Physiological and Biochemical Processes of *Magnolia wufengensis* in Response to Foliar Abscisic Acid Application during Natural Cold Acclimation

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**Abstract.** The rare species *Magnolia wufengensis* frequently suffers from freezing injury in northern China. To investigate the influence of exogenous abscisic acid (ABA) application on the natural cold acclimation of *M. wufengensis*, physiological and biochemical changes in field-grown *M. wufengensis* seedlings subjected to foliar ABA treatments at four concentrations (0, 300, 600, and 900 mg·L⁻¹) were evaluated from Sept. 2012 to Jan. 2013. The optimum foliar application concentrations of ABA for *M. wufengensis* were between 600 and 900 mg·L⁻¹, which led to faster shoot growth cessation, leaf senescence, and development rates of bud endodormancy level and shoot freezing tolerance. The improved freezing tolerance under exogenous ABA application was associated with promoted dehydration and accumulation of soluble protein, and certain soluble sugars such as glucose and fructose. Foliar ABA treatments initiated a cascade of steps for advancing the cold-acclimation process of *M. wufengensis*. We suggest that exogenous ABA application may be used on *M. wufengensis* grown in northern China, where there are short growing seasons and early fall frost events.

*Magnolia wufengensis,* a new *Magnolia* species (Magnoliaceae), was discovered in Wufeng in Hubei Province, southern China (Ma et al., 2006a, 2006b). Increasingly popular for urban greening, *M. wufengensis* has considerable ornamental and economical value owing to its colorful flower (pure, dark, or pale red) and varied flower petal numbers (9–25, 32, or 46) (Sang et al., 2011). However, because of the biological characteristics and anthropogenic disturbances, its distribution is mainly limited to mountainous areas at 1400 to 2000 m elevation in western Wufeng, and its current population of less than 2000 individuals continues to decline (Sang et al., 2011). *Magnolia wufengensis* has been introduced to many parts of China in recent years to expand its geographic distribution. However, freezing injury has been a major constraining factor to the sustainability and profitability of *M. wufengensis* seedling production and has caused huge economic losses in northern China.

Freezing injury affects the growth, productivity, and geographical distribution of horticultural plants (Pearce, 2001; Weiser, 1970). The susceptibility of plants to freezing injury may be attributable not only to insufficient freezing tolerance, but also to the timing and rate of cold acclimation (Suojala and Lindén, 1997). Cold acclimation, the process by which plants transit from a cold-sensitive to cold-hardy state (Zabadal et al., 2007), is essential for the survival of woody plants growing in temperate regions (Teets et al., 1989). Cold acclimation usually develops in two stages. In the first stage, short-day induces growth cessation, periderm formation, and certain development of freezing tolerance (Davis and Evert, 1970; Palva et al., 2002; Wolpert and Howell, 1986). Decreasing air temperature observed in the second stage increases freezing tolerance to the maximum level (Artlip and Wisniewski, 1997; Stergios and Howell, 1977). Additionally, there is a series of physiological and biochemical responses during cold acclimation, including the modification of membrane lipid composition, synthesis of protective proteins, increase of compatible compounds, and enhancement of antioxidative mechanisms (Guy, 1990; Thomashow, 1999; Welling and Palva, 2008).

The phytohormone ABA plays an important role in plant cold acclimation by facilitating the acquisition of freezing tolerance, which has been reported in many plant species (Churchill et al., 1998; Dallaire et al., 1994; Mora-Herrera and Lopez-Delgado, 2007). Exogenous ABA application can initiate cold acclimation like low temperature (Mohaptra et al., 1988). Three unique C-repeat/­DRE Binding Factor genes are up-regulated by low temperature or exogenous ABA treatments, and these genes are conserved across both cold-tolerant and cold-sensitive cultivars (Xiao et al., 2006). Furthermore, exogenous ABA treatments and cold acclimation have similar effects on cell wall deformation including decreasing cell-wall pore size and increasing cell-wall strength (Rajashekar and Lafla, 1996). The improved cold-hardiness of plants under exogenous ABA application has been linked to physiological and biochemical changes such as reducing water content and oxidative damage (Kumar et al., 2008) and producing carbohydrates (Meng et al., 2008).

However, to our knowledge, no published reports are available on the influence of exogenous ABA application on *Magnolia* plants. Thus, this study begins to shed light on this unknown field. Physiological and biochemical responses of field-grown *M. wufengensis* seedlings to foliar ABA treatments during cold acclimation were investigated in this study. Our hypothesis stated that ABA would advance the cold-acclimation process in *M. wufengensis* seedlings through physiological and biochemical regulations that mimicked environmental cues including short-day and/or low temperature. The following questions were addressed: 1) What is the optimum foliar ABA application concentration for advancing the cold
acclimation process and ultimately improving the freezing tolerance of *M. wufengensis*?

2) Does freezing tolerance correlate with dehydration and the accumulation of compatible compounds in *M. wufengensis*

### Materials and Methods

**Plant materials and experimental treatments.** In early Apr. 2012, 2-year-old *M. wufengensis* seedlings were removed from the Rare Plant Institute of Forestry Bureau of Wufeng County in Wufeng, Hubei Province, China (lat. 29º56’ N, long. 110º15’ E), and transplanted to our study site, the Puzhaoyuan Nursery of Jiefeng Experimental Station of Beijing Forestry University in Beijing, China (lat. 39º48’ N, long. 116º28’ E). The seedlings were randomly grown in four plots in our study site. Each plot had an area of 15 × 3 m and contained 30 plants. From April to August, the seedlings were well irrigated and protected from bacterial pathogens and weed competition. Artificial irrigation was stopped after 31 Aug. In our pre-experiment, early September was shown to be the optimum time for ABA application, and ABA concentrations above 1000 mg L⁻¹ were observed to cause leaf damage (data not shown). Thus, plants in each plot were subjected to four concentrations of ABA consisting of 0 mg L⁻¹ (T0), 300 mg L⁻¹ (T1), 600 mg L⁻¹ (T2), and 900 mg L⁻¹ (T3) between 1600 and 1800 HR on 1 Sept. There were seven plants per ABA treatment in each plot. Whole seedlings were sprayed with ABA solutions to runoff with a 5-L handheld sprayer averaging a spray volume of 0.5 L/seedling. The ABA sample was 10% (v/v) S-ABA provided by Lomon Bio Corporation (China). The air temperature conditions in the site during our experimental period (from Sept. 2012 to Jan. 2013) were obtained from the Jiefeng Experimental Station (Fig. 1).

**Determination of shoot growth.** Shoot length was measured everyday from 1 to 30 Sept. Ten representative seedlings under each treatment were selected and one healthy upper-crown shoot facing the sun on each representative seedling was selected for shoot length measurement. The relative growth rate (RGR) of shoot length was determined by the formula, RGR = (lnv2 – lnv1)/(t2 – t1), where v1 is the value of shoot length in the first measuring day, v2 is the value of shoot length in the last measuring day, t1 is the first measuring day (1 Sept.), and t2 is the last measuring day (30 Sept.). Days to growth cessation (DGC) of shoot length, assessed as the number of days since 1 Sept. to shoot growth cessation, were recorded.

**Determination of leaf senescence.** Leaf senescence, assessed as the leaf photosynthetic rate, was determined once per 10 d from 1 Sept. to 31 Oct. Ten representative seedlings under each treatment were selected and one healthy upper-crown leaf facing the sun on each representative seedling was selected for leaf photosynthetic measurement. The net photosynthetic rate (PN, μmol CO₂ per m⁻²·s⁻¹) was investigated using an LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE). Data were recorded between 0900 and 1100 HR in each measuring day. Air cuvette irradiance, temperature, and carbon dioxide (CO₂) concentration were maintained at 1000 μmol·m⁻²·s⁻¹, 25 °C, and 400 μmol·mol⁻¹, respectively.

**Determination of bud dormancy.** Bud dormancy, assessed as days to 50% budburst (D50B), was determined monthly from 30 Sept. 2012 to 30 Jan. 2013. Ten representative seedlings under each treatment were selected and one healthy upper-crown shoot facing the sun on each representative seedling was excised into a foam medium and then placed in a plastic tray filled with water. Trays were then placed into a growth chamber with the following settings: 24-h photoperiod with 500 μmol·m⁻²·s⁻¹, 22 °C, and 80% relative humidity. Budburst was observed everyday. The greater D50B indicates a higher dormancy level.

**Determination of shoot freezing tolerance.** Freezing tolerance of shoots, assessed as the low temperature where 50% injury occurred (LT50), was determined monthly from 30 Sept. 2012 to 30 Jan. 2013. Ten representative seedlings under each treatment were selected and one healthy upper-crown shoot facing the sun on each representative seedling was selected for LT50 measurement. There are seven designed temperatures—0, –5, –10, –15, –20, –25, and –30 °C—in our freezing test. The shoots were cooled at a rate of 5 °C·h⁻¹ until the target temperatures were reached and were maintained for 2 h at each target temperature. LT50 was measured according to the method of Jun et al. (2012).

**Determination of physiological and biochemical parameters in the shoots.** Physiological and biochemical parameters in the shoots were determined monthly from 30 Sept. 2012 to 30 Jan. 2013. Ten representative seedlings under each treatment were selected and one healthy upper-crown shoot facing the sun on each representative seedling was selected for physiological and biochemical measurements. The water content was measured using the formula: water content = (fresh weight – dry weight)/fresh weight. The proline content was measured according to the method of Bates et al. (1973). The soluble protein content was measured according to the method of Bradford (1976). The total soluble sugar content was measured according to the method of Li (2000), and the glucose and fructose contents were measured according to the method of Liu et al. (2004).

### Statistical analysis.

All data were analyzed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL), including a one-way analysis of variance for main effects of different treatments and a correlation analysis within cold-hardiness and other physiologically and biochemical parameters. All tables and figures were produced using Microsoft Word 2007 and Microsoft Excel 2007 (Microsoft Inc., Redmond, WA), respectively.

### Results

**Shoot growth.** Both RGR and DGC values of shoot length were significantly greater under T1 and their lowest under T2 and T3 (Fig. 2A and B). As compared with the seedlings under T0, the RGR of shoot length increased by 0.0008 mm·d⁻¹ under T1 and decreased by over 0.0015 mm·d⁻¹ under T2 and T3. The shoots under T2 and T3 stopped elongating before 13 Sept., but shoot growth cessation under T0 and T1 did not occur until late September.

**Leaf senescence.** The seedlings under T0 held a steady Pn through September and then trended downward in October. The Pn value under T1 also decreased in October but rose during September. Leaf photosynthetic rates under T2 and T3 remained stable during early September and decreased drastically after mid-September (Fig. 3). The decreased rates of Pn between 1 Sept. and 31 Oct. were 0.1035, 0.0852, 0.1652, and 0.1695 μmol CO₂ per m²·s⁻¹ per day under T0, T1, T2, and T3, respectively. By 31 Oct., the Pn values were 4.70, 5.77, 1.02, and 0.73 μmol CO₂ per m²·s⁻¹ under T0, T1, T2, and T3, respectively. In addition, leaf abscission was also accelerated by T2 and T3 (data not shown).

**Bud dormancy.** D50B under each treatment continued to increase during cold acclimation (Fig. 4). Based on the D50B values, the seedlings under T2 and T3 displayed significantly deeper bud dormancy levels than the others. The increased rates of D50B during cold acclimation were 3.0, 3.2, 4.0, and 4.2 d per month under T0, T1, T2, and T3, respectively. In January, the buds reached the maximum dormancy level.
In December, the shoots contained their maximum soluble protein levels (286.96, 268.69, 416.64, and 433.91 mg·g⁻¹ DW under T0, T1, T2, and T3, respectively).

**Total soluble sugar content in the shoots.** The total soluble sugar content in the shoots under each treatment rapidly increased, reached its maximum level in December, and then decreased in January (Fig. 9). The total soluble sugar content between late Oct. 2012 and late Jan. 2013 significantly increased under T2 and T3. In December, the shoots contained their maximum soluble sugar content levels (44.10, 45.08, 53.91, and 56.00 mg·g⁻¹ DW under T0, T1, T2, and T3, respectively).

**Glucose and fructose contents in the shoots.** Two major soluble sugars, glucose and fructose, were detected during cold acclimation. Both glucose and fructose contents in the shoots under each treatment kept increasing, reached their maximum levels in December, and then decreased in January (Fig. 10A and B). The contents of these two soluble sugars between late Nov. 2012 and late Jan. 2013 significantly increased under T2 and T3. In December, the shoots contained their maximum glucose content (20.64, 18.89, 24.46, and 25.72 mg·g⁻¹ DW under T0, T1, T2, and T3, respectively) and fructose content (24.33, 22.90, 26.66, and 27.64 mg·g⁻¹ DW under T0, T1, T2, and T3, respectively) levels.

**Correlation between freezing tolerance and the water, proline, soluble protein, and soluble sugar contents.** A significant correlation was detected between freezing tolerance and the water, proline, soluble protein, total soluble sugar, glucose, and fructose contents in the shoots of *M. wufengensis* seedlings under each treatment (Table 1). The LT50 value positively correlated with water content and negatively correlated with proline, soluble protein, total soluble sugar, glucose, and fructose contents.

**Discussion**

The initiation of cold acclimation and extent of winter-hardiness can be influenced by the onset of senescence (Guy, 2003; Levitt, 1980). Vegetative growth in late fall has been shown to negatively correlate with winter-hardiness (Dhont, 2006). Our experimental results showed that the shoot growth of *M. wufengensis* was promoted by ABA treatment of 300 mg·L⁻¹ and was inhibited by treatments of 600 and 900 mg·L⁻¹ (Fig. 2A). This corroborates studies that reported that ABA could promote plant growth at a low dosage and inhibit growth at a high dosage (Kamuro, 1994, 2005). The conflicting effects of ABA on growth are linked to the interactions between ABA and other plant hormones (Hansen and Grossmann, 2000; Hoffmann-Benning and Kende, 1992; Vysotskaya et al., 2009). Additionally, ABA treatments of 600 and 900 mg·L⁻¹ stimulated the growth cessation of *M. wufengensis* seedlings by 9 to 11 d (Fig. 2B). This suggests that the seedlings under these two ABA concentrations underwent cold acclimation earlier, because growth cessation is an early physiological step in the cold acclimation process (Williams et al., 1972).
Growth cessation is followed by leaf senescence, which is another early step in the cold acclimation process (Kozlowski and Pallardy, 2002), indicating nutrient remobilization from leaves to overwintering tissues. Degradation of chlorophyll occurs in mesophyll cells during leaf senescence, which can lead to a decreased Pn value because chlorophyll is an important determinant of photosynthetic rate (Mao et al., 2007). The Pn values of *M. wufengensis* seedlings showed an ABA concentration dependence that increased under low concentration (300 mg·L⁻¹) and decreased under high concentrations (600 and 900 mg·L⁻¹) (Fig. 3). The dual role of ABA in photosynthesis has been previously reported (Li et al., 2006; Zhou et al., 2006) and may be caused by different stress factors, ABA concentrations, and treatment times (Mclaren and Smith, 1977). The link between ABA and leaf senescence has been demonstrated in *Arabidopsis thaliana* with the up-regulation of two senescence-associated mRNA, pSEN4 and pSENS, after exogenous ABA application (Park et al., 1998). Furthermore, this observation might be an early sign that the seedlings treated with ABA application of 600 to 900 mg·L⁻¹ had become more cold-hardy than the others, because cold-hardy species usually begin leaf senescence earlier than cold-sensitive species (Ma et al., 2010).

Bud dormancy in woody species is categorized into three types based on the inhibitory sources: paradormancy (inhibition from distal organs), endodormancy (inhibition from internal bud signals), and ecodormancy (inhibition from unfavorable environmental conditions) (Lang et al., 1987). Buds initially experience endodormancy and then ecodormancy during cold acclimation after the chilling requirement has been satisfied. It can be concluded that ABA application of 600 and
900 mg·L⁻¹ played a role in inducing the endodormancy of M. wufengensis (Fig. 4), because single cuttings (not paradormant) were used in favorable growing environments (not ecodormant) (Zhang et al., 2011). Increased endogenous ABA contents have been suggested to be involved in controlling dormancy (Alvim et al., 1976; Kucera et al., 2005). Thus, we speculate that exogenous ABA application may increase endogenous ABA level in M. wufengensis seedlings during cold acclimation.

Similar to other woody perennials, the freezing tolerance of M. wufengensis was strengthened during cold acclimation (Lenahan et al., 2010; Lim and Arora, 1998). However, the seedlings treated with ABA application of 600 and 900 mg·L⁻¹ showed more cold-hardiness than the others during cold acclimation (Fig. 5). In addition, relatively more shoots of the seedlings without ABA application were damaged in January (visual observation), indicating the effectiveness of ABA on increasing the freezing tolerance of M. wufengensis as well. Exogenous ABA application has previously been shown to increase freezing tolerance in many plants, but this is the first report demonstrating that exogenous ABA application increased the freezing tolerance of Magnolia plants.

Increased freezing tolerance is commonly connected with reduced water content (Guy, 2003), which was well exhibited in our study. We detected that there was a significant relationship between water content and freezing tolerance (Table 1) in M. wufengensis seedlings. Plant cells are usually dehydrated under the extracellular freezing process, and dehydration-induced freezing tolerance has been well illustrated (Pearce, 2001). Thus, the observed differences in water content among treatments (Fig. 6) may explain, at least partly, the superior freezing tolerance under ABA application of 600 and 900 mg·L⁻¹. It has been demonstrated in grapevine that water stress in late fall can help plants acquire early cold acclimation and dormancy, which may also be the consequence of the induced ABA synthesis caused by water stress (Keller, 2010). Furthermore, an ABA-responsive protein kinase was involved with dehydation and cold acclimation in Triticum aestivum L. (Holappa and Walkersimmons, 1995).

Our study also confirmed a significant correlation between freezing tolerance and proline content in M. wufengensis seedlings (Table 1). As an amphiphilic molecule, proline can bind to hydrophobic surfaces using its hydrophobic moieties, thus converting them to hydrophilic ones. Such conversions enable the cell to preserve the structural integrity of cytoplasmic proteins under the dehydration conditions that develop under drought, salinity, and frost stresses (Papageorgiou and Murata, 1995). Moreover, there is evidence that proline functions as a free radical scavenger (Matysik et al., 2002) and accumulate during cold acclimation and/or freezing stress in plants such as Prunus persica (Wisniewski et al., 1999), Arabidopsis thaliana (Nylander et al., 2001), and Rhododendron (Marjan et al., 2003).

Soluble sugar can influence freezing tolerance by facilitating the deep supercooling of Magnolia.
plant tissues (Kasuga et al., 2007), decreasing the freezing point of intracellular water (Morin et al., 2007), and preventing membrane and macromolecule injuries from freeze-induced dehydration (Krasensky and Jonak, 2012; Shao et al., 2006). Exogenous ABA application can induce sugar accumulation in grape (Koussa et al., 1998), wheat (Kerepesi et al., 2004), and gentian (Suzuki et al., 2006). A significant correlation between freezing tolerance and total soluble sugar content was observed in *M. wufengensis* (Table 1), suggesting that the improved freezing tolerance in the *M. wufengensis* seedlings treated with ABA application of 600 and 900 mg·L⁻¹ was the result of soluble sugar accumulation (Fig. 9).

Among the major soluble sugars, glucose, fructose, and sucrose can act in water replacement to maintain membrane phospholipids in the liquid–crystalline phase and to prevent structural changes in soluble proteins when cells suffer from a frost-induced water deficit (Bravo et al., 1998). Glucose and fructose contents in *M. wufengensis* seedlings highly correlated with LT50 values (Table 1), indicating a putative role for these two soluble sugars in the freezing tolerance of *M. wufengensis*. Indeed, the accumulation patterns of glucose and fructose have frequently been shown to correlate with freezing tolerance in herbaceous plants (Gusta et al., 2004; Guy et al., 1992), whereas in woody plants, the majority of studies has found no implication of hexoses in cold acclimation (Cox and Stushnoff, 2001; Kasuga et al., 2007). Hence, the alterations in glucose and fructose concentrations in the shoots of *M. wufengensis* under ABA application (Fig. 10A and B) are noticeable.

In conclusion, an optimum foliar application concentration of ABA between 600 and 900 mg·L⁻¹ initiated a cascade of steps for advancing the cold acclimation process of *M. wufengensis*. An improved freezing tolerance under ABA application was associated with dehydration and the accumulation of proline, soluble protein, and certain soluble sugars such as glucose and fructose. We suggest that foliar ABA application may be used on *M. wufengensis* grown in northern China where there are short growing seasons and early fall frost events.

### Table 1. Correlation coefficients between freezing tolerance (estimated as LT50) and the water, proline, soluble protein, soluble sugar, glucose, and fructose contents in the shoots of *Magnolia wufengensis* under abscisic acid (ABA) treatments.

| Variable          | T0  | T1  | T2  | T3  |
|-------------------|-----|-----|-----|-----|
| Water             | 0.98** | 0.96** | 0.99** | 0.98** |
| Proline           | −0.80* | −0.78* | −0.91** | −0.89** |
| Soluble protein   | −0.91** | −0.87** | −0.95** | −0.92** |
| Soluble sugar     | −0.89** | −0.86** | −0.89** | −0.89** |
| Glucose           | −0.78* | −0.81* | −0.88** | −0.90** |
| Fructose          | −0.94** | −0.92** | −0.86** | −0.86** |

*T0, T1, T2, and T3 indicate ABA application of 0, 300, 600, and 900 mg·L⁻¹, respectively. * and ** indicate significant at P < 0.05 and at P < 0.01, respectively.

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![Fig. 9. Dynamics of total soluble sugar content in the shoots of *Magnolia wufengensis* under abscisic acid (ABA) treatments. T0, T1, T2, and T3 indicate ABA application of 0, 300, 600, and 900 mg·L⁻¹, respectively. Data are presented as means ± sds (n = 10).](image)

![Fig. 10. (A) Dynamics of glucose content in the shoots of *Magnolia wufengensis* under abscisic acid (ABA) treatments. (B) Dynamics of fructose content in the shoots of *Magnolia wufengensis* under ABA treatments. T0, T1, T2, and T3 indicate ABA application of 0, 300, 600, and 900 mg·L⁻¹, respectively. Data are presented as means ± sds (n = 10).](image)
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