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Trehalose Changes Hydraulic Conductance of Tissue-cultured Soybean Embryos

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Abstract

The hydraulic properties of tissue-cultured soybean (*Glycine max* [L.] Merr.) embryos grown on trehalose-containing culture media were investigated. Water potentials of culture media ranged from -0.13 to -0.65 MPa when sucrose and/or trehalose concentrations were altered. The wall extensibility was greatly larger than hydraulic conductance, and the size of the effective turgor was much smaller than that of the growth-induced water potential, indicating that hydraulic conductance and the growth-induced water potential were predominantly regulating growth of soybean embryos under tissue culture conditions. The hydraulic conductance of soybean embryos grown on trehalose-containing media became smaller than that in sucrose-containing media. When both sucrose and trehalose were added to the culture media, the hydraulic conductance became an intermediate between only sucrose-containing and only trehalose-containing treatments. It is suggested that trehalose might reduce hydraulic conductance in soybean embryos, resulting in growth retardation of soybean embryos.

1. Introduction

The non-reducing disaccharide trehalose, *i.e.*, α-D-glucopyranosyl-[1-1]-α-D-glucopyranoside, is known to enhance tolerance against desiccation and high temperature probably by protecting membranes and enzymes from various stresses (Crowe *et al.*, 1984; Lee *et al.*, 1989; Hottinger *et al.*, 1994). The tobacco plants which were introduced the gene encoding the trehalose-6-phosphate synthase subunit accumulated trehalose under drought stress condition (Holmström *et al.*, 1996; Goddijn *et al.*, 1997; Romero *et al.*, 1997). Holmström *et al.* (1996) suggested that stabilization of cellular structures and macromolecules by trehalose may underlie both the improved water retention and enhanced desiccation tolerance. Thus, it seems water loss from cells might be inhibited by changes in hydraulic properties for the water relations of tobacco plants.

On the contrary, Veluthambi *et al.* (1982) claim that trehalose may be toxic for plant tissues when it is supplied exogenously. Thus, growth of tissue-cultured soybean stems may be inhibited by the addition of trehalose in tissue-culture medium. It was found that cell wall synthesis was inhibited upon trehalose feeding in *Cuscuta reflexa* Roxb. cells (Veluthambi *et al.*, 1982), and growth of the shoot tip of the species was completely suppressed *in vitro* (Veluthambi *et al.*, 1981). Our previous work (Ikeda *et al.*, 1999a) found that growth of soybean stems was inhibited under osmotic and temperature stresses and by the addition of plant growth regulators in tissue culture condition, and that the stem elongation rate was primarily regulated by hydraulic properties. Thus, in the present work, effects of trehalose on hydraulic conductance and wall extensibility were investigated in tissue-cultured soybean embryos.

Growth of plants is attributed to cell elongation which is driven by water absorption by cell and cell wall extension (Lockhart, 1965a, b). Water absorption to the cell is governed by the growth-induced water potential (the difference of water potential between the water source and the elongating cells) (Molz and Boyer, 1978; Nonami and Boyer, 1987, 1993; Nonami *et al.*, 1997; Ikeda *et al.*, 1999a, b). The relationship between relative growth rate (G; unit: s^{-1}) and the size of the growth-induced water

potential ($\Delta \Psi_G$; unit: MPa) can be modeled by a simple linear relation as follows;

$$G = L(\Delta \Psi_G) = L(\Psi_O - \Psi_W) \quad (1)$$

where L (s^{-1} MPa^{-1}) is a hydraulic conductance associated with a growth process, and Ψ_O and Ψ_W are water potential of the water source and that of expanding cells, respectively.

In a cell elongation process, cell wall must be extended towards the outside by cell turgor. The equation considers turgor (Ψ_P; unit: MPa) to bring about steady enlargement (expressed as the relative growth rate) according to the extensibility of the wall (m; unit: s^{-1} MPa^{-1}) as given by Green *et al.* (Green *et al.*, 1971; Ray *et al.*, 1972);

$$G = m(\Psi_P - Y) \quad (2)$$

where Y is the yield threshold turgor below which the force on the wall is too small to enlarge the wall irreversibly. Thus, (Ψ_P-Y) is the growth-effective turgor. When G is plotted as a function of Ψ_P, the slope of the line is m and, Y is the intercept of the Ψ_P axis when G = 0.

Eqns. 1 and 2 can be combined by applying the relation of $\Psi_W = \Psi_S + \Psi_P$ (Ψ_S; osmotic potential), because plant cells elongate due to simultaneous water uptake and wall extension. Hence,

$$G = \frac{mL}{m+L}(\Psi_O - \Psi_S - Y) \quad (3)$$

The Eqn. 3 is known as Lockhart's combined equation developed from a theory of cell enlargement (Lockhart, 1965a, b), and is re-interpreted in the tissue growth in the zone of elongation (Boyer, 1985; Nonami and Boyer, 1990).

Simultaneous determinations of all parameters in Eqn. 3 are not experimentally easy, and therefore, Eqn. 3 can be re-arranged as follows;

$$\frac{G}{L} + \frac{G}{m} = (\Psi_O - \Psi_W) + (\Psi_P - Y) \quad (4)$$

Equation 4 is shown as algebraic summation of Eqns. 1 and 2. In order to study which component contributes more predominantly to growth of tissue-cultured plants, parameters of (Ψ_O-Ψ_W) and (Ψ_P-Y) were linearly separated and measured when G was altered under various environmental stress conditions. Because Ψ_W and Ψ_P in the zone of elongation and Ψ_O can be measured in the same tissue by using psychrometers, G could be plotted against (Ψ_O-Ψ_W) and Ψ_P. By assuming that Y was not altered signifi cantly when G was changed, Y was determined at G=0 from the plot of G against Ψ_P. Although the valid domain of Eqn. 4 may not be completely overlapping with Eqns. 1 and 2, we assume that both water potential gradient and cell turgor are contributing growth simultaneously, and thus, $\Psi_O \geq \Psi_W$ and $\Psi_P \geq Y$ under all conditions. This also means L > 0 and m > 0. Because L and m are denominators in Eqn. 4, $L \neq 0$ and $m \neq 0$.

2. Materials and Methods

2.1. Plant Materials

Embryos were taken out from soybeans (*Glycine max* [L.] Merr. cv Fuki; Takii & Company, Ltd., Kyoto, Japan) under sterile conditions after the seeds were imbibed for 6 hours in running tap water and disinfected in 1% ($v \cdot v^{-1}$) NaOCl solution for 10 min, and transplanted on media in individual vessels.

First, in order to investigate effects of sucrose concentrations on soybean stem growth, the concentration of MS (Murashige and Skoog, 1962) was kept at 50 % of MS which is the optimum concentration (Ikeda *et al.*, 1999a), *i.e.*, the composition and concentrations were as follows; 10.3 mM NH_4NO_3; 9.4 mM KNO_3; 0.63 mM KH_2PO_4; 1.5 mM $CaCl_2$; 0.75 mM $MgSO_4$; 0.05 mM H_3BO_3; 0.05 mM Na_2SO_4; 0.05 mM $FeSO_4$; 0.05 mM $C_{10}H_{14}N_2O_8Na_2$; 0.28 mM $C_6H_6(OH)_6$; 46.0 μM $MnSO_4$; 15.0 μM $ZnSO_4$; 13.5 μM H_2NCH_2COOH; 2.5 μM KI; 0.5 μM Na_2MoO_4; 1.5 μM N:CHC (COOH):CHCH:CH; 1.00 μM $C_8H_{11}NO_3HCl$; 0.6 μM $C_{12}H_{17}N_4OSClHCl$; 0.05 μM $CoCl_2$; 0.05 μM $CuSO_4$. The concentration of sucrose ranged from 0 to 60 g l^{-1} in agar-solidified MS culture media (*i.e.*, -0.13 to -0.65 MPa of water potential). Agar (Wako Pure Chemical Ind. Ltd., Japan) at 8 g l^{-1} was added to each medium prior to autoclaving. Ten m*l* of culture solution was dispensed in a tissue culture tube (22 mm the inner diameter and 100 mm height, TEST-F 25-100, Iwaki Glass Co. Ltd., Chiba, Japan). The media were then autoclaved for 15 min at 103 kPa and 121 ℃.

The soybean embryos were grown at 25 ± 1 ℃ in the dark. About 100-200 plantlets were cultured for each treatment.

Second, soybean embryos were also cultured on agar-solidified MS media containing trehalose (Hayashibara Biochemical Labs., Inc., Shimoishii, Okayama, 700-0907 Japan). The concentration of trehalose ranged from 0 to 75 g l^{-1} in agar solidified MS media (*i.e.*, -0.13 to -0.64 MPa of water potential). The culture condition was the same as the sucrose experiment.

Additionally, both sucrose and trehalose were included in agar-solidified MS media as follows;

30 g l^{-1} sucrose and 0 g l^{-1} trehalose, 20 g l^{-1} sucrose and 10 g l^{-1} trehalose, 15 g l^{-1} sucrose and 15 g l^{-1} trehalose, 10 g l^{-1} sucrose and 20 g l^{-1} trehalose, 0 g l^{-1} sucrose and 30 g l^{-1} trehalose. Because sucrose and trehalose have the same molecular weight, the water potential of the media with the above chemical compositions should be similar. The culture medium with 50 % of MS salt and 30 g l^{-1} sucrose contains 0.135 osmoles of solutes, and thus, osmotic pressure should be 0.34 MPa, which was calculated with van't Hoff's equation. When the water potential of that medium was measured with the isopiestic psychrometer, it was -0.35 MPa. The slight difference between the calculated value and the measured one was due to the matric potential of agar (-0.01 MPa).

Soybean samples for the water status measurements were taken from 6-day-old plants. Growth of plants in tissue culture vessels was measured with a ruler. Growth rates were determined from changes in stem length as a function of time. The relative growth rate was calculated by dividing the growth rate by the length of the zone of elongation as described by Ikeda *et al.* (1999a).

2.2. Water status measurements with isopiestic psychrometers

The water status of plant tissues and culture media was determined by using the isopiestic psychrometer (Boyer, 1995). The elongation region (about 5 mm) and mature region were placed on the bottom of the psychrometer sampling chamber. Prior to the sampling, thermocouple chambers were coated with melted and resolidified petrolatum (Boyer, 1995). The chambers were loaded with plant tissues immediately after their excision. After water potential measurements, the osmotic potential of tissue were determined on the same tissues by freezing at -30 ℃ and thawing (Ehlig, 1962). Turgor was calculated by subtracting the osmotic potential from the water potential (Nonami *et al.*, 1987). Water status measurements were duplicated 5-10 times for each treatment.

3. Results

3.1. The water status of soybean stems under various sucrose concentrations

The growth-induced water potential (**Fig. 1C**) was determined by differences between the water potential of the elongation zone and the mature zone of soybean stems (**Fig. 1A**). The growth-induced water potential was the largest at -0.35 MPa of water potential (i.e., 30 g l^{-1} sucrose) in culture medium (**Fig. 1C**), and the growth rate became also

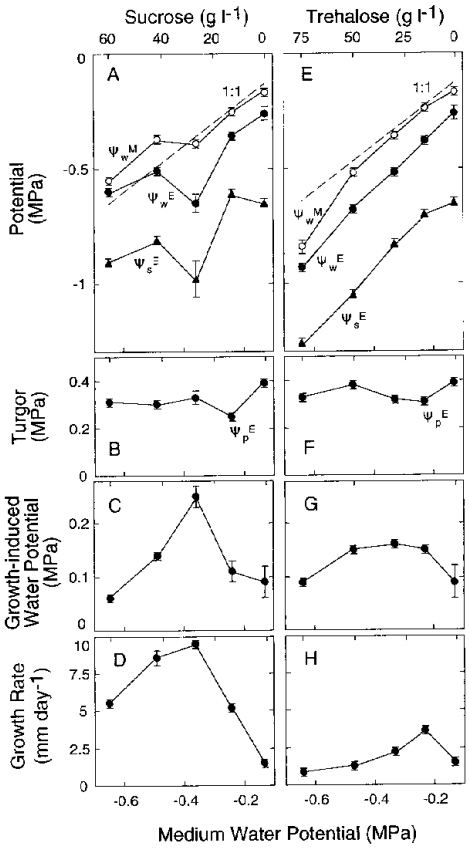

Fig. 1. Water potentials of the mature zone (Ψ_W^M ; ○) and elongation zone (Ψ_W^E ; ●), and osmotic potentials of the elongation zone (Ψ_S^E ; ▲) (A, E), turgor of the zone of elongation (Ψ_P^E) (B, F), the growth-induced water potential (C, G) and growth rates (D, H) when soybean embryos were grown on tissue culture media having different water potentials on sucrose (A-D) and trehalose (E-H) treatments. The growth-induced water potential was calculated by subtracting the water potential of the elongation region from that of the mature region. Slanting dotted lines with 1:1 in A and E indicate equipotential lines of the medium water potential. Vertical bars indicate the standard errors. The upper x-axis indicates concentrations of sucrose (A-D) and trehalose (E-H) in culture media.

the highest (**Fig. 1D**). However, turgor of the zone of elongation (**Fig. 1B**) did not proportionally correspond to the size of the growth rate (**Fig. 1D**).

3.2. Response to trehalose in tissue-cultured soybean embryos.

When no sugar was supplied to culture media, growth of soybean embryos was reduced to 1.48 mm day^{-1} (**Fig. 1D** and **H**). When 15 g l^{-1} trehalose was added to culture media, the growth rate was slightly increased, indicating that trehalose was used as the source of carbon for growth of soybean

embryos. However, when trehalose concentration was increased from 15 g l^{-1} to 30 g l^{-1}, the growth rate was reduced, although trehalose might not be toxic. When trehalose was further added to culture medium as the carbon source, the growth was extremely reduced **(Fig. 1H)**. Also, the growth-induced water potential was reduced **(Fig. 1G)**. Turgor of the zone of elongation **(Fig. 1F)** did not proportionally correspond to the size of the growth rate **(Fig. 1H)**.

In order to obtain the hydraulic conductance and wall extensibility in elongating soybean stems, relationships between relative growth rates and the growth-induced water potential **(Fig. 2A)** and relationships between the relative growth rates and turgor **(Fig. 2B)** were plotted. The relative growth rate had a linear relationship with the growth-induced water potential through the origin, and the hydraulic conductance was 5.03 x 10^{-5} s^{-1} MPa^{-1} for sucrose treatment (● ; **Fig. 2A**) and 1.74 x 10^{-5} s^{-1} MPa^{-1} for trehalose treatment (▲ ; **Fig. 2A**).

The slope of plots formed by the relative growth rates and turgor was -1.72 x 10^{-4} s^{-1} MPa^{-1} for sucrose treatment (● ; **Fig. 2B**) and was -5.81 x 10^{-5} s^{-1} MPa^{-1} for trehalose treatment (▲ ; **Fig. 2B**).

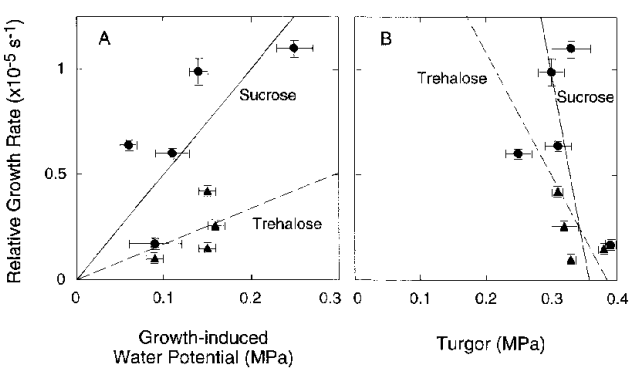

Fig. 2. Relationships between the growth-induced water potential and the relative growth rate (A) and relationships between turgor and the relative growth rate (B) when soybean embryos were grown on the medium having different water potentials in sucrose (●) and trehalose (▲) treatments. Vertical and horizontal bars indicate standard errors, and data points were taken from **Figs. 1D** and **1H** for calculation of the relative growth rate. The regression lines are; (A) y = 5.03x with r = 0.608 for the sucrose treatment (solid line) and y = 1.74x with r = 0.590 for the trehalose treatment (dashed line), and (B) y = -17.2 x + 6.16 with r = 0.357 for the sucrose treatment (solid line) and y = -5.81x + 2.23 with r = 0.594 for the trehalose treatment (dashed line). In **A**, L was obtained from the slope of the line. In **B**, m was considered to be infinitely large, because m must be positive.

In **Fig. 2B**, the slope became negative when the slope was calculated statistically (for sucrose treatment (●) and trehalose treatment (▲) in **Fig. 2B**). The wall extensibility must be positive for the validity of Eqn. 4, and consequently, m is postulated to be $+\infty$.

3.3. Growth on sucrose- and trehalose-containing media

Since the total sugar concentrations were kept equal among culture media, *i.e.*, 30 g l^{-1}, the water potential of the culture medium was -0.35 MPa under various culture media with trehalose and/or sucrose mixture **(Fig. 3A)**. The growth of soybean embryos was reduced as the amount of trehalose added to the culture media was increased **(Fig. 3D)**. Because the osmotic shock effect on growth of soybean embryos could be nullified, it is evident

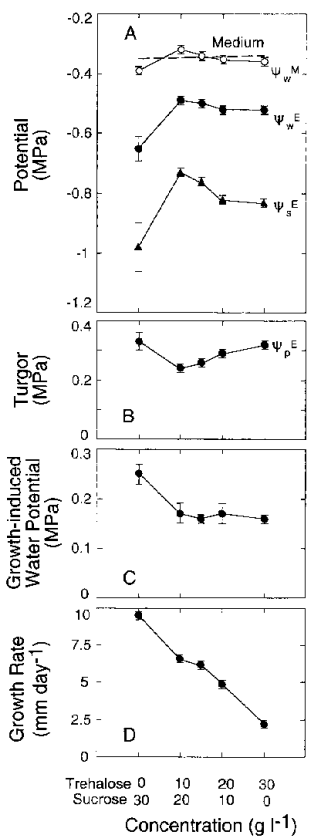

Fig. 3. Water potentials of the mature zone (Ψ_W^M ; ○) and elongation zone (Ψ_W^E ; ●), and osmotic potentials of the elongation zone (Ψ_S^E ; ▲) (A), turgor of the zone of elongation (Ψ_P^E) (B), the growth-induced water potential (C) and growth rates (D) when soybean embryos were cultured on trehalose and/or sucrose containing culture media. The growth-induced water potential was calculated by subtracting the water potential of the elongation region from that of the mature region. A dashed line in **A** indicates the medium water potential. Vertical bars indicate the standard errors.

that a decrease in growth rates was caused by the addition of trehalose in culture media.

The growth-induced water potential (**Fig. 3C**) was determined by differences between the water potential of the zone of elongation and that of maturation (**Fig. 3A**). The growth-induced water potential was the largest when 30 g l^{-1} sucrose was added to culture medium (**Fig. 3C**) and growth rate became also the highest (**Fig. 3D**). However, turgor of the zone of elongation did not proportionally correspond to the size of the growth rate (**Fig. 3B**).

When relationships between relative growth rates and the growth-induced water potential were plotted, the relative growth rate had a linear relation with the growth-induced water potential through the origin, and the hydraulic conductance was 3.83 x 10^{-5} s^{-1} MPa^{-1} (**Fig. 4A**). When both sucrose and trehalose were added to the medium, the hydraulic conductance became an intermediate between only sucrose-containing and only trehalose-containing media (**Fig. 4A**). The slope of plots formed by the relative growth rates and turgor in **Fig. 4B** was -5.32 x 10^{-3} s^{-1} MPa^{-1} (**Fig. 4B**). Since the wall extensibility must be positive for the validity of Eqn. 4, m is postulated to be $+\infty$.

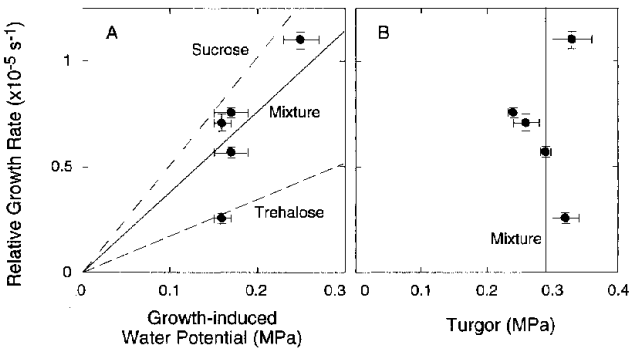

Fig. 4. Relationships between the growth-induced water potential and the relative growth rate (A) and relationships between turgor and the relative growth rate (B) when sucrose and trehalose were added to culture medium. Vertical and horizontal bars indicate standard errors, and data points were taken from **Fig. 3D** for calculation of the relative growth rates. The regression lines are; (A) y = 3.83x with r = 0.732 (solid line) and (B) y = -531.92 x + 153.87 with r = 0.015. In **A**, L was obtained from the slope of the line. A dotted line and a dashed line in **A** indicate sucrose and trehalose treatments adopted from **Fig. 2A**, respectively. In **B**, m was considered to be infinitely large, because m must be positive.

4. Discussion

Our previous work (Ikeda *et al.*, 1999a) showed that sizes of the water potential gradient between the water source and elongating cells correlated to the speed of growth rates under nutrient deficiency, salt stress, growth retardation induced by plant growth regulators, low temperature and high temperature conditions, indicating that cell expansion rates were mainly associated with how much water could be absorbed by elongating cells regardless of kinds of given environmental stress conditions. Also, Ikeda *et al.* (1999b) observed that the same principle was applicable to growth of the tissue-cultured carnation stems and callus tissue. In the present paper, the growth-induced water potential was also associated with growth of soybean embryos cultured on sucrose-containing and/or trehalose-containing media.

The hydraulic conductance of tissue-cultured soybean embryos cultured on trehalose-containing media was 1.74 x 10^{-5} s^{-1} MPa^{-1}, whereas it was 5.03 x 10^{-5} s^{-1} MPa^{-1} when they were cultured on sucrose-containing media (**Fig. 2A**). Ikeda *et al.* (1999a) reported 5.10 x 10^{-5} s^{-1} MPa^{-1} of the hydraulic conductance under salt stress condition and 4.31 x 10^{-5} s^{-1} MPa^{-1} of the hydraulic conductance in additions of 2,4-dichlorophenoxyacetic acid and benzylaminopurine to culture media, respectively, when soybean embryos were cultured on sucrose-containing media. Comparing the hydraulic conductance of the previous work (Ikeda *et al.*, 1999a) with that of the present work in the trehalose treatment, it became apparent that trehalose reduced hydraulic conductance significantly when soybean embryos were cultured on trehalose-containing media. Water loss was inhibited at transgenic tobacco cells which accumulated trehalose under water stress conditions (Holmström *et al.*, 1996; Romero *et al.*, 1997). This tolerance against water stress might be attributed to a decrease in the hydraulic conductance. When both sucrose and trehalose were added to the medium, the hydraulic conductance became an intermediate between only sucrose-containing and only trehalose-containing media (**Fig. 4A**). This indicated that the hydraulic conductance was altered by the addition of trehalose to the medium, and consequently, metabolic changes might occur in cell plasma membrane. Crowe *et al.* (1984) reported that trehalose substitutes for water molecules in the membrane during dehydration and thus helps to maintain membrane integrity. In the present study, when trehalose concentration was increased in the culture media, osmotic shock was enhanced in the root zone of soybean embryos. Thus, it is likely that dehydration might be induced in soybean embryos at low water

potentials, resulting in substitution of some water molecules with trehalose in the membrane and inducing a decrease in hydraulic conductance in order for soybean embryos to prevent dehydration.

Dupray *et al.* (1995) observed that osmoregulation is promoted by trehalose in *Salmonella manhattan* at low water potentials. However, in *Myrothamus flabellifolia* Welw., it did not accumulate trehalose further at low water potentials when its leaves were desiccated even though sucrose and arbutin were significantly accumulated more at low water potentials than the hydrated control (Bianchi *et al.*, 1993). Holmström *et al.* (1996) described that the water stress tolerance was not induced by osmotic adjustment with trehalose in transgenic tobacco plants because the trehalose concentrations seemed too low for osmotic adjustment, *i.e.*, ≤ 5 mM in the cytosol. Because turgor was almost kept similarly under various trehalose concentrations (**Fig. 1F**), and also because osmotic potential of the zone of elongation decreased significantly at low water potentials (**Fig. 1E**), it is obvious that the osmotic adjustment occurred in soybean embryos at low water potentials when trehalose was added in culture media. Thus, trehalose seemed to help osmotic adjustment in soybean embryos significantly at low water potentials.

Veluthambi *et al.* (1982) found that cell wall synthesis was inhibited upon 2 % trehalose feeding in *Cuscuta reflexa* Roxb. cells. In this experiment, soybean embryos cultured on 15 g l^{-1} (1.5 %) and 30 g l^{-1} (3 %) trehalose-containing media grew better than those cultured on media without sugars (**Fig. 1H**), and thus, it was not evident that inhibition of cell wall synthesis took place. Rather, the wall extensibilities of soybean embryos cultured on only sucrose-containing, only trehalose-containing and sucrose/trehalose-containing media were similar, and were considered to be infinitely large (**Figs. 2B** and **4B**). Thus, it is more likely that the wall extensibility was not a limiting factor for growth in soybean embryos when trehalose was added to the culture media in the present study.

Consequently, we concluded that trehalose feeding modified hydraulic conductance in soybean embryos, and hence, reduced growth. Also, osmotic adjustment was promoted at low water potentials when trehalose was added to culture media. The growth-induced water potential was a regulating factor for growth of soybean embryos cultured on sucrose and/or trehalose-containing media. However, the wall extensibility and the effective turgor were not limiting factors for growth of soybean embryos cultured on sucrose and/or trehalose-containing media.

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