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Water Potential Associated with Cell Elongation and Cell Division of Tissue-Cultured Carnation Plants

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Abstract

This work was undertaken to determine the growth parameters of Lockhart's equation for finding which component was predominantly contributing to the cell expansion rates of tissue-cultured carnation plants (*Dianthus caryophyllus L.*). The water potential of the culture media ranged from −0.02 to −0.51 MPa so that water stress conditions could be applied. Cell expansion could be inhibited completely by adding 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BA) to the culture media to form callus tissue. The sizes of the water potential gradient between the water source and the elongating cells correlated to the speed of growth rates under nutrient deficiency and growth retardation induced by the plant hormones, indicating that cell expansion rates were mainly associated with how much water could be absorbed by the elongating cells regardless of changes in growth under osmotic stress and growth retardation induced by addition of 2,4-D and BA.

1. Introduction

In tissue culture, both auxins and cytokinines are applied to culture media for the induction of callus tissue in many plant species (George *et al.*, 1987). Even though callus tissue can be induced by adding auxins and cytokinines into media, concentrations and combinations of the plant hormones necessary for such a condition in carnation plants have been reported differently (e.g., 0.1 mg l^{-1} naphthylacetic acid (NAA) and 3 mg l^{-1} benzylaminopurine (BA) (Leshem, 1986); 4 mg l^{-1} indoleacetic acid and 2.5 mg l^{-1} kinetin (Petru and Landa, 1974); 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg l^{-1} BA (Palet *et al.*, 1991)). In these reports (Leshem, 1986; Palet *et al.*, 1991; Petru and Landa, 1974), the standard concentration of MS (Murashige and Skoog, 1962) salts for callus induction was used. For induction of rice (Chen, 1977) and red fescue (Torello *et al.*, 1984) callus, 50 % of MS salts was the optimum concentration. When callus tissue is formed, cell division rather than cell elongation occurs. However, it is not known how the water status of callus tissue is affected by the concentration of plant hormones and MS salts during callus induction periods under different mixture compositions of the culture media. The water status of callus tissue has not been measured critically and the cell division in callus tissue has not been compared with cell elongation from the viewpoint of the plant-water relationship.

For stem elongation to occur in tissue-cultured plantlets, the optimum concentration of MS salts and sucrose varies in many plant species (George *et al.*, 1987). In general, the standard concentration of MS salts has been used in many kinds of plant species (George *et al.*, 1987). Sometimes, the reduced MS salt content was used (e.g., 50 % of MS and 15 g l^{-1} sucrose in wheat (Ahloowalia, 1982) and *Atropa belladonna* (Lorz and Potrykus, 1979) and 25 % of MS and 20 g l^{-1} sucrose in *Gasteria verrucosa* (Beyl and Sharma, 1983)). For carnation plants in vitro, Kakehi (1979) reported that the standard concentration of MS salts with 30 g l^{-1} sucrose, 1 mg l^{-1} NAA and 1 mg l^{-1} BA was the optimum for stem elongation. Kozai and Iwanami (1988) found that the optimum culture medium was the standard concentration of MS with 10 g l^{-1} sucrose under an enriched CO_2 environment.

Even though the optimum concentration of MS salts and sucrose for plant growth has been reported elsewhere (Ahloowalia, 1982; Beyl and Sharma, 1983; Kakehi, 1979; Kozai and Iwanami, 1988; Lorz and Potrykus, 1979), rates of stem elongation may be affected by differences in concentrations of MS salts and/or sucrose. The objective of this study is to determine growth parameters modeled by Lockhart (1965a, 1965b) for tissue-cultured carnation plants from the viewpoint of plant-water relation in cell dividing callus and cell elongating stem tissue.

Since water typically constitutes 80 to 95 % of the

mass of growing plant tissues, increases in cell volume under non-transpiring conditions in tissue culture can be considered as nearly equivalent to the amount of water taken up by elongating cells. Because water must flow into cells during cell elongation, a water potential gradient must exist between the water source and the elongating cells. In growing tissues, a potential field has been proposed as necessary for the uptake of water from the xylem by cells in the growing region (Boyer, 1985; Molz and Boyer, 1978; Nonami and Boyer, 1987, 1993; Nonami *et al.*, 1997). Such a water potential field associated with cell elongation is called a growth-induced water potential (Boyer, 1985; Ikeda *et al.*, 1996; Molz and Boyer, 1978 ; Nonami and Boyer, 1987, 1993; Nonami *et al.*, 1997). The size of the growth-induced water potential can be determined by the difference in water potential between elongating cells and the water source. The relationship between the relative growth rate (G; unit : s-1) and the size of the growth-induced water potential ($\Delta\Psi_G$; unit: MPa) can be expressed as;

$$G=L(\Delta\Psi_G)=L(\Psi_o-\Psi_w) \qquad (1)$$

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

During cell elongation, the cell wall must be extended outward by cell turgor, and the relation of relative growth rate to turgor (Ψ_p; unit: MPa) and extensibility of the wall (m; unit: s^{-1} MPa^{-1}) is (Green *et al.*, 1971; Ray *et al.*, 1972);

$$G=m(\Psi_p-Y) \qquad (2)$$

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 (p, the slope of the line is m and, Y is the intercept of the Ψ_p axis when G=0.

Because plant cells elongate due to simultaneous water uptake and wall extension, Eqs. I and 2 can be combined by applying the relation of $\Psi_w=\Psi_s+\Psi_p$ assuming that water potential in cytoplasm is locally equilibrated with that in the wall space (Molz and Boyer, 1978; Nonami and Boyer, 1987). Hence,

$$G=\frac{ml}{m+L}(\Psi_o+\Psi_s-Y) \qquad (3)$$

The Eq. 3 is known as Lockhart's combined equation developed from a theory of cell enlargement (Lockhart, 1965a, 1965b), and is re-interpreted for tissue growth in the zone of elongation (Boyer, 1985; Nonami and Boyer, 1990). Simultaneous determinations of all parameters in Eq. 3 are not experimentally easy, and therefore, Eq. 3 can be re-arranged as follows;

$$\frac{G}{L}+\frac{G}{m}=(\Psi_o-\Psi_w)+(\Psi_p-Y) \qquad (4)$$

Equation 4 is shown as algebraic summation of Eqs. 1 and 2. In order to study which component contributes more predominantly to growth of tissue-cultured plants, parameters of ($\Psi_o-\Psi_w$) 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 ($\Psi_o-\Psi_w$) and Ψ_p. By assuming that Y was not altered significantly in this study when G was changed, Y was determined at G = 0 from the plot of G against Ψ_p. Although the valid domain of Eq. 4 may not be completely overlapping with Eqs. 1 and 2, we assume that both the water potential gradient and cell turgor are contributing to 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 Eq. 4, $L\neq0$ and $m\neq0$.

In this study, growth parameters were analyzed by applying Lockhart's equation to plants grown under tissue culture conditions when concentrations of culture media were changed. Furthermore, plant hormones were added to the culture media so that cell elongation was inhibited by formation of callus tissue.

2. Material and Method

2.1 Plant Materials

Virus-free meristem tip-propagated carnation plants (*Dianthus caryophyllus* L. cv Silver pink) cultured in test tubes were obtained from Daiichi Seed Co. Ltd., Japan. Segments (terminal 10 mm) of carnation plants which were further multiplied by tissue culture in our laboratory to ensure uniform growth were used.

In order to test the effect of salt and plant hormone concentrations for callus formation, 1 mg l^{-1} of 2,4-D and 1 mg l^{-1} of BA were added to the standard MS solution in order to test the effect of salt and plant hormone concentrations on callus formation. The plant hormones and sucrose were diluted with the same ratio of the standard MS medium to have 4 different media. *i.e.*, 1) 30 g l^{-1} of sucrose, 1 mg l^{-1} of 2,4-D and 1 mg l^{-1} of BA at 100% MS media. 2) 18 g l^{-1} of sucrose, 0.6 mg l^{-1} of 2,4-D and 0.6 mg l^{-1} of BA at 60 % of MS media. 3) 9 g l^{-1} of sucrose, 0.3 mg l^{-1} of 2,4-D and 0.3 mg l^{-1} of BA at 30 % of MS media. 4) 0 g l^{-1} of sucrose, 0 mg l^{-1} of 2,4-D and 0 mg l^{-1} of BA at 0 % of MS media. The pH of all media was set at 5.8 using 1N NaOH. Agar (Wako Pure Chemical Ind. Ltd., Japan) at concentration of 8 g l^{-1} was added to each medium prior to autoclaving. Thirty

ml of medium was dispensed into each 180 m*l*-glass bottle which was sealed with aluminum foil. The media were then autoclaved for 15 min at 103 kPa and 121°C.

The carnation segments were grown at 25±1°C and 105 μmol m^{-2} s^{-1} photosynthetically active photon flux density. Thirty plantlets were cultured for each treatment. Carnation samples for the water status measurements were taken from 20-day-old plants, and 3 to 5 water status measurements were conducted for each treatment.

Additionally, in order to check the effects of various concentrations of MS salts on cell elongation of carnation plants, the standard MS solution with 30 g l^{-1} of sucrose was diluted to 10 % intervals without the addition of the plant hormones. The growth conditions were similar to those of the previous experiment.

2.2 Measurement of growth

The length between the tip of plants and the agar surface in tissue culture vessels was measured as the plant height with a ruler. The growth region of the stem was limited in the terminal 10 mm of stem from the tip. The growth rate was determined from changes in the plant height between 18 and 20 days after initiation of culture. Thus, the relative growth rate was calculated by dividing the growth rate by the length of the elongating region, i.e., 10 mm. The cell sizes of cortex for stem and callus were measured under a microscope (IMT-2, Olympas Optical Co., Japan) after tissue was sliced with a razor blade. The cell volume was calculated as a cylinder for stem after the width and length of cells were measured and as a sphere for callus after the diameters were measured under the microscope.

2.3 Water status measurement with isopiestic psychrometers

A thermocouple chamber (Boyer and Knipling, 1965) was coated with melted and resolidified vaseline (Boyer, 1967) and loaded with 6 to 8 segments which were excised from the zone of elongation (terminal 8 to 10 mm), maturation (basal 10 to 15 mm) of stems and callus tissue. The water potentials were measured by using the isopiestic technique (Boyer and Knipling, 1965). A thermocouple bearing a sucrose solution of known water potential could be placed in the thermocouple chamber for measurement of tissue water potential. The measurement was isopiestic, i. e., the vapor pressure of the solution was the same as that of the tissue and no net vapor exchange took place. This prevented errors caused by the diffusive resistance problem of the tissue to water vapor transfer and assured that the tissue neither hydrated nor dehydrated during the course of the measurement (Boyer and Knipling, 1965).

Also, water potentials of media were measured with the isopiestic psychrometer (Boyer and Knipling, 1965).

After water potential was measured, the osmotic potential of the tissue in the thermocouple chamber was determined in the same tissue immediately after freezing at −70°C and thawing (Ehlig, 1962). The turgor was calculated by subtracting the osmotic potential from the water potential.

3. Results

3.1 Growth inhibition induced by plant hormones

When plant hormones were applied to culture media, 95%, 100% and 85% of the explants developed into callus tissue at concentrations of 2.7, 5.4 and 9.0 μM of the plant hormones, respectively, although the control did not form callus tissue. The average cortical cell volume of the elongating region in the control grown without the addition of plant hormones in the medium was 66.9 ±16.6 p*l*. When callus tissue was formed, cell division took place, but the cell size of callus was 6.12 p*l* in the average, which was about 1/10th of that of normal plant (*i.e.*, 66.9 p*l*).

When callus tissue was formed, the water potential of callus tissue became almost the same as that of the culture medium (**Fig. 1-A**), and thus, the growth-induced water potential was considered to be very small, and the growth-induced water potential of callus tissue (**Fig. 1-C**) was estimated from the differences between water potential of the medium and that of tissue (**Fig. 1-A**). The size of the growth -induced water potential was correlated with the size of the growth rate of the tissue (**Figs. 1-C and 1-D**). Turgor was significantly higher in callus tissue than in the expanding cells although callus tissue had continuous cell division (**Fig. 1-B**). Because the culture media, in which the callus tissue was grown, had higher salt concentration (**Fig. 1-A**), it is evident that callus cells accumulated solutes actively at low water potentials, resulting in high turgor in the cells. Thus, osmoregulation took place in callus tissue under osmotic stress conditions.

3.2 Growth inhibition under nutrient deficiency

The growth-induced water potential (**Fig. 2-C**) was determined from the difference of water potential between the zone of elongation and that of maturation (**Fig. 2-A**). When the size of growth-induced water potential became smaller, the growth rates became smaller proportionally (**Figs. 2-C** and **2-D**). However, turgor of the zone of elongation did not correspond to the size of the growth rates when carnation

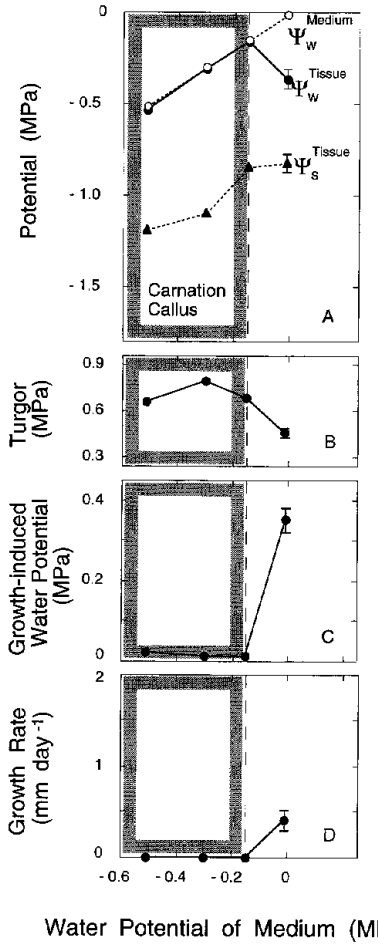

Fig. 1 Water potentials of plant tissue (Ψ_w^{Tissue}; ●) and culture media (Ψ_w^{Medium}; ○), and osmotic potentials of the elongation zone (Ψ_s^{Tissue}; ▲) (A), turgor of the zone of elongation (B), growth-induced water potential (C) and growth rate (D) when carnation plant segments were grown on tissue culture media having different water potentials together with the plant hormones. The growth-induced water potential was calculated by subtracting the water potential of plant tissue from of culture medium. The callus tissue was formed on the media under −0.15 MPa of water potential, which is shown in regions encircled by shadowed boxes in A, B, C and D. Vertical bars indicate the standard errors.

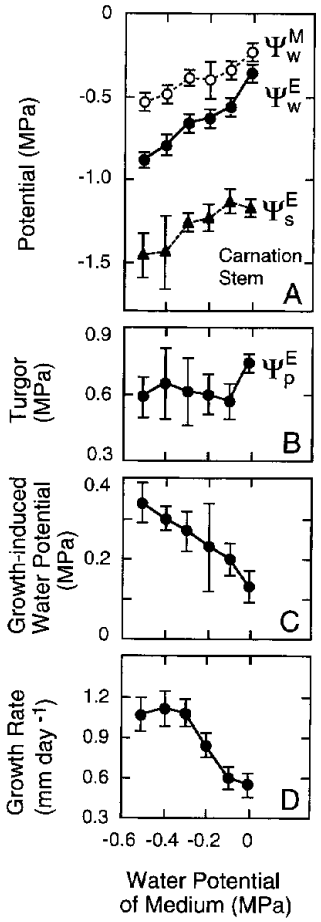

Fig. 2 Water potentials of the mature zone (Ψ_w^M; ○) and elongation zone (Ψ_w^E; ●), and osmotic potentials of the elongation zone (Ψ_s^E; ▲) (A), turgor of the zone of elongation (Ψ_p^E) (B), growth-induced water potential (C) and growth rate (D) when carnation plant segments were grown on tissue culture media having different water potentials. The growth-induced water potential was calculated by subtracting the water potential of the elongation region from that of mature region. Vertical bars indicate the standard errors.

plants were grown under tissue culture conditions (**Fig. 2-B**). Decreases in growth rates at higher water potentials of culture media (**Fig. 2-D**) indicate that plants were suffering from nutrient deficiency. Even though plants did not receive sufficient nutrients from the medium, maintenance of turgor in the zone of elongation (**Fig. 2-B**) indicates that the cells adjusted osmotically and did not lose turgor when the plants were grown under nutrient deficiency conditions.

3.3 Growth parameters in carnation plants

In order to obtain hydraulic conductance and wall extensibility in growing carnation plants, the relationship between relative growth rates and the growth-induced water potential (**Fig. 3-A**), and the relationship between relative growth rates and turgor (**Fig. 3-B**) were plotted. The relative growth rate had a linear relationship with the growth-induced water potential through the origin, and the hydraulic conductance was 8.06×10^{-6} s^{-1} MPa^{-1} from the slope (**Fig. 3-A**). The size of the growth-induced water potential ranged from 0.01 MPa to 0.34 MPa (**Figs. 1-C, 2-C** and **3-A**).

In **Fig. 3-B**, the slope of a line formed by plots of the relative growth rates against turgor became negative when the slope was calculated statistically (a

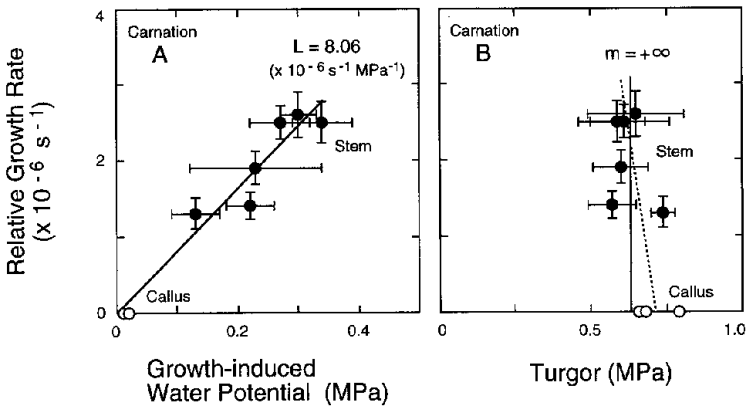

Fig. 3 Relationship between the growth-induced water potential and relative growth rate (A) and relationship between turgor and relative growth rate (B) when segments of carnation plants were grown on the medium having different water potentials (●) and having different water potentials containing plant hormones (○). Vertical and horizontal bars indicate standard errors, and data points were taken from **Figs. 1** and **2** for calculation of relative growth rates. The regression lines are; (A) $y=8.06x$ with $r=0.974$, and (B) $y=-25.6x+18.2$ with $r=0.604$. 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

dashed line in **Fig. 3-B**). Because the wall extensibility must be positive for the validity of Eq. 4, and further because the standard errors overlap each other, the line was drawn as a vertical line which goes through the average value of turgor at the intercept of the x-axis (**Fig. 3-B**). Thus, the yield threshold was estimated to be 0.65 MPa, and the growth-effective turgor was considered to be negligibly small, and $m=+\infty$.

3.4 Factors Controlling Cell Elongation

By applying Lockhart's equation (Lockhart, 1965a, 1965b) to plants growing under tissue culture conditions, growth parameters were determined when growth of the plants was altered by subjecting them to nutrient deficiency and different concentrations of plant hormones. Under all stress conditions, m was greatly larger than L (**Fig. 3**), and thus the coefficient of Eq. 3 can be modified as follows;

$$\frac{mL}{m+L}\approx\frac{mL}{m}=L \quad \text{when} \quad m>>L$$

Furthermore, sizes of the growth-induced water potential were much larger than those of the growth-effective turgor, and additionally the growth-effective turgor was near zero, *i.e.*, $(\Psi_o-\Psi_w)>>(\Psi_p-Y)\approx 0$. By using the above approximation, Eq. 3 can be modified as follows;

$$F=\frac{mL}{m+L}(\Psi_o-\Psi_s-Y)=\frac{mL}{m+L}\{(\Psi_o-\Psi_w)(\Psi_p-Y)\}$$
$$\approx L(\Psi_o-\Psi_w)$$

Thus, Eq. 3 can be approximated to be equivalent to Eq. 1 when the growth parameters were measured in tissue-cultured plants grown under environmental stresses. This indicates that cell elongation under tissue culture conditions was predominantly regulated by the size of the growth-induced water potential regardless of culture conditions.

4. Discussion

In this study, plant hormones were added to the media to induce callus formation. When cells stop elongation by forming callus tissue, the growth-induced water potential becomes zero. When stem growth rates of tissue-cultured carnation plant were reduced under nutrient deficient conditions, sizes of the growth-induced water potential were linearly related to cell elongation rates of tissue-cultured carnation plantlets. Ikeda *et al.* (1996) showed that this principle was also applicable in the growing of tissue-cultured soybean stems and roots. Therefore, it is safe to conclude that the rate of water uptake by the elongating cell was the regulating factor for cell elongation rates of plants grown under tissue culture conditions regardless of which kind of environmental stresses were given to plants. When cell expansion is taking place, the cell wall must be pushed outward, and thus, the presence of turgor in the cell is essential for cell expansion to supply a force to push the wall toward the outside. Although turgor in the cell is required, the growth-effective turgor was negligibly small. Thus, turgor was not the limiting factor for the cell expansion in the case of carnation plants grown under tissue culture conditions in this experiment.

Under water stress conditions, plants accumulate solutes in cells in order to maintain the size of cell volume and turgor against dehydration caused by low water potentials. This phenomenon is known as osmoregulation (Morgan, 1984). Osmoregulation has been observed in stems (Meyer and Boyer, 1972, 1981; Nonami and Boyer, 1990), leaves (Munns *et al.*,

1979; Westgate and Boyer, 1985), roots (Ikeda *et al.*, 1996; Sharp *et al.*, 1990; Voetberg and Sharp, 1991) and fruits (Fukuyama, 1990; Yakushiji *et al.*, 1996). In the present study, osmoregulation was associated with decreases in osmotic potential (**Figs. 1-A** and **2-A**) and turgor maintenance (**Figs. 1-B** and **2-B**) under osmotic stress applied to tissue-cultured plants. These results show that osmoregulation not only occurred in stems but also in callus tissue, which can be considered as dividing cells. Meyer and Boyer (1972) described cell elongation that had either the same sensitivity as cell division or was more sensitive to low water potentials than cell division in soybean hypocotyls. In this study, the growth-induced water potential and cell expansion rates of callus tissues were small. The water potential of callus tissue was close to that of the medium (**Fig. 1-A**), and thus, the callus tissue was not significantly absorbing water although cell division took place. However, active osmoregulation took place because the size of turgor became much higher than elongating cells (**Fig. 1-B**). Brown and Thorpe (1980) observed osmoregulation in tobacco callus when callus was grown in higher concentrations of culture media. When concentrations of culture media were either decreased or increased, growth of callus tissues was inhibited, and then, turgor of callus tissues whose growth was inhibited was higher than that of callus tissues growing actively at the optimal concentration of culture medium (Brown and Thorpe, 1980). Thus, they observed that increased turgor was associated with growth inhibition and osmoregulation of tobacco callus tissues (Brown and Thorpe, 1980).

Because the tissue culture vessel is closed, the humidity in the vessel is almost saturated, and thus, it can be considered that evaporation and transpiration from plant tissues are negligibly small. When plants are exhibiting slow growth, it is possible that the tissue culture medium is inducing a significant water stress on plants. Horticultural crops grown in tissue culture, such as orchids and cyclamens, take several weeks to several months to grow in the same media before the plants are taken out from the culture vessel (Reinert and Mohr, 1967; Wainwright and Harwood, 1985). Under such conditions, differences in a fraction of water potentials of the medium may yield a significant growth difference after the tissue culture is completed, because the cumulative effects of water stress on plant growth become significant in several months. Thus, we suggest that measurements of the growth-induced water potential of tissue-cultured plantlets should supply the optimum condition of medium for tissue-cultured plantlets.

Finally, we conclude that the cell elongation rate is primarily regulated by how much water can be absorbed by elongating cells and the size of the water potential difference between elongating cells and the water source under osmotic stresses, and also during callus formation.

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