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Research Article

Improvement of *ex vitro* acclimatization of mulberry plantlets by supplement of abscisic acid to the last subculture medium

Yoon Sun Huh · Joung Kwan Lee · Sang Young Nam

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Abstract Mulberry (*Morus* sp.) of the family Moraceae is very economically important in Asian countries including Korea, because its leaf and fruit have been commercially used in sericulture and horticultural industries. Therefore it is necessary to develop the optimal production system for rapid and cost-effective propagation of mulberry. Our studies focused on establishing an acclimatization method for the successful plantlet production of new cultivar 'Cheongsu' which was transferred *ex vitro* after *in vitro* culture. In particular, effect of abscisic acid (ABA) addition into the last subculture medium on plantlet response to subsequent *ex vitro* transfer and its growth was investigated. During acclimatization, stomatal conductance and transpiration rate of ABA-pretreated plantlets were significantly lower than those of non-treated plantlets. Net photosynthetic rate of ABA-pretreated plantlets decreased after *ex vitro* transfer but increased after 14 days, and it was mostly higher than that of non-treated plantlets. Moreover, relative water content as well as chlorophyll contents and its ratio were also higher in ABA-pretreated plantlets. On the other hand, proline was considerably higher than in control plantlets. After 1 month of *ex vitro* transfer, survival rate of ABA-pretreated plantlets was 85.6%, which increased by 29.1% in comparison with control (56.5%). More vigorous growth was also observed in ABA-pretreated plantlets. From these results, it was found that application of ABA to the last subculture medium could improve acclimatization and promote survival of mulberry plantlets after *ex vitro* transfer, inducing water stress tolerance and alleviating abiotic stresses.

Keywords Mulberry, Abscisic acid, *Ex vitro* transfer, Acclimatization, Water stress

Y. S. Huh (✉) · J. K. Lee · S. Y. Nam
Horticultural Research Division, Chungcheongbuk-do Agricultural Research and Extension Services, Cheongju, 28130, Korea
e-mail: yshuh2@korea.kr

Introduction

Mulberry (*Morus* sp.) is an economically important tree grown in India, China, Korea and several Asian countries where its foliage is used as food for silkworms in sericulture industry (Vijayan et al. 2012). It is also commercially valuable in the horticulture, food and cosmetic industries, in particular, its phytochemical and medicinal properties such as antioxidants (Yen et al. 1996) and hypoglycemia compounds (Kelkar et al. 1996) have been widely used as healthcare products. These days it is cultivated for fruit production, which is used for human consumption including jam, jelly, frozen desserts, pulp, juice and wine (Koyuncu 2004). Mulberry fruit is regarded as a traditional medicine for dysentery, constipation and avulsed teeth due to rich phenolic acids and flavonoids (Arfan et al. 2012; Lee and Bae 2011).

Plant micropropagation has been widely applied to agriculture industry for large scale production of economically important and valuable species. But its commercial use is limited in many species due to the low plantlet survival rates during the acclimatization (Pospisilova et al. 2009a, b), which are known to be related to an abnormal morphology, anatomy and physiology under *in vitro* culture conditions (Dias et al. 2013a). Poor functioning of overall water housekeeping system such as deficient stomatal control and cuticular abnormalities, which is involved in the drastically different vapor pressure between *in vitro* and *ex vitro* conditions, can induce the water deficit and plant dehydration during *ex vitro* transfer and acclimatization. Higher irradiance can also increase the harmful stress and restrict a plantlet growth, resulting in the photoinhibition or generation of reactive oxygen species (ROS). Therefore, the regulation of transpiration and stabilization of water status as well as adequate operation of photosynthetic mechanism are very important for plant survival and its further growth during an adaptation period to the new growing environment (Desjardins et al. 2009; Pospisilova et al. 2009b).

Abscisic acid (ABA) plays a critical role in many physiological processes of plants including water balance and in the adaptation of plants to stress environments (Finkelstein and Gibson 2002; Hetherington 2001). It is transported via xylem to the shoot, where it regulates transpiration loss of water and leaf growth (Hronkova et al. 2003). Various stresses induce ABA synthesis and it is considered as a plant stress hormone (Tuteja 2007). Role of abscisic acid on tolerance to abiotic stresses has also been reported when tissue cultured plantlets are transferred directly to the field (Aguilar et al. 2000). It can act as an anti-transpirant during the acclimatization of tissue-cultured plantlets and reduce the relative water loss of the leaves of micropropagated plantlets during transplantation even when non-functional stomata are present (Pospisilova et al. 1999, 2007). Several studies reported that an exogenous ABA addition into the culture medium could reduce the programmed cell death, known as apoptosis, in cultured cells and tissues (Carimi et al. 2003; Pennell and Lamb 1997; Wang et al. 1999). Therefore acclimatization can also be improved by the positive effect of ABA on chlorophyll contents and other photosynthetic parameters as well as on plant growth.

In these studies, we tried to investigate the effect of an exogenous ABA addition to the last culture medium on photosynthetic parameters, proline content and survival of mulberry plantlets during acclimatization after *ex vitro* transfer, and furthermore, to propose the importance of ABA treatment prior to transplanting for large scale propagation.

Materials and Methods

Plants and cultivation

Mulberry plantlets (*Morus alba* L. cv Cheongsu) were cultured in 450 ml glass culture vessels (Φ80 x h131 mm) under 23 ± 1°C and 16h photoperiod (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity). At the last subculture stage, 10 uM ABA was added into the medium. Control plants were cultured on the same medium without ABA. After 4 weeks of last subculture, mulberry plantlets (6 cm high) were taken out of the culture vessels and washed several times with distilled water to remove traces of medium on plantlet surfaces. Then, they were transferred to pots with a mixture of common horticultural substrates and perlite (1:1), and placed in the acclimatization room, which minimum and maximum air temperatures were kept between 16 and 28°C, and relative humidity was gradually decreased from 90 to 60%. After acclimatization and hardening phase for 1 month, the general plant growth characteristics including shoot number, length and survival rate were measured. This experiment was designed randomly. Each treatment had ten replicates and was conducted three times.

Determination of relative water content and photosynthetic parameters

Leaves were sampled from mulberry plantlets before *ex vitro* transfer as well as 1, 2, 3 and 4 hours after *ex vitro* transfer for calculating their relative water content (RWC). RWC was measured gravimetrically in leaf discs (about 0.5 cm^2) which were water-saturated by immersing into holes of fully wet polyurethane foam under dark according to Catsky (1960). It was calculated form gradual decrease in fresh mass of initially water-saturated leaves and dry mass.

Net photosynthesis (P_N), transpiration rate (Tr), and stomatal conductance (C_s) were measured before *ex vitro* transfer as well as 7, 14, 21 and 28 days after *ex vitro* transfer using a portable open photosynthesis system (LI-6400, LI-COR, USA). All measurements were taken with leaf temperature maintained at 20°C, relative humidity between 50% and 65%, and a leaf to air vapor pressure deficit (VPD) of 0.8 ~ 1.2 kPa. CO_2 concentration within the chamber was maintained at 370 $\mu mol \cdot mol^{-1}$, and light intensity was maintained at 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Measurement of leaf chlorophyll content

Leaves were sampled from mulberry plantlets before *ex vitro* transfer and at 7, 14, 21, 28 days after *ex vitro* transfer. Leaf discs (about 0.8 cm in diameter) were weighted and incubated with 3 ml dimethyl sulfoxide (DMSO) in a test tube at 65°C until the tissue became colorless. The absorbance at 664.9 and 648.2 nm of the DMSO extract was determined with a spectrophotometer (SoftMax Pro, Molecular Device Co., USA), and the chlorophyll a and b concentrations of the leaves were calculated according to Barnes et al. (1992) method.

Proline assay

Leaves were sampled from mulberry plantlets before *ex vitro* transfer and at 7, 14, 21, 28 days after *ex vitro* transfer for proline analysis. Proline content was measured according to methodology described by Bates et al. (1973). 0.2 g of fresh leaf samples were homogenized in 3 mL of 3% aqueous sulphosalicylic acid and the residue was removed by centrifugation at 12,000 g for 10 min. 2 mL of the homogenized

supernatant was reacted with 1 mL acid-ninhydrin and 1 mL of glacial acetic acid for 1 hour at 100°C, and this reaction was terminated in an ice bath. The reaction mixture was extracted with 2 mL toluene, mixed vigorously and left at room temperature for 30 min until separation of the two phases. The chromophore-containing toluene (1 mL, upper phase) was warmed to room temperature and its optical density was measured at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve using L-Proline and calculated as umol·g^{-1} FW.

Statistics

All analytical experiments were repeated twice. In each experiment a set of 20 plants were used for determination of each parameter. Means and standard error (SE) were calculated using SigmaStat (Windows, version 3.1.).

Results and Discussion

Effect of ABA on relative water content and photosynthetic parameters during acclimatization of *in vitro* cultured mulberry plantlets

Figure 1 showed the relative water content and photosynthetic parameters of mulberry plantlets pretreated with or without ABA during acclimatization after *ex vitro* transfer. Relative water content (RWC) was respectively measured in ABA-treated and control plantlets taken out of culture vessels before and after *ex vitro* transfer. It decreased more slightly in ABA-treated plantlets in comparison with non-treated plantlets, which means that ABA-treated plantlets might be much less dehydrated right after *ex vitro* transfer. On the contrary, control plantlets showed a steeper reduction of RWC after *ex vitro* transfer, indicating that these plants might be under a severe water stress conditions. ABA-treated plantlets showed lower stomatal conductance (Cs) and transpiration rate (Tr) than control plantlets, these reduction of Cs and Tr values means that ABA-pretreated plants exhibited a better water status. But Cs and Tr increased rapidly until 14 days after *ex vitro* transfer and decreased gradually afterward in control plantlets. Net photosynthetic rate (Pn) of ABA-treated plantlets was rather higher, and its drastic reduction was not observed. On the other hand, in control plantlet, it fell down considerably until 14 days after *ex vitro* transfer and increased since then. From these results, ABA pretreatment might reduce Cs and Tr, induce higher Pn, and strengthen adaptation capacity of plantlets after *ex vitro* transfer.

During acclimatization, plantlets are subjected to various stresses in response to changes in *ex vitro* environmental conditions, due to impaired stomata function and reduced

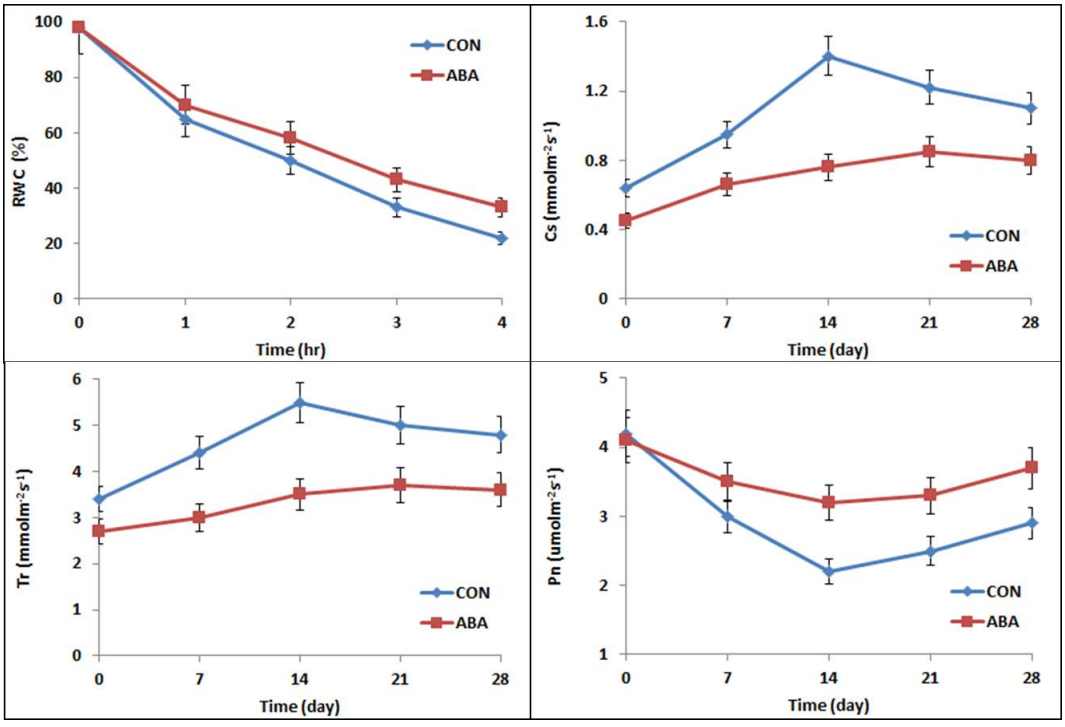

Fig. 1 Relative water content (RWC), stomatal conductance (Cs), transpiration rate (Tr) and net photosynthetic rate (Pn) of mulberry plantlets treated with or without ABA during the last subculture stage before *ex vitro* acclimatization. Each parameters were measured before and after *ex vitro* transfer. Means ± SE, n = 20

cuticle deposition on cells, sudden dehydration and increase in ROS production (Batkova et al. 2008). *In vitro* cultured plantlets are characterized by deficient functionality of stomata, which is gradually recovered during *ex vitro* acclimatization (Dias et al. 2013a, b; Hazarika 2006; Pospisilova et al. 2009b). However, ABA application strongly reduced water loss even when stomata did not function normally (Pospisilova et al. 2007), which decreased water stress during acclimatization of *Ulmus minor*, indicating a faster and efficient improvement of the water control mechanisms. Stomatal closure is considered as one of the first defense strategy against drought damage under water stress conditions, protecting the plants from dehydration and eventually from sudden death (Chaves et al. 2003; Dias and Bruggemann 2007). But stomatal closure unavoidably limits the CO_2 availability in the intercellular spaces of the mesophyll cells. Therefore, it is very critical to find the adequate acclimatization condition to prevent water loss and enhance photosynthesis efficiency for improvement of plant ability to deal with *ex vitro* environmental stresses. Dias et al. (2014) reported that foliar application of ABA promoted net CO_2 assimilation rate, plant dry mater accumulation and antioxidant enzyme activity. Several studies also demonstrated that ABA pre-treatment ameliorated negative effect of water stress in naturally grown barley, bean, maize, sugar beet and tobacco (Agarwal et al. 2005; Haisel et al. 2006; Mizrahi et al. 1974).

In our results, it was found that ABA could efficiently play a role in alleviating the negative shock from *ex vitro* transplantation, reducing the water loss and relieving the risk of dehydration for successful survival and vigorous growth of mulberry plantlets during acclimatization.

Effect of ABA on chlorophyll contents and its ratio during acclimatization of *in vitro* cultured mulberry plantlets

Figure 2 showed the chlorophyll contents and chlorophyll a/b ratio of mulberry plantlets pretreated with or without ABA during *ex vitro* acclimatization. Significant and rapid increase of chlorophyll a content was observed in ABA-treated plantlets, of which chlorophyll a+b content was also higher than that of control plantlets. Chlorophyll a/b ratio rose considerably until 7 days after *ex vitro* transfer, but did not increase remarkably afterward.

Chlorophyll content is one of the most important parameters for evaluation of the plant hardening after acclimatization (Gour et al. 2007). It could be higher or lower in leaves of *in vitro* cultured plantlets than in corresponding *ex vitro* plantlets, depending on irradiance, medium composition and CO_2 concentration. Exogenous ABA application increased chlorophyll a and chlorophyll b levels in the tomato leaf tissue (Barickman et al. 2014). Chlorophyll a+b were higher in ABA-treated plants during *ex vitro* transfer of micro-

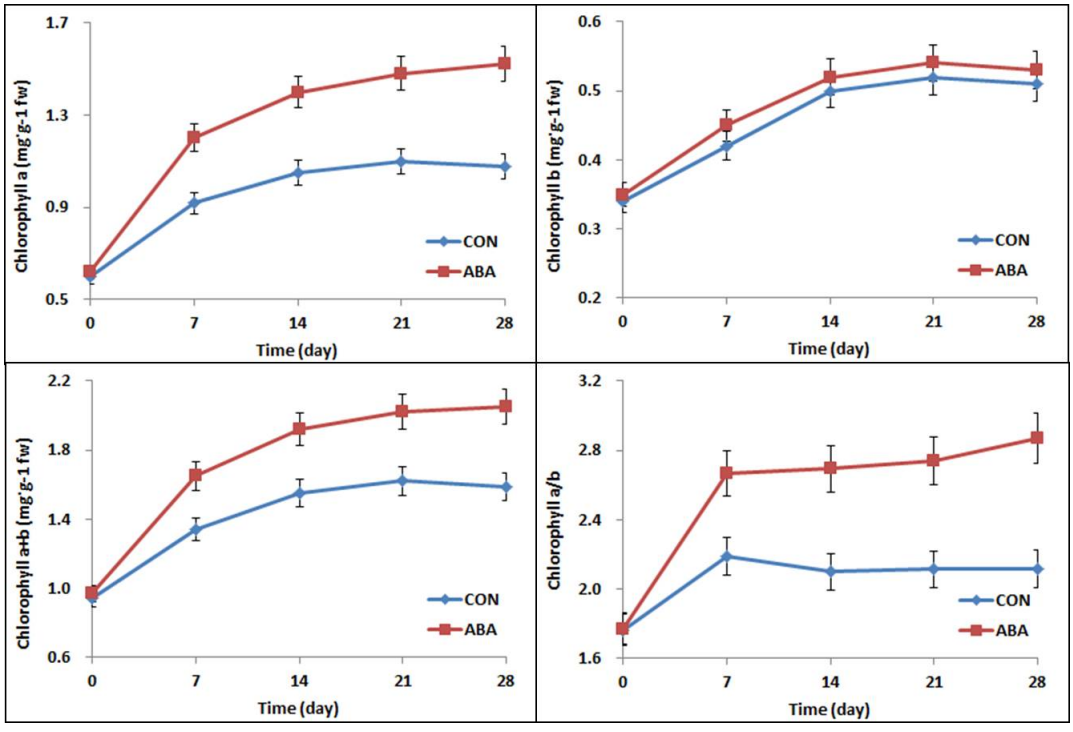

Fig. 2 Chlorophyll contents and chlorophyll a/b ratio of mulberry plantlets treated with or without ABA during the last subculture stage before *ex vitro* acclimatization. Each parameters were measured before *ex vitro* transfer and after *ex vitro* transfer. Means ± SE, n = 20

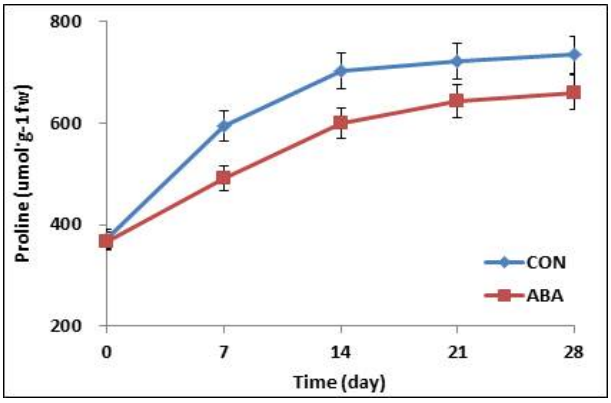

Fig. 3 Proline content of mulberry plantlets treated with or without ABA during the last subculture stage before *ex vitro* acclimatization. Each parameters were measured before *ex vitro* transfer and after *ex vitro* transfer. Means ± SE, n = 20

propagated tobacco plantlets (Pospisilova et al. 2009a). Higher chlorophyll a+b content was also observed when ABA was applied immediately after *ex vitro* transfer (Pospisilova et al. 2009a). Chlorophyll a+b content in ABA-treated plants increased not only in persistent leaves but also new leaves developed at 2 weeks after *ex vitro* transfer (Pospisilova et al. 1998). In particular, increase in chlorophyll a/b ratio induced by ABA treatment may lead to decrease in light-harvesting complex associated with photosystem II, which can help in its photoprotection (Spundova et al. 2003). Chlorophylls in leaf tissue, induced by ABA treatments, can increase the antioxidant capacity of plants to abiotic-induced stress (Barickman et al. 2014). Our studies also indicated that ABA application led to increase of chlorophyll contents and its ratio, which could assign high adaptability to plantlets under unfavorable *ex vitro* environmental condtions.

Effect of ABA on proline content during acclimatization of *in vitro* cultured mulberry plantlets

Higher proline content was observed in control plantlets, which increased rapidly until 14 days after *ex vitro* transfer (Fig. 3). It was supposed that reduction of water stress induced by ABA pretreatment could alleviate the risk of plant dehydration and promote plant survival, resulting in decrease in proline accumulation. In addition, it was found that proline could be an indicator showing plant stress levels after *ex vitro* transfer, and its rapid accumulation also meant a severe water stress conditions of plants during acclimatization.

In acclimatization, several metabolic changes can be activated in an attempt to neutralize the damages caused by hydric stress including the accumulation of different compatible solutes (Hoekstra et al. 2001; Mohammadkhani and Heidari 2008). Proline is considered as the primary accumulated metabolite in different stress conditions, which contributes to maintain the water absorptive capacity and improve the drought tolerance for plant survival and establishment (Kishor et al. 1995; Liang et al. 2013; Molinari et al. 2007). Proline accumulation may occur due to the physiological responses induced by osmotic stress in plants (Zhang et al. 2000). The increase in proline synthesis in transgenic tobacco (*Nicotiana tabacum*) overproducing proline by elimination of feedback inhibition of P5CS, which catalyzes the proline biosynthesis, caused higher tolerance to its hyperosmotic stress (Hong et al. 2000; Kishor et al. 1995). Carvalho et al. (2013) also demonstrated that proline could modify the expression of genes related to the plant responses to water deficit.

Effect of ABA on general growth characteristics after 1 month of acclimatization of *in vitro* cultured mulberry plantlets

General growth characteristics of mulberry plantlets was evaluated on 1 month after *ex vitro* transfer (Table 1), the survival rate of ABA-pretreated plantlets was 85.6%, which increased by 29.1% in comparison with non-treatment (56.5%), moreover, ABA-pretreated plantlets grew more vigorously. After 6 months, most plantlets also survived successfully (Fig. 4).

Our results showed that application of ABA to the last subculture medium might improve the adaptable capacity of mulberry plantlets to *ex vitro* environment during acclima-

Table 1 The general growth characteristics of mulberry plantlets pretreated with or without ABA on 1 month after *ex vitro* acclimatization

Treatment	Plant height (cm)	Stem diameter (mm)	Survival rate (%)	Lateral branch		
				No. (/plant)	Length (cm)	Diameter (mm)
Control	9.3±0.5[z]	3.3±0.1	56.5±3.0	2.6±0.2	3.2±0.1	2.1±0.1
ABA-treatment	10.5±0.6	3.4±0.1	85.6±3.6	3.6±0.2	3.5±0.2	2.1±0.1

[z]Each value represents the mean±SE.

Fig. 4 Mulberry plants on 1 (left) and 6 (right) months after acclimatization and *ex vitro* transfer. They were pretreated with ABA during the last subculture stage before acclimatization

tization and promote more vigorous plantlet growth, due to its positive effects including the induction of stronger drought tolerance and better photoprotection.

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