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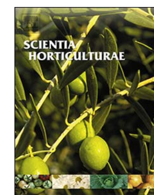

Freezing stress deteriorates tea quality of new flush by inducing photosynthetic inhibition and oxidative stress in mature leaves

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ABSTRACT

The unprecedented early spring frost that appears as a freezing stress can cause significant yield losses in tea [*Camellia sinensis* (L.) O. Kuntze]; however, its effect on the quality of newly emerged tea leaves and the underlying physiological mechanisms still remain unclear. In the present study, two years-old tea seedlings were exposed to freezing temperature (−5 °C) for 3, 6 and 12 h, designated as mild, moderate and severe freezing stress, respectively; and gas exchange, chlorophyll fluorescence, water use efficiency and reactive oxygen species accumulation were analyzed after a period of recovery. The results showed that net photosynthetic rate, stomatal conductance, transpiration rate, water use efficiency and maximal photochemical efficiency of photosystem II significantly decreased but accumulation of hydrogen peroxide increased in mature leaves with increasing freezing duration after 2 day recovery. Even after 15 day recovery, water content and dry weight of one bud and one leaf in newly emerged tea flush significantly decreased, showing the lowest decrease (2.7% and 23.7%) by mild and the highest decrease (10.6% and 66.4%) by severe freezing stress, respectively. Meanwhile, tea polyphenol concentration increased but amino acid concentration decreased in young leaves, resulting in a gradual increase (48.9–83.1%) in polyphenol to amino acid ratio with increasing freezing duration. These results suggest that freezing-induced photosynthetic inhibition and oxidative stress in mature leaves eventually decrease both yield and quality of young leaves and thus indicating a profound effect of source leaves that experienced stress on the quality of newly emerged young tea leaves.

1. Introduction

Tea is the most popular non-alcoholic health drink, produced from young leaves and buds of tea plants [*Camellia sinensis* (L.) O. Kuntze], an evergreen woody perennial plant species belonging to Theaceae family (Shen et al., 2015). Although tea can be grown in diverse agro-climatic regions, thermophilic nature of tea plants limits its geographical distribution particularly in the temperate climate. Furthermore, due to poor tolerance of tea plants to low temperature, every year sudden frost in fall or early spring that appears as freezing stress causes a significant loss of tea production (Shen et al., 2015; Zheng et al., 2015; Wang et al., 2017). Therefore, it is indispensable to better understand the physiological response of tea plants to freezing stress in order to improve freezing tolerance in the face of climate change.

Freezing generally refers to a temperature below zero (subzero, <0) °C, which is unbearable to the majority of tropical plant species (Hashempour et al., 2014). However, in many regions across the world, plants have to cope with freezing stress (Mayr and Améglio, 2016). Plants grown in temperate regions can enhance their tolerance to freezing temperature following prior exposure to a mild low temperature through a process known as cold acclimation (Zhan et al., 2015). Tea plants endure winter dormancy, but do not show deciduous habit (Mukhopadhyay et al., 2016). Metabolite profiling of tea leaves in winter reveals that divergences in low temperatures alter metabolic pathways differently (Shen et al., 2015). Freezing mainly causes injury to the cell membranes (Hashempour et al., 2014). Because, intercellular ice formation causes destruction of tonoplast, leading to cell death if rapid cooling occurs (Mayr and Améglio, 2016). In plants, the immediate consequences of low temperatures include mechanical blockades, alterations in the activity of macromolecule and reductions in osmotic potential in the cellular environment (Xiong et al., 2002). Freezing-induced damage is associated with an increased production of the reactive oxygen species (ROS) that cause damage to biomolecules such as lipids, protein and DNA in plants (Mittler, 2002; Hashempour

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et al., 2014). Excessive ROS generation creates an imbalance between oxidants and antioxidants, a condition commonly known as oxidative stress (Mittler, 2002; Foyer et al., 2017). Freezing-induced ROS generation is also responsible for the membrane lipid peroxidation, a key indicator of oxidative stress (Hashempour et al., 2014). Nevertheless, abiotic stress-induced such alterations in biochemical, physiological and molecular processes ultimately affect the growth and productivity of tea (Upadhyaya and Panda, 2013).

Being the most temperature sensitive process in plants, photosynthesis is drastically inhibited by freezing stress. Freezing-induced depletion in photosynthesis is attributable to a reduction in photochemical activity and/or stomatal conductance (Ploschuk et al., 2014). Chilling or freezing temperatures enhance photoinhibiton, the so called loss of photosystem II (PSII) function (Foyer et al., 2017). The degree of photoinhibition basically represents the balance between the rate of PSII damage and the rate of repair. Recent studies suggest that the repair of PSII is inhibited under environmental stress, since stress downregulates the production of D1 protein by inactivating translation machinery and/or affecting the CO_2 fixation. However, an interruption in CO_2 fixation also results in the generation of ROS that halt PSII protein synthesis in chloroplasts (Takahashi and Murata, 2008).

Tea quality is a cumulative attribute, which indicates an optimal combination of a range of metabolites. One of the key indices determining tea quality is the ratio of total polyphenol to amino acid (Han et al., 2016). Polyphenols are mostly flavonoid metabolites, which are thought to have anti-cancer, anti-mutagenic, anti-microbial, anti-inflammatory, and anti-atherosclerotic properties in animals (Ravindranath et al., 2006; Tounekti et al., 2013). Health benefits of tea are largely associated with the composition of tea polyphenol. In plants, flavonoids have multifaceted functions, including their role in signaling and stress tolerance (Ahmed et al., 2015). Diverse environmental cues stimulate the amounts and varieties of flavonoids differently and the mechanisms underlying are still being elucidated. An increased production of flavonoids is a sign of enhanced defense response of plants for stress protection (Tounekti et al., 2013), however, too much polypehonols makes tea bitter and thus lessens its quality (Li et al., 2016a). Moreover, the concentration of polyphenol greatly varies between young and mature leaves (Li et al., 2016b). Since the first two leaves and a bud are plucked for manufacturing tea, the quality of tea basically refers to the compositional features of young leaves and bud of tea plants. When frost occurs, apparently the entire foliar portion of tea plants experiences stress, however, the effect of such freezing temperature on the quality of new flushes that appear after a recovery period still remains largely unknown. Nonetheless, the duration of stress and developmental stages of plants largely affect stress-induced changes in multiple plant processes (Upadhyaya and Panda, 2013).

In this study, we attempt to investigate the effect of three duration of freezing periods such as 3, 6 and 12 h, designated as mild, moderate and severe freezing stress, respectively on gas exchange, chlorophyll fluorescence, water use efficiency and reactive oxygen species accumulation in mature leaves that experience stress. In addition, we determined the effect of the freezing stress on water content, dry weight, polyphenol and amino acid concentration of one bud and one leaf that emerged after the freezing stress. Our results suggest that freezing-induced damage to photosynthetic apparatus of mature leaves alters both yield and quality of newly emerged young leaves.

2. Methods

2.1. Plant materials and treatments

Two years-old tea seedlings of Longjing 43 [*Camellia sinensis* (L.) O. Kuntze] were exposed to freezing temperature (−5 °C) for 3, 6 and 12 h by placing them into a programmable test chamber for whole plant freezing treatment at night commencing from 10:00 p.m. during early spring. Inside the chamber, relative humidity was kept at 45–50% and darkness was maintained. Afterward, tea plants were returned to normal growth conditions maintaining the photosynthetic photo flux density (PPFD) at 600 mmol m^{-2} s^{-1}, photoperiod was 14/10 h (day/night), day/night air temperature was 26/22 °C and relative humidity was 85% in controlled-environment growth chambers (Conviron, Winnipeg, Canada). Control tea plants (CK) were grown under the same conditions without prior freezing treatment. Two days after recovery, gas exchange, water use efficiency, chlorophyll fluorescence and reactive oxygen species accumulation were analyzed in mature leaves, whereas water content, dry weight of one bud and one leaf, polyphenol and amino acid concentration were measured after 15 day recovery when new shoot appeared.

2.2. Measurements of gas exchange parameters

Gas exchange parameters were measured in mature fully expanded tea leaves after 2 d recovery from freezing stress. Net photosynthetic rate, stomatal conductance and transpiration were analyzed within the time period from 9:00 a.m. to 11:00 a.m. using an open-flow infrared gas analyzer adapted with light and temperature control systems (Li-COR 6400, Li-COR, Lincoln, NE, USA) as described previously (Ahammed et al., 2015). Water use efficiency (WUE) was calculated as the ratio of net photosynthetic rate to transpiration rate.

Chlorophyll fluorescence was measured in the same leaves that were used for gas exchange measurements after 30 min of dark adaptation period using an imaging-PAM chlorophyll fluorimeter equipped with a computer-operated PAM-control unit (IMAG-MAXI; Heinz Walz, Effeltrich, Germany) as described previously (Ahammed et al., 2015).

2.3. Detection of hydrogen peroxide (H_2O_2) accumulation in tea leaves

H_2O_2 in leaves was visually detected by staining with 3,3-diaminobenzidine (DAB) (Thordal-Christensen et al., 1997). Tea leaves were submerged in 1 mg mL^{-1} solution of DAB (pH 3.8) and incubated for 6 h under light at 25 °C. Afterward, leaves were bleached by immersion in boiling ethanol (96%) for 10 min. This treatment decolorized the leaves except for the deep brown polymerized product produced by the reaction of DAB with H_2O_2. Ascorbic acid was used as antioxidant to confirm that brown spots correspond to H_2O_2 formation. After cooling, the leaves were extracted at room temperature with fresh ethanol. Later on those DAB stained leaves were observed and photographed with a light microscopy system (Leica DM4000B & DFC425, Leica microsystem Ltd., Heerbrugg, Germany).

2.4. Measurement of water content and dry weight in tea buds and leaves

Relative water content (RWC) was measured using the following equation: RWC (%) = [(fresh weight − dry weight)/(turgid weight − dry weight)] × 100. To determine turgid weight, leaf samples were placed in distilled water at 4 °C in darkness for 12 h to reach a constant weight, whereas dry weight was determined after placing the samples in an oven at 70 °C for 48 h (Flexas et al., 2006).

2.5. Determination of tea polyphenol and total amino acid quantification

Total tea polyphenol was extracted and determined spectrophotometrically according to the method of the International Organization for Standardization (ISO) 14502-1 as described by (Han et al., 2016). Gallic acid was used as standard. In brief, the diluted sample extract (1.0 mL) was transferred to tubes in duplicate, where each tube contained 5.0 mL of a 1/10 dilution of Folin-Ciocalteu's reagent in water. Afterward, 4.0 mL sodium carbonate solution (7.5% w/v) was added into each tube. The tubes were kept at room temperature for 60 min before absorbance at 765 nm was measured against water.

Amino acids from tea leaf sample (0.5 g) were extracted in 80% ethanol at 80 °C. Following evaporation, dried samples were dissolved

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