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# Sodic alkaline stress mitigation by exogenous melatonin in tomato needs nitric oxide as a downstream signal

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ABSTRACT

The present study was designed to determine the interactive effect of exogenous melatonin and nitric oxide (NO) on sodic alkaline stress mitigation in tomato seedlings. It was observed that exogenous melatonin treatment elevated NO levels in alkaline–stressed tomato roots. However, exogenous NO had little effects on melatonin levels. Importantly, melatonin–induced NO generation was accompanied by increased tolerance to alkaline stress. Chemical scavenging of NO reduced melatonin–induced alkaline stress tolerance and defense genes' expression. However, inhibition of melatonin biosynthesis had a little effect on NO-induced alkaline stress tolerance. These results strongly suggest that NO, acting as a downstream signal, is involved in the melatonin–induced tomato tolerance to alkaline stress. This process creates a new signaling pathway for improving stress tolerance in plant.



## 1. Introduction

As the sessile organisms, plants are often highly challenged by disparate and changeable environments, among which soil salinity and alkalinity negatively affect plant growth and development. All over the world, there are 831 million hectares of soils suffering from salinity, and more than half of them are caused by the accumulation of sodic alkaline (Gong et al., 2014a). Indeed, the damage of soil alkalinization resulting from $NaHCO_3$ and $Na_2CO_3$ may be more deleterious than the damage induced by the neutral salts, such as NaCl and $Na_2SO_4$ (Gong et al., 2013). It is well known that the destructive effects of neutral salt mainly arise from water deficit due to osmotic stress and ion–specific effects caused by excess sodium ions absorption (Zhang and Blumwald, 2001). Excepting these, high pH of alkaline stress is another limiting factor for plant growth and development, which directly affects the uptake of mineral elements and disturbs the ion homeostasis (Gong et al., 2014b). Both alkaline and saline conditions influence photosynthesis and metabolism, but they also involve different physiological and molecular mechanisms (Gong et al., 2014c). To date, there are a large number of reports related with physiology mechanism of salt tolerance. However, it was only recently that the physiological mechanism of plant tolerance to alkaline stress was concerned.

Melatonin (N-acetyl-5-methoxytryptamine), an indoleamine molecule with low molecular weight, has been widely discovered in animal and plant kingdoms, and its physiological function has drawn increasing attention from animals to plants. In plants, increasing evidence, indicate that melatonin plays significant roles in regulating various physiological processes including seed germination, growth of explants, flower development, and root system architecture (Murch and Saxena, 2002; Hernández-ruiz et al., 2005; Wang et al., 2012). As a ubiquitous and versatile molecule, melatonin has a series of the desirable properties of a perfect antioxidant and ideal free radical scavenger such as its high lipophilicity and part hydrophilicity as well as regeneration after radical quenching (Zhang et al., 2014a,b). So, it is not surprising that melatonin plays a crucial role in protecting plants against oxidative damage under environmental stress (Arnao and Melatonin, 2014). In animals, components involved in signal pathways modulated by melatonin has been widely investigated, which include receptors, intracellular cyclic nucleotides (cAMP and cGMP) (Mazzon et al., 2006), calcium signal (Bazwinsky-wutschke et al., 2014), mela-

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tonin's metabolites (AFMK and AMK) (Galano et al., 2013), protein phosphorylation, and many other hormones (Pandi-perumal et al., 2008). On the contrary, the melatonin–regulated signal in plants is just at the beginning. Recently, it was observed that a series of signal molecules such as abscisic acid, gibberellins, salicylic acid, ethylene, and indole-3-acetic acid, were involved in melatonin–regulated physiological processes including development, abiotic and biotic stress (Lee et al., 2014; Zhang et al., 2014a,b; Kostopoulou et al., 2015; Shi et al., 2015). These results may imply that melatonin is a unique regulator like other confirmed plant hormones.

Nitric oxide (NO) is a hydrophobic, highly diffusible gaseous and ubiquitous bioactive molecule in organisms. It takes part in a broad spectrum of regulatory functions in plants, such as seed germination, abiotic and biotic stress tolerance, flowering and senescence (Wimalasekera et al., 2011; Tanou et al., 2012). From previous studies, it was found that there existed crosstalk between melatonin and nitric oxide in animals (Sarti et al., 2013; Tain et al., 2014). To our knowledge, there were no reports about relationships between melatonin and nitric oxide in plants. However, some shared physiological functions of melatonin and nitric oxide can be found in their regulation of plant tolerance to stress conditions. Especially both were involved in reactive oxygen species signal pathways in plants (Scheler et al., 2013; Park et al., 2013).

It has been observed that exogenous melatonin and NO application could decrease damage induced by alkaline stress in plants, respectively (Liu et al., 2015; Gong et al., 2014d). Therefore, in this study, we hypothesized that they might have an interactive effect on tomato tolerance to sodic alkaline stress. Interestingly, alkaline stress mitigation by melatonin in tomato was inhibited by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). While the mitigative effect of NO could not be removed by melatonin synthesis inhibitor *p*-chlorophenylalanine (p-CPA). Thus, NO may act as a downstream signal of melatonin in regulation of tomato plants adaption to alkaline stress. In the present study, the roles of melatonin and NO in regulating $NaHCO_3$-induced oxidative stress were assessed in tomato seedlings. To our knowledge, this is the first evidence that NO is involved in melatonin regulating plants tolerance to abiotic stress as a downstream signal.

## 2. Materials and methods

### 2.1. Plant materials and treatments

Tomato seeds (*Solanum lycopersicum* L.) were germinated in the incubator at 28 °C and sown in vermiculite. After the third true leave emerging, seedlings were selected and transferred into plastic container filled with 5 L of half–strength Hoagland solution. The containers were brushed with black paint to protect the root systems from light exposure, and were cultivated in a glasshouse (22–28 °C/ 15–18 °C day/night). After 10 days of precultivation, the treatments were started.

The experiment included six treatments: (i) control, tomato plants cultivated with only Hoagland nutrient solution; (ii) alkalinity treatment, Hoagland nutrient solution plus 75 mM $NaHCO_3$; (iii) Hoagland nutrient solution plus 0.5 μM melatonin (MT) and 75 mM $NaHCO_3$; (iv) Hoagland nutrient solution plus 100 μM sodium nitroprusside (SNP; a NO donor) and 75 mM $NaHCO_3$; (v) Hoagland nutrient solution plus 75 mM SNP, 10 μM p–Chlorophenylalanine (p-CPA, which blocks conversion from serotonin to melatonin) and 75 mM $NaHCO_3$; (vi) Hoagland nutrient solution plus 0.5 μM MT, 10 μM 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; a specific scavenger of NO) and 75 mM $NaHCO_3$. The experiment was arranged in a randomized complete block design with five replicates, and the solution was replaced every two days. After 10 days of treatment, all physiological indices were assessed.

### 2.2. Determination of plant growth and potassium and sodium contents

Net photosynthesis was evaluated with a portable photosynthetic system (Li-6400; Li-COR Biosciences, Lincoln, USA). Shoot heights were measured from 10 plants per treatment. Chlorophyll was extracted in 80% (w/v) acetone and determined by the method described by Inskeep and Bloom (1985). After half an hour in the dark, chlorophyll fluorescence parameter of the third fully expanded leaf was quantified by chlorophyll fluorescence imaging (FluorCam7, Photon Systems Instruments, USA) (Maxwell and Johnson, 2000). Shoots and roots were separated and weighed after being washed with distilled water. Then they were dried in an oven at 105 °C for 15 min, 75 °C for 72 h, and weighed. The drying samples were analyzed for $Na^+$ and $K^+$ content by an atomic absorption spectrophotometer (TAS-990, Purkinje General, China).

### 2.3. Determinations of lipid peroxidation, electrolyte leakage and proline

The level of lipid peroxidation was analyzed by measuring the peroxidation products, thiobarbituric acid reactive substances (TBARS). TBARS was quantified by the method of Hodges et al. (1999), and Electrolyte leakage (EL) was quantified by using the method of Dionisio-Sese and Tobita (1998). Proline (Pro) was quantified by using the ninhydrin assay according to Bates et al. (Bates et al., 1973). 0.5 g of samples was homogenized in 10 ml of 3 % (w/v) sulfosalicylic acid and the homogenate filtered through qualitative filter paper. Two milliliters of filtrate was used for Pro determination at 520 nm.

### 2.4. Assays of ROS accumulation

According to the previous research (Jabs et al., 1996; Thordal-Christensen et al., 1997), the histochemical staining of $O_2^{\bullet-}$ and $H_2O_2$ was accomplished by vacuum infiltrating with 0.1 mg $ml^{-1}$ nitrobluetetrazolium (NBT) in 25 mM K-Hepes buffer (pH 7.8) and 0.1 mg $ml^{-1}$ DAB in 50 mM Tris-acetate (pH 3.8), respectively. $O_2^{\bullet-}$production rate was quantified by hydroxylamine oxidation assay (Rauckman et al., 1979). The concentration of $H_2O_2$ was extracted with 5% (w/v) trichloroacetic acid and was determined according to Patterson et al. (Patterson et al., 1984).

### 2.5. Determinations of antioxidant metabolites and antioxidant enzyme activities

Reduced ascorbic acid (ASA) and oxidized ascorbic acid (DHA) were measured by homogenizing with 1.5 ml of ice–cold 6% (v/v) $HClO_4$. Reduced GSH and GSSG were by extracting with 1.5 ml of 5% (w/v) sulfosalicylic acid. The concentrations of ASA, DHA, GSH, and GSSG were determined by the method of Hodges et al. (1996) and Griffith (1980).

The total protein was quantified by the method of Peterson (1983). For analysis of antioxidant enzyme activity, 0.3 g fresh samples were ground in an ice–cold mortar with 3 ml ice–chilled potassium phosphate buffer (50 mM, pH 7.8) containing 0.2 mM ethylene diaminetetraacetic acid (EDTA), 2 mM ascorbate and 2% (w/v) polyvinylpyrrolidone (PVP). All homogenates were centrifuged at 12,000 × *g* (4 °C) for 20 min and the supernatants were used for the determination of antioxidant enzyme activities. Superoxide dismutase (SOD) activity was analyzed by determination of its ability to inhibit the photochemical reduction of NBT (Stewart and Bewley, 1980). Catalase (CAT) activity was determined by the