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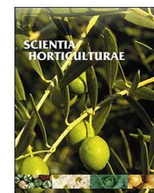

Research paper

Nitric oxide synthase-dependent nitric oxide production enhances chilling tolerance of walnut shoots *in vitro* via involvement chlorophyll fluorescence and other physiological parameter levels

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ABSTRACT

The role of nitric oxide (NO) in response to chilling stress in walnut (*Juglans regia* L.) seedlings was investigated. Walnut shoots were treated *in vitro* with sodium nitroprusside (SNP), NO scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), NO synthase (NOS) inhibitor *N*-nitro-$_{L}$-Arg-methyl ester ($_{L}$-NAME), and nitrate reductase (NR) inhibitor sodium tungstate. Their effects on chilling tolerance, the contents of soluble sugar, proline, and total phenol, reduced glutathione (GSH) and redox state (GSH/GSSG), and the activities of NOS and NR and NO level were analyzed. The results showed that low temperatures at 4 °C induced electrolyte leakage, lipid peroxidation and photosynthetic efficiency suppression, which were dramatically alleviated by exogenous application of SNP. The levels of soluble sugar, proline, total phenol, GSH and GSH/GSSG increased evidently in the presence of SNP under chilling stress. The above protective effect of SNP could be reversed by NO scavenger PTIO or NOS inhibitor. However, NR inhibitors did not affect the protective effect of SNP. Moreover, measurements of NOS activity and NO production showed that both NOS activity and endogenous NO content increased markedly under chilling stress. The NO production was markedly reduced by NOS inhibitors, but not affected by NR inhibitors in the presence or absence of chilling stress. Taken together, these results suggest that NOS-dependent NO production may confer chilling tolerance in walnut shoots *in vitro* by enhancing the levels of soluble sugar, proline, total phenol, GSH and GSH/GSSG to alleviate electrolyte leakage, lipid peroxidation and photosynthetic efficiency suppression induced by chilling stress.

1. Introduction

The global aim that agricultural productivity increased by 70% by the year 2050 is facing sever obstructions due to increasing abiotic stress factors (FAO, 2009). Among various abiotic stresses, chilling stress is one of the most important factors limiting the productivity and distribution of plants. It affects a range of physiological and biochemical activities in plant system depending on the severity and duration of the chilling stress (Karimi and Ershadi, 2015). Chilling stress increase membrane permeability and disrupt membrane integrity resulting membrane damage which can be measured as a level of electrolyte leakage (Zhou and Guo, 2009). Under conditions of chilling stress, one of the most susceptible cellular organelles is the chloroplasts. Chilling stress leads to the decrease in photosystem II maximum photochemical efficiency, which is generally expressed as variable to maximum fluorescence ratio (F_v/F_m) (Wang et al., 2016a,b). Exposure to low temperature induces the overproduction of reactive oxygen species (ROS) which could cause lipid peroxidation, DNA damage and protein denaturation. The level of lipid peroxidation can be monitored using the peroxidation product, malondialdehyde (MDA), as a marker (Liu et al., 2011). To overcome chilling stress, plants can trigger a cascade of events that induce biochemical and physiological modifications to enhance their tolerance (Theocharis et al., 2012). This phenomenon is known as chilling or cold acclimation. These adaptations include changes in the membrane composition, the induction of antioxidative systems and the synthesis of protective molecules, such as sugar, proline, reduced glutathione (GSH) and polyamines (Meng et al., 2015; Theocharis et al., 2012; Zeng et al., 2011).

Nitric oxide (NO), which was first identified as a unique diffusible molecular messenger in animals, plays important roles in plant various

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physiological processes, including seed germination and dormancy, growth and development of plant tissue, plant cell maturation and senescence, flowering, hormone responses, stomatal closure and programmed cell death (Arc et al., 2013; Kong et al., 2016; Mur et al., 2013). Furthermore, NO is suggested to be involved in regulating the multiple plant responses to a variety of abiotic stresses, such as drought, salinity, extreme temperature, UV radiation, mechanical injury, herbicide, heavy metal and pathogen attach (Fancy et al., 2017; Fatma et al., 2016; Jian et al., 2015,). Due to its biological action, NO has been considered as either stress-inducing or a protective agent. For example, under environmental stress, application of sodium nitroprusside (SNP), a NO donor, to plants resulted in increased tolerance to environmental stresses, thereby alleviating injury and promoting plant growth (Asgher et al., 2017; Bai et al., 2015). NO may react directly with radical species, such as superoxide anion (O_2^{-}) and intermediates in lipid peroxidation, acting as antioxidant and breaking lipid peroxidation process (Simontacchi et al., 2013). NO is also involved in proline accumulation that is required for cold tolerance and is achieved after the induction/repression of genes of proline metabolism (Puyaubert and Baudouin, 2014). Moreover, NO positively functions in maintaining the stability of chlorophyll and thylakoid membranes during leaf senescence (Liu and Guo, 2013).

Several studies have addressed the sources of NO production in plants. Besides the non-enzymatic NO production, to date, seven potential enzymatic sources of NO have been identified in plants (Gupta et al., 2011; Freschi, 2013). Increasing evidences indicate that, among them, NO synthase (NOS) activities and nitrate reductase (NR) are currently considered as the most likely candidates for the production of NO under physiologically relevant conditions (Asgher et al., 2017; Mur et al., 2013). NO is generated primarily by NOS in animals (Zhao et al., 2007). NOS-like activity has been detected widely in plants, and mammalian NOS inhibitor *N*-nitro-$_L$-Arg-methyl ester ($_L$-NAME) were also found to inhibit NO generation and NO mediated responses in plants, suggesting that NOS activity may also occur in plants (Gupta et al., 2011; Mur et al., 2013). Although a canonical NOS gene or a mutant deficient in NOS dependent NO production has not been identified in higher plants yet (Freschi, 2013), a NOS-like enzyme has been reported in the algae *Ostrococcus tauri* (Foresi et al., 2010). On the other hand, a great deal of evidence has indicated NR as one of the major plant biosynthetic sources of NO (Jian et al., 2015; Rosales et al., 2011). Both pharmacological and genetic evidence support a role for NR in NO production in plants. NR inhibitors such as tungstate, sodium azide and potassium cyanide has been shown to inhibit NO production in plants (Fancy et al., 2017; Sang et al., 2008; Wang et al., 2010). Of course, the source of NO may be different depending on the plant species, type of tissues or cells, and plant growth conditions. More evidence is required for the elucidation of how NO is made by different plant cells, in different external situations. Furthermore, the source of NO production induced by chilling stress in many plants is not very clear. It is still not known that NO production is mainly from NOS-like activity or NR under conditions of chilling stress and how the source of NO is associated with the plant responses to chilling.

Walnut (*Juglans regia* L.), which has been cultivated for both its edible nuts and timber, is one of the most valuable and widely cultivated horticultural commodities in the world. Its nuts are used worldwide in human nutrition as it contains high amounts of proteins, fats, essential dietary fatty acids, vitamins, and minerals (Ikhsana et al., 2016). However, walnut seedlings are susceptible to chilling injury in northern China. Therefore, it is necessary to explore the mechanism underlying acclimation to cold temperatures in walnut. In the present study, the role of NO on chilling resistance, chlorophyll fluorescence and other physiological parameter levels were investigated in walnut shoots *in vitro* under conditions of chilling stress. We used NO donors, NO scavengers, NOS inhibitors and NR inhibitors to determine whether endogenous NO is associated with chilling stress-induced physiological responses in walnut shoots *in vitro*. Furthermore, the origin of NO responsible for potential endogenous NO concentrations change in response to chilling stress was also evaluated. Firstly, the results presented here provide theoretical basis and technical support for walnut chill-proof growing in real field conditions. Secondly, in order to reduce individual error walnut shoots *in vitro* which are obtained through vegetative propagation were used as experimental materials in this research. Vegetative propagation is used to produce progeny plants, which are genetically identical to a single source plant.

2. Materials and methods

2.1. Plant material and treatments

The walnut cultivar 'Jinlong 2' was cultured aseptically on proliferation medium (full-strength DKW supplemented with 4.4 μM 6-benzyl-aminopurine and 4.9 nM 3-indole-butyric acid) as described previously (Pei et al., 2007). The medium was autoclaved at 121 °C and 124 kPa for 20 min before use. Subculturing was performed every 20 days. Shoot proliferation was performed in 350-mL cylindrical bottles. Each bottle contained four shoot clusters during subculture. The cultures were incubated at 25 ± 3 °C under a 16 h photoperiod (120 $\mu mol\ m^{-2}\ s^{-1}$) provided by cool-white fluorescent tubes. As shown in Fig. 1, shoots, 4.5–5.0 cm in length, were excised and used for all investigations.

$_L$-NAME was used as the NOS inhibitor and sodium tungstate as the NR inhibitor. To elaborate the influence of exogenously applied $_L$-NAME on NOS activity or exogenously applied sodium tungstate on NR activity, walnut shoots, 4.5–5.0 cm in length, were treated with $_L$-NAME of different concentration (0, 100, 200, and 400 μM) or sodium tungstate of different concentration (0, 100, 200, and 400 μM) and then cultured in 25 or 4 °C for 3 d.

SNP was used as the NO donor and 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO) as the NO scavenger. $_L$-NAME was used as the NOS inhibitor and sodium tungstate as the NR inhibitor. Walnut shoots, 4.5–5.0 cm in length, were excised and transferred into different proliferation medium containing the following: (1) sterilized water (control), (2) 100 μM SNP, (3) 200 μM PTIO, (4) 200 μ $_L$-NAME, (5) or 200 μM sodium tungstate. Half of the plants in each treatment were cultured at 25 °C, the other half of plants were exposed to 4 °C. After 3 d of culture, the third leaves were sampled. Fresh leaves were used for electrolyte leakage, chlorophyll content and chlorophyll

Fig. 1. *In vitro* walnut shoots of 4.5–5.0 cm height used for all investigations.

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