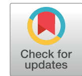
Scientia Horticulturae 238 (2018) 356–362

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Alleviation of cold damage by exogenous application of melatonin in vegetatively propagated tea plant (*Camellia sinensis* (L.) O. Kuntze)



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ARTICLE INFO

Keywords:
Tea plant
Melatonin
Glutathione
Chilling

ABSTRACT

Melatonin is involve in plant development and abiotic stress responses. In the present study, the effects of exogenous melatonin on chilling in tea plant were investigated. Compared to control, decreasing chlorophyll contents and increasing malondialdehyde levels in leaves were observed in tea plants treated with chilling at 4 °C. Interestingly, compared to chilling treatment, chlorophyll contents were increased significantly and malondialdehyde levels were dramatically decreased in tea leaves after melatonin treatments. Moreover, exogenous melatonin enhanced the anti-oxidation ability by increasing the activities of superoxide dismutase, catalase and peroxidase. It was also observed that there is increased the contents of reduced glutathione (GSH), decreased the contents of oxidized glutathione (GSSG), increased the ratio of GSH/GSSG, and increased the activity of ascorbate peroxidase and glutathione reductase in tea leaves after melatonin treatments. Meanwhile, the genes expression of *CsGSHS*, *CsGR*, and *CsAPX* were significantly up-regluated after the treatment of melatonin comparison with chilling treatment. Therefore, these data indicated that exogenous melatonin can attenuate the cold damage via enhancing anti-oxidation ability in tea plant.

1. Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is the most popular health drink in the world (Liang et al., 2007). The tea plant, is an important economic crop, and has been cultivated for nearly 2000 years in China (Chen et al., 2009). As a perennial evergreen woody crop, tea plant is constantly exposed to abiotic stresses such as cold, drought, salt and flooding. As a typical warm-climate plants, the quality and yield of tea plants are significantly influenced by sudden chilling. Hence, the utilization of tea is limited by low temperature and the shoots wither in late autumn and winter. Thus, seeking an effective way of attenuating cold damage in tea plants is in demand.

Cold stress has diverse effects on plant physiology, biochemistry and molecular biology (Shi et al., 2015). Cold stress can induce membrane damage to plants. Malonaldehyde (MDA) content is usually considered an indicator of membrane structural integrity. The content of MDA is significantly increased after low temperature treatment, resulting in decrease of plant membrane fluidity, and destruction of ion homeostasis in plants (Beck et al., 2004; Hou et al., 2010). Chlorophyll is the most crucial pigments related to photosynthetic, which is extremely sensitive to cold stress. Because low temperature almost destroys all the major components of photosynthesis (Allen and Ort, 2001). Moreover, chilling inhibit plant growth via different mechanisms, such as photosynthesis, photorespiration, and respiration, and result in inducing excessive production of reactive oxygen species (ROS), which cause injury to plants (Apel and Hirt, 2004; Jaspers and Kangasjärvi, 2010). To cope with oxidative stress under low temperature condition, plants have evolved efficient antioxidant systems to scavenge ROS. Antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) provide an effective protection mechanism against oxidative stress, and the activities of these enzymes increased dramatically under cold stress (Ao et al., 2013; Baek and Skinner, 2003; Shi et al., 2015). To improve the plant tolerance to chilling and thus raise crop productivity, the application of exogenous substances has been employed widely (Han et al., 2016; Posmyk et al., 2009a; Shu-Ming, 2012). One of the exogenous substances tested is melatonin, which has been widely used to help plants cope with various stress conditions, including cold stress (Posmyk and Janas, 2009; Zhang et al., 2015c).

Melatonin (N-acetyl-5-methoxytryptamine) is a well-known hormone that mediates many biological processes in animals such as

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; MDA, malonaldehyde; POD, peroxidase; SOD, superoxide dismutase

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https://doi.org/10.1016/j.scienta.2018.04.068
Received 5 January 2018; Received in revised form 28 April 2018; Accepted 28 April 2018
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circadian rhythms and sleep (Hardeland et al., 2012). Since firstly detected in higher plants in 1995 (Dubbels et al., 1995; Hattori et al., 1995), the study of melatonin in plants has attracted remarkable attention, and additional studies found that melatonin is widely distributed in leaves, roots, stems, fruits, and seeds of all plant species examined (Arnao, 2013; Arnao and Hernándezruiz, 2007; Kolar and Machackova, 2005; Manchester et al., 2000; Shi et al., 2015). Subsequently, melatonin has been demonstrated to be a ubiquitous modulator in growth, development and stress responses in multiple plants. Previous studies have proved that melatonin protects plants against a variety of abiotic stress such as cold stress (Bajwa et al., 2014; Kang et al., 2010; Posmyk et al., 2009a), excess copper (Posmyk et al., 2009b), high temperature (Byeon and Back, 2014), salt stress (Li et al., 2012), drought stress (Wang et al., 2014), and UV radiation (Afreen et al., 2006). All these stresses lead to over-production of ROS. Recent studies have reported that melatonin alleviates oxidative damage by scavenging ROS and stimulating antioxidant systems under various abiotic stresses (Manchester et al., 2015; Zhang et al., 2015c).

To remove and detoxify excess ROS which is harmful to plant tissue, plants form both enzymatic and nonenzymatic antioxidant defences. Among of these, The ascorbate–glutathione (AsA–GSH) cycle is an important and efficient ROS-scavenging pathway (Palma and Río, 2006). Moreover, reduced glutathione (GSH), a cellular antioxidants, is of great importance in preserving a wide range of metabolic processes involved in the AsA–GSH cycle (Drążkiewicz et al., 2003). It is associated with the cellular redox balance, and the ratios of GSH/GSSG is crucial in the induction of tolerance (Wang et al., 2013a). The antioxidant enzymes of the AsA–GSH cycle, ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione S-transferase (GST), provide endogenous defence against the accumulation of harmful concentrations of ROS(Mittler et al., 2004). Previous studies have revealed that *CsGSHS*, which encodes a key enzyme in GSH biosynthetic, has a major role in preserving GSH content for plants in response to abiotic stress in plants (Kang et al., 2013; Mohanpuria et al., 2007). Recently, many researchers have demonstrated that melatonin also motivates antioxidant enzymes and augmenting antioxidants involved in the AsA-GSH cycle to scavenge excess ROS (Zhang et al., 2015a; Zhao et al., 2016).

Although remarkable progress has been made in elucidating the role of melatonin in responses to abiotic stress in a number of species in recent years, studies on the effect of melatonin in tea plant against cold stress have been rarely investigated. In the present study, we investigated the impact of melatonin on cold stress in tea plant. Hence, physiological, molecular and metabolic methods were employed to discover the potential mechanism of melatonin involved in resisting cold stress in tea plants.

2. Materials and methods

2.1. Plant materials, growth conditions and treatment

Two-year-old vegetatively propagated cuttings of tea plant (*C. sinensis cv*. 'Longjing 43') were grown in a chamber at the Tea Research Institute of Nanjing Agricultural University (Nanjing, China). Tea plants were grown in a growth chamber under 25 °C, 75% relative humidity, 200 μmol $m^{-2} s^{-1}$ light intensity and a photoperiod of 12 h light and 12 h darkness, and allowed to acclimatize for one week before treatment.

The tea plants were randomized into seven groups. The melatonin treatment groups were exposed to cold stress at 4 °C sprayed with melatonin (Sigma-Aldrich, St. Louis, MO, USA) concentrations (5, 50, 100, 500 and 1000 μM) (Li et al., 2016; Zhang et al., 2015b) at 24 h after cold treatment. The chilling group was under cold conditons and sprayed with deionized water (0 μM melatonin). While the control group (CK) were treated with 25 °C and then sprayed with deionized water. Leaf samples were harvested at 48 h after melatonin treatment, immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis of physiological indicators, cold resistance antioxidant enzymes, and gene expression of *CsGR, CsGSHS, CsAPX, CsGST* and *CsGPX*.

2.2. Determination of biological indicators

For chlorophyll assay, 100 mg of fresh bud tissue was ground with 80% acetone and estimated following the method described by Lichtenthaler (Lichtenthaler, 1987). Protein content was estimated by the method described by Bradford (BradfordMM, 1976) using bovine serum albumin as a standard protein. Soluble sugar content was determined using the anthrone colorimetric method described by Yemm and Willis (Yemm and Willis, 1954). The level of lipid peroxidation in leaves was assessed by measuring the MDA content using 2-thiobarbituric acid as described by Hodges et al (Hodges et al., 1999).

2.3. Extractions and assays of the antioxidant enzymes

Fresh leaves (0.5 g) were ground on ice with 0.5 g quartz sands and 5.0 mL of 50 mM precooled phosphate buffer (pH 7.5) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) and 5.0 % (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged (16,000*g*, TGL-10C, Anting, Shanghai, China) at 4 °C for 15 min (Pereira et al., 2002). The supernatant was used for the analysis of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione S-transferase (GST). All the above antioxidant enzymes were determined by using the reagent kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instruction.

Glutathione reductase (GR) was assayed by the method of Smith et al (Thorne, 1988). The reaction mixture contained 1.0 mL of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 mL of 3.0 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 mL of 2.0 mM NADPH, 0.1 mL enzyme extract and distilled water to make up a final volume of 2.9 mL. The reaction was initiated by adding 0.1 mL of 2 mM oxidized glutathione (GSSG). The increase in absorbance at 412 nm was recorded spectrophotometrically at 25 °C over a period of 5 min. The activity was expressed as absorbance change (ΔAbs_{412nm}) g dry mass^{-1} s^{-1}.

2.4. Glutathione contents assay

For the measurement of GSH, GSSG, 0.2 g of leaf tissue was homogenized in 2 mL of 5% sulfosalicylic acid containing 2 mM EDTA and centrifuged at 4 °C for 20 min at 15,000*g*, and the supernatant was used for total and GSSG determinations by the DTNB/GR recycling procedure, as described by Nagalakshmi and Prasad (Nagalakshmi and Prasad, 2001). A standard curve prepared by using GSH and GSSG was used in the calculation of the amounts of total glutathione, reduced GSH and GSSG.

2.5. Total RNA extraction cDNA synthesis, and qRT-PCR assay

Total RNA was extracted using the Quick RNA Isolation Kit (Huayueyang, Beijing, China). The RNA concentration was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cDNA of tea leaves was synthesized using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China), in accordance with the manufacturer protocols. Quantitative real-time PCR (qRT-PCR) was performed on an IQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The reaction program was set as follows: 95 °C for 30 s; followed by 40 cycles at 95 °C for 5 s and 55 °C for 25 s. The reaction volume was 20 μL, which contained 2 μL diluted cDNA strand, 7.2 μL ddH2O, 10 μL SYBR® Premix Ex Taq(TaKaRa, Dalian, China), and 0.4 μL each primer. All experiments were repeated three

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