the Fv/Fm of leaf discs punched out with cork borer (diam. 15 mm), placed into tube and frozen in low temperature chamber, were a little lower than those of whole leaf. In the case of leaf discs, on thawing, moisture was leaked out from tissues more easily and desiccation of tissues might have occurred. It is very convenient to punch out leaf discs, place into tube and freeze samples in cold chamber in comparison with treating a whole leaf. However, the former needs prevention from desiccation of tissues during thawing.

Comparison of Fv/Fm of tea leaves grown in different growing conditions

Native land races are distributed under very different vegetation sites. Tea plants grow principally in shrubs or under bamboo trees. Therefore, the light conditions for growth and development are very variable. Tea leaves of University tea farm with nonshading and those of Damyang and Okkwa with 70~80% shading were sampled and exposed to strong light of 1,500 μmol $m^{-2}s^{-1}$ of PAR for 1~4h, and the Fv/Fm was measured.

Fig. 4 shows a maximal fluorescence yield (Fm) in tea leaves sampled from different growth conditions and exposed to different durations of strong light. The Fm values photoinhibited for 1h were reduced to 32~42% of the control, and then decreased slightly with longer exposure of strong light.

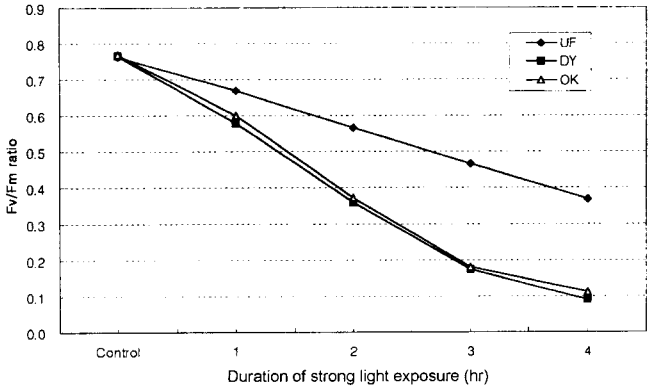

Fig. 5. Comparison of Fv/Fm ratios in tea leaves sampled from different growth conditions on 22 June in 2000. UF: University farm, nonshading, DY: Damyang, 70~80% shading. OK: Okkwa 70~80% shading.

The changes in Fm values were greater in tea leaves sampled from shading than nonshading conditions.

As shown in Fig. 5, Fv/Fm values under different growing conditions differed more remarkably, and decreased linearly with increasing duration of strong light exposure (r=-0.99** ~-1.0**). The Fv/Fm for nonshading condition decreased less sharply than that for shading, indicating that the plants grown under nonshading had greater tolerance to photoinhibition in winter season. This result suggested that the tea plants grown in naturally open field would have had greater freezing tolerance than those grown under shrubs or bamboo trees.

The Fv/Fm values of tea leaves treated with different freezing temperatures for 2h, and thawed at 3°C overnight were measured (Fig. 6). The Fv/Fm values were very similar

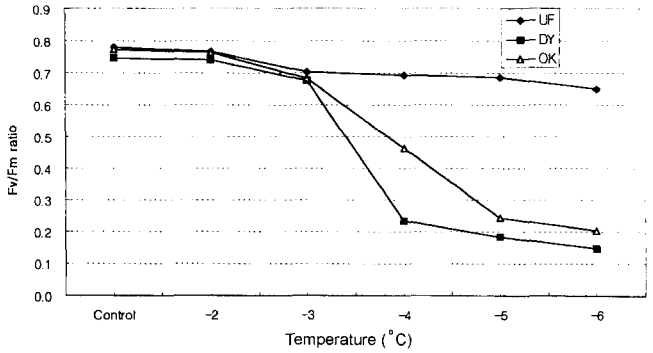

Fig. 6. Comparison of Fv/Fm ratios by tea leaves treated with different low temperatures for 2 hours on 22 June in 2000. UF: University farm, nonshading, DY: Damyang, 70~80% shading, OK: Okkwa 70~80% shading.

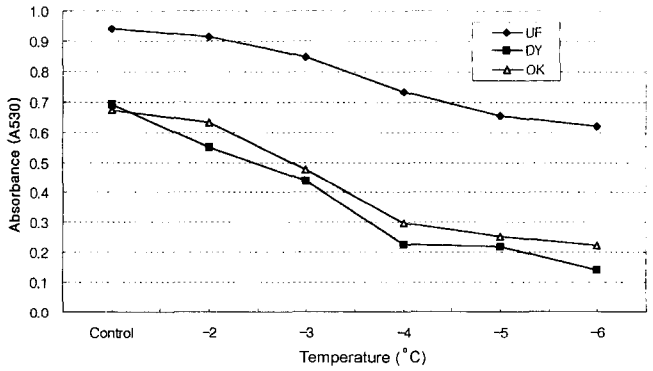

Fig. 7. Comparison of absorbance (A530 nm) by tea leaves treated with different low temperatures on 22 June in 2000. Tea leaves were treated with different low temperatures (2hr), and overnight (3°C) and dark-adapted (30 min.) under room temperature.
UF: University farm, nonshading,
DY: Damyang, 70~80% shading.
OK: Okkwa 70~80% shading.

in both growth conditions down to -3°C, but the values showed drastic difference between the two conditions at -4~-6°C plots. The Fv/Fm for nonshading at University tea farm had decrease of 16% at -6°C plot, but Fv/Fm at shading, Damyang or Okkwa regions, had decrease of 74~80%. The plots grown at nonshading survived at -6°C, but the plants grown at shading died even at -4°C (LT_{50}). The results of TTC test were very similar to the change of Chl fluorescences (Fig. 7). The absorbance of TTC reduction solution for nonshading had decrease of 34% even at -6°C, but that for shading had decrease of 56~68%.

Those results indicate that test on freezing resistance in germplasm of native land races should be done in consideration of both genetic and environmental factors. Tea plant is a evergreen tree. If temperature and light conditions do not fall down to limiting level, the plants continue to keep photosynthesis even though in very low level and accumulate sucrose, and freezing tolerance will be enhanced.

A simple method to test freezing resistance

When establishing a new tea garden, out-pollinated seeds have been used as a propagator. Tea plants have characteristics of self-incompatibility and each seed has different genetic background. Therefore, to get enough materials is very important and limited to test freezing resistance in germplasm and early hybrid generations.

As for breeding a new tea variety with freezing resistance, lots of F1 hybrid seeds are made for diverse variations because hybrid F1 plants are propagated vegetatively and evaluated for yield, quality and other traits. To test freezing resistance in early hybrid generation in order to screen a superior line and propagate vegetatively, with small materials as possible, it is necessary to establish a simple method for freezing resistance.

The TTC test (absorbance of TTC reduction solution) and Chl fluorescence measurements (Fv/Fm) were done with 20 hybrid lines in order to select a new superior line (Table 1). The absorbance value of TTC reduction solution for -5°C was 51% as compared with the control, 45% for -6°C plot and the coefficients of variation were 13.6%~16%.

The Fv/Fm value for -4°C plot was 47% as compared with

Table 1. Means, standard deviations (SD) and CVs of absorbance of TTC reduction solution and Fv/Fm ratio in 20 tea hybrids grown up for 5 years and sampled on 30 May, 2000.

Treatment	Absorbance (A530)				Fv/Fm ratio			
	Mean	SD	CV(%)	Ratio(%)	Mean	SD	CV(%)	Ratio(%)
Control	0.663	0.080	12.1	100	0.739	0.017	2.3	100
-3°C	0.510	0.088	17.3	76.9	0.588	0.123	21.0	79.6
-4°C	0.402	0.054	13.4	60.6	0.345	0.090	26.1	46.7
-5°C	0.341	0.046	13.6	51.4	0.219	0.069	31.6	29.6
-6°C	0.272	0.044	16.0	41.0	0.155	0.037	23.8	21.0

Table 2. Simple linear regression equations for low temperatures, absorbance of TTC reduction solution and chlorophyll fluorescence (Fv/Fm) in 20 tea hybrids grown up for 5 years and sampled on 30 May, 2000.

Simple regression equation	R^2
TTC = 0.562 + 0.041**LT	0.757
CHL = 0.600 + 0.064**LT	0.740
TTC = 0.204 + 0.571**CHL	0.784

**Significant at 1% level.
TTC; Absorbance of TTC reduction solution, LT; Low temperature, CHL; Chlorophyll fluorescence (Fv/Fm)

the control, 30% for -5°C plot with CV of 31.6% for -5°C plot. Also, The low temperatures explained the variations of absorbance of TTC reduction solution and Chl fluorescence (Fv/Fm) highly significantly, and also the absorbance and Fv/Fm showed very close linear relationship (Table 2). The second leaves were sampled from 20 different plants of 3 years old at University farm, and measured for the absorbance of TTC reduction solution and Chl fluorescence. To test with small materials as possible, only the control and -5°C treatments were investigated for TTC test, and the control and 6h photoinhibition for Chl fluorescence. As shown in Table 3, the Fv/Fms for 6h photoinhibition showed highly significant correlations with the absorbance of TTC reduction solution for-5°C plot (r=0.709**~0.801**). Especially, Spearman's rank correlation between data from the two different methods was very close (r_s=0.910**), revealing that the lines with high reduction capacity had high Chl fluorescence (Fv/Fm).

The Fv/Fm at 6h photoinhibition (x) explained the variation of absorbance of TTC reduction solution at -5°C plot highly significantly (R^2=0.64**, Fig. 8), suggesting that Chl fluorescence (Fv/Fm) is a very useful simple method to test freezing resistance in germplasm, and early hybrid generations in tea plants.

Those results were similar to those reported by Chun *et al.* (1997), and Binder & Fielder (1996), and it have some merits for testing freezing resistance in case that lots of lines are

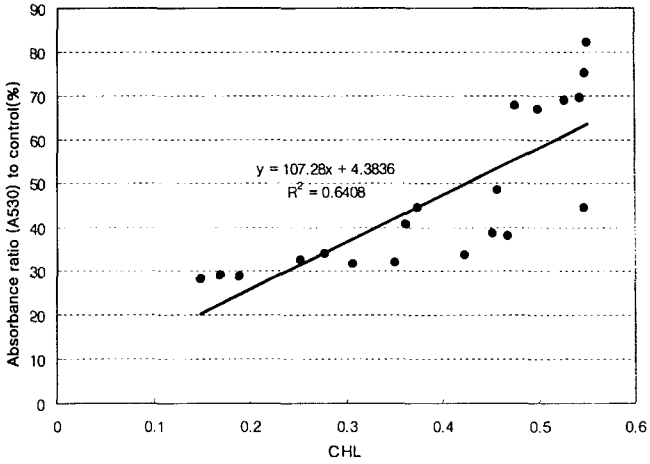

Fig. 8. Relationship between absorbance (A530 nm) of TTC reduction solution and chlorophyll fluorescence (Fv/Fm; CHL) in 20 native tea leaves.

to be evaluated in short time with a leaf.

ACKNOWLEDGEMENTS

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Table 3. Simple correlation coefficients and Spearman's rank correlation between absorbance of TTC reduction solution (TTC) and chlorophyll fluorescence (CHL) in 20 native tea trees grown up for 3 years with naturally pollinated seeds.

Characteristic	Correlation coefficient			
	CHL	CHC	TTC	TTR
CHL; Fv/Fm photoinhibited for 6 hr	-	0.994**	0.709**	0.801**
CHC; CHL ratio to control		-	0.723**	0.799**
TTC; Absorbance of -5°C plot			-	0.870**
TTR; TTC ratio to control				-
Rank correlation with CHL				0.910**

**Significant at 1% level.

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