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Factors Influencing the Induction of Freezing Tolerance by Abscisic Acid in Cell Suspension Cultures of *Bromus inermis* Leyss and *Medicago sativa* L.[1]

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ABSTRACT

A 2-gram fresh weight inoculum of bromegrass (*Bromus inermis* Leyss. culture BG970) cell suspension culture treated with 7.5×10^{-5} molar abscisic acid (ABA) for 7 days at 25°C survived slow cooling to −60°C. Over 80% of the cells in ABA treated cultures survived immersion in liquid N_2 after slow cooling to −40 or −60°C. In contrast, a 6-gram fresh weight inoculum only attained a hardiness level of −28°C after 5 days of ABA treatment. Ethanol (2×10^{-2} molar) added to the culture medium at the time of ABA addition, inhibited the freezing tolerance of bromegrass cells by 25°C. A 6-gram inoculum of both control and ABA treated bromegrass cells altered the pH of the medium more than a 2-gram inoculum. ABA inhibited the increase in fresh weight of bromegrass by 20% after 4 days. Both control and ABA (10^{-4} molar) treated alfalfa cells (*Medicago sativa* L.) grown at 25°C hardened from an initial LT_{50} of −5°C to an LT_{50} of −23°C by the third to fifth day after subculture. Thereafter, the cells dehardened but the ABA treated cells did not deharden to the same level as the control cells. ABA inhibited the increase in fresh weight of alfalfa by 50% after 5 days.

ABA has been demonstrated to induce hardening to freezing temperatures in plants (9), plant parts (3), and in plant tissue culture (4). Chen and Gusta (4) demonstrated that tissue culture from five species capable of cold hardening, increased in hardiness upon treatment with ABA at nonhardening temperatures. Whereas, tissue culture from five species that could not cold harden, did not harden in the presence of ABA. However, exogenous application of ABA to plants has failed to increase hardiness (8) or has produced only limited hardiness. Orr *et al.* (14) were not able to demonstrate an increase in the cold hardiness of alfalfa cells grown in the presence of ABA at 25°C, but were able to if the cultures were grown at 2°C. However, Chen and Gusta (4) reported that alfalfa cells hardened when treated with ABA at nonhardening temperatures.

This study was conducted to determine the factors which limit the ABA induced hardening of cells at nonhardening temperatures. The effect of ethanol, culture size, changes in culture medium due to growth of cells, and stage of growth were investigated in relation to hardening.

MATERIALS AND METHODS

Source of Culture. Cell suspension cultures of bromegrass (*Bromus inermis* Leyss. culture BG970) and alfalfa (*Medicago sativa* L. culture Alf-Wis) were obtained from the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada. The cells were grown according to Gamborg and Wetter (7) in a modified Erickson's medium containing 3% sucrose, B5 micronutrients and B5 hormone supplements. The cultures were grown in 50 ml liquid culture medium in 250 ml Erlenmeyer flasks shaken at 25°C in fluorescent light with an intensity of 71 W m^{-2} for 16 h and at 20°C in the dark for 8 h. Cells were transferred to fresh Erickson's medium weekly unless otherwise noted.

Preparation of Culture Medium. ABA (Calbiochem) was added to 10 ml of double distilled H_2O and dissolved by dropwise addition of 0.2 N KOH. During the addition of KOH, pH did not exceed 12. Ethanol was added to the culture medium either before sterilization at 121°C for 15 min or after autoclaving. Ethanol added after autoclaving was sterilized with a millex-GS 0.22 μm filter unit (Millipore Corporation, Bedford, MA).

Freezing Conditions and Viability Assays. Freezing conditions were similar to those of Chen and Gusta (4), except freezing was initiated at −3°C and the temperature lowered at a rate of 4.8°C/h from −3 to −20°C, at 9.6°C/h from −20 to −30°C and 19°C/h from −30 to −60°C. Samples frozen in liquid N_2 were cooled as described above to either −40 or −60°C before submersion in liquid N_2. After 10 min in liquid N_2, samples were held at −40°C for 30 min and thawed at 4°C. Viability was determined by TTC[2] reduction, by fluorescein diacetate staining, and by regrowth after 3 weeks as described by Chen and Gusta (4). Ethanol was added after the culture period in order to demonstrate any toxic effects ethanol might have on frozen cultures. Cultures were held in the presence of ethanol for 1 h and then used for freeze tests as described above (4).

Fresh and Dry Weight of Tissue Culture. The fresh weight of culture was determined by aspirating the culture medium using a pipette connected to a vacuum line. Loss of tissue was prevented by placing the pipette tip against the bottom of the flask.

Dry weights of aspirated cultures were determined after removing the cells from the flasks with 50 ml of distilled H_2O and filtering the cells over Miracloth. Cells were washed with 100 ml of distilled H_2O and dry weight was determined gravimetrically after holding the samples at 45°C for 48 h.

RESULTS

Freezing Tolerance and Growth. Bromegrass cells, maintained in Erickson's media at a nonhardening temperature of 25°C, had an LT_{50} ranging from −7 to −11°C over a 7-d period (Fig. 1). A 2 g inoculum of cells treated with 7.5×10^{-5} M ABA had a LT_{50} of −15°C after 2 and 4 d of treatment. After the 7th d the ABA

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[2] Abbreviation: TTC, 2,3,5-triphenyl tetrazolium chloride.

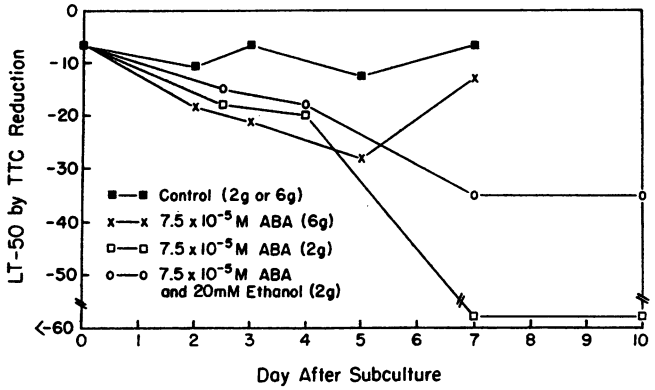

FIG. 1. Effect of ethanol (2×10^{-2} M) and inoculum size on ABA (7.5×10^{-5} M) hardening of bromegrass tissue culture. Ethanol was added before sterilization of the culture medium. Cultures were grown in 50 ml liquid culture medium in 250 ml Erlenmeyer flasks shaken at 25°C in fluorescent light of 71 W m^{-2} for 16 h and at 20°C in the dark for 8 h. Each flask was inoculated with 2 or 6 g of bromegrass suspension culture. Viability was determined by TTC reduction method. In the freeze test, freezing was initiated at −3°C and samples were cooled at −4.8C/h to −20°C, −9.6°C/h to −30°C, and −19°C/h to −60°C.

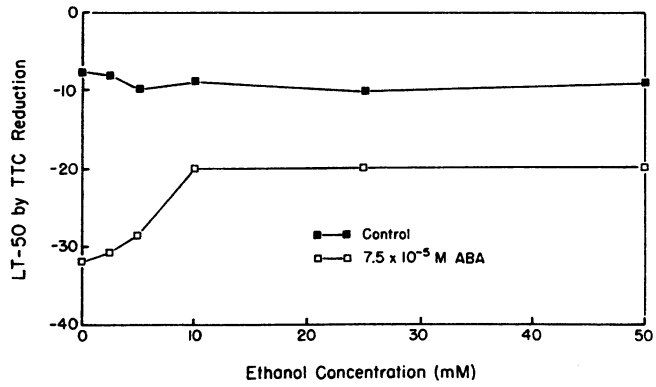

FIG. 2. Effect of ethanol on ABA (7.5×10^{-5} M) induced hardening of bromegrass tissue culture. Ethanol was added after sterilization of the culture medium. Cultures were grown in 50 ml liquid culture medium in 250 ml Erlenmeyer flasks shaken at 25°C in the dark. Each flask was inoculated with 1 g of bromegrass suspension culture. Freezing conditions and viability assessment as described in Figure 1.

treated cells survived −60°C. Over 80% of the cells survived immersion in liquid nitrogen, as determined by fluorescein diacetate staining, if precooled slowly to −40 or −60°C. These cells maintained the same hardiness from the 7th to 10th d of treatment. To study the effect of ethanol on hardening, ethanol was added to the culture medium after autoclaving (Fig. 2). Ethanol ($2.5–50 \times 10^{-3}$ M), added at the beginning of the culture period, had no effect on the cold hardiness of control cultures. However, ABA treated cultures only hardened to −20°C (LT_{50}) in the presence of 12.5×10^{-3} M ethanol. Cultures grown in the absence of ethanol but treated with up to 50×10^{-3} M ethanol immediately before freezing showed no loss of hardening with respect to controls (data not shown).

A 6 g inoculum of bromegrass cells treated with 7.5×10^{-5} M ABA did not attain the same level of freezing tolerance as a 2 g inoculum. After 5 d of treatment, the cells had an LT_{50} of −25°C, but on the 7th d the cells were reduced in hardiness and had an LT_{50} of −12°C.

The fresh weight of bromegrass cells (6 g inoculum) increased steadily over a 7 d period (Fig. 3A). Cells treated with 7.5×10^{-5} M ABA increased in fresh weight similar to the control cells for the first 4 d but thereafter no significant increase occurred. The relative dry matter content of the control remained relatively constant during the 7 d period (Fig. 3B). Growth as determined on a dry weight basis, was steady for the first 5 d and then stopped in both the control and ABA treated cells. The ABA treated cells accumulated more dry matter than the control by the 5th d after subculturing (Fig. 3C).

In this study, alfalfa cells were not as homogeneous as the bromegrass cells in all parameters that were studied. This was reflected in the survival of cells following controlled freezing. Bromegrass cells were killed over a narrow temperature range (2–5°C), whereas in alfalfa cells this temperature range was much larger. An LT_{80} was found to be less variable than an LT_{50}, and was therefore used to determine the hardiness of alfalfa cells.

A time course study on the freezing resistance of alfalfa cells treated with and without ABA (10^{-5}), revealed that both cultures attained a similar level of frost tolerance (LT_{80} = −20°C) after 3 d of culture (Fig. 4). Thereafter, the cells lost freezing tolerance. However, the control cells lost freezing resistance at a faster rate than the ABA treated cells. Fourteen d after subculture, the control cells could not tolerate temperatures colder than −3°C, whereas culture grown in the presence of 10^{-4} M ABA could tolerate −12°C (LT_{80}).

The freezing resistance of alfalfa cells treated with 10^{-4} M ABA for 5 d was −22.6°C (LT_{80}) as compared to −16.3°C (LT_{80}) for the control cells (Table I). Cells grown in the presence of 20 mM ethanol did not harden either in the presence or absence of ABA and could only tolerate −16°C (LT_{80}). The growth rate of alfalfa cell suspension cultures, determined from the fresh weight after 5 d of culture, was similar for both the ABA and control cells treated with 20 mM ethanol (Table II). However, the growth rate was only 75% compared to the controls. The growth rate of cells treated with ABA in the absence of ethanol, was 33% of the control. Reproducible results for changes in relative dry matter content could not be obtained for alfalfa suspension cultures due to the large coefficient of variation (9.7%). In contrast, the coefficient of variation of the relative dry matter content of bromegrass cells was 3.1%.

Medium pH Changes. The initial pH of the culture medium was 5.8. Upon sterilization of the medium at 121°C for 15 min, the pH declined to 5.5 and was not adjusted thereafter. After 2 d of growth of a 6 g initial inoculum of bromegrass culture, the pH of the medium decreased to 4.3 in the control and to 4.4 in the ABA (7.5×10^{-5} M) treated cells (Fig. 5A). After 5 d of culture, the pH of the medium increased to 6.7 and 6.9 for the control and ABA treated cells, respectively. A 2 g inoculum of bromegrass cells had a less pronounced effect on the pH of the medium as compared to the 6 g inoculum (Fig. 5B). In a 2 g culture treated with ABA, the pH of the medium increased to 6.3 after 1.5 d and then decreased to 5.2 after 6 d. After 10 d, the pH of the medium had risen to 5.8. In control cultures the pH declined to 4.7 after 3.5 d and then increased to 5.7 after 7 d.

DISCUSSION

Exogenous applications of ABA to cell suspension cultures at nonhardening temperatures induces freezing tolerance in cells capable of cold hardening at a low temperature. The cells treated with ABA cold hardened faster and attain a greater degree of freezing tolerance than cells hardened at cool temperatures (4). There is now substantial evidence indicating that ABA is involved in the hardening process and substitutes for the cold requirement in triggering the genetic system to induce the hardening process (3, 4, 9, 14, 17).

Bromegrass cell suspension cultures grown at 25°C for 7 d can only tolerate −8°C, whereas cells grown in the presence of ABA for 7 d can tolerate −60°C and liquid N_2 temperatures if first cooled slowly to −40 or −60°C. In this study, ABA was dissolved

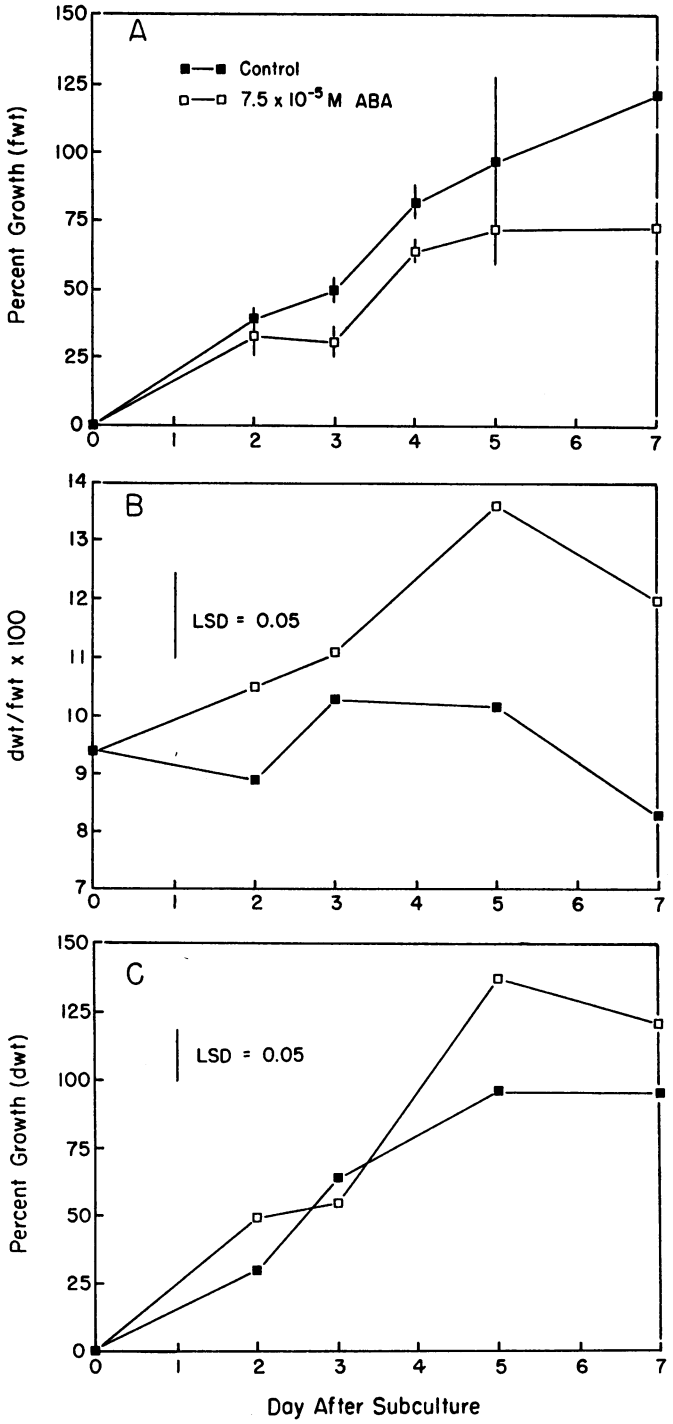

FIG. 3. Effect of ABA (7.5×10^{-5} M) on (A) the increase in fresh weight of bromegrass tissue culture. Culture maintenance conditions are described in Figure 1. Fresh weight was measured at inoculation of the culture and was approximately 6 g. Error bars represent $LSD_{P=0.05}$ based on individual standard deviations of 8 samples; (B) the ratio of dry weight to fresh weight of bromegrass cultures (the relative dry matter content expressed as a percentage). Error bar represents the $LSD_{P=0.05}$ based on pooled standard deviation of 5 samples inoculated with 6 g; and (C) the increase in dry matter. These values were calculated from paired samples used to create A and B (dry wt/fresh wt) × fresh wt increase. Error bar represents the $LSD_{P=0.05}$ based on a pooled standard deviation of samples inoculated with 6 g ($n = 5$).

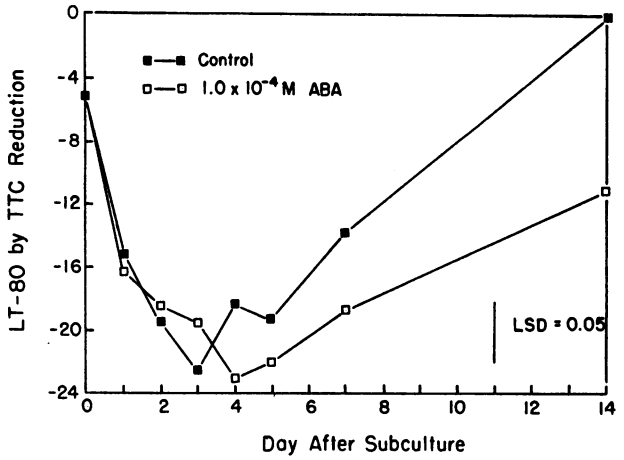

FIG. 4. The effect of ABA (1×10^{-4} M) on the hardening of alfalfa tissue culture. Flasks were inoculated with 2 to 3 g of alfalfa tissue culture. Culture conditions are as described in Figure 1. Viability was determined by the TTC reduction method. Freezing conditions were identical to those described in Figure 1. The error bar represents $LSD_{P=0.05}$ based on pooled standard deviation ($n = 4$).

Table I. *Effect of Ethanol and ABA (1×10^{-4} M) on Freezing Resistance of Alfalfa Suspension after 5 Days*

Ethanol was added before sterilization of the culture medium. Cultures were grown in 50 ml culture medium as described in Figure 1. Each flask was inoculated with 3 g of alfalfa suspension culture. Viability was determined by the TTC reduction method. Freezing conditions are as described in Figure 1.

	LT_{80}	
	2×10^{-2} M Ethanol	No Ethanol
	°C[a]	
ABA, 1×10^{-4} M	−16.0 ± 1.51	−22.6 ± 1.18
Control	−15.0 ± 2.0	−16.3 ± 3.2

[a] Confidence limits for 95% confidence interval. Each value represents the mean LT_{80} of 4 flasks.

Table II. *Effect of Ethanol and ABA on the Fresh Weight of Alfalfa Suspension Cultures*

Culture maintenance conditions are described in Figure 1. Fresh weight was measured at inoculation of the culture and was approximately 3 g.

	Fresh Weight Growth	
	2×10^{-3} M Ethanol	No Ethanol
	(fresh wt d 5 − fresh wt d 0)/fresh wt day 0 × 100%[a]	
ABA, 1×10^{-4} M	33.0 ± 20.3	78.6 ± 11.5
Control	36.6 ± 15.8	121.2 ± 30.7

[a] Confidence limits for the 95% confidence interval. Each value represents the mean of 25 samples from 5 flasks.

in dilute KOH instead of ethanol as reported previously by Chen and Gusta (4).

No effect of ethanol was found on survival if the ethanol was added immediately before freezing. Therefore, the addition of ethanol (20×10^{-3} M) to the culture medium before autoclaving reduced the effectiveness of ABA in inducing freezing tolerance as these cultures could only tolerate −35°C after 7 d. If ethanol (12.5×10^{-3} M) was added to the culture medium after autoclaving the bromegrass cells only attained an LT_{50} of −20°C after 7 d. The concentration of ethanol present after autoclaving me-

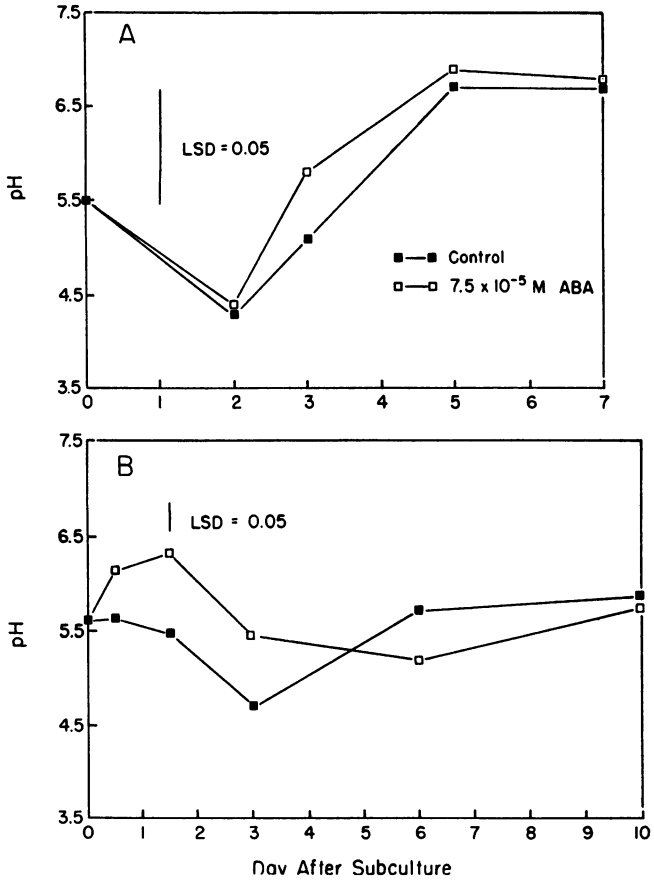

FIG. 5. The effect of a 6 g (fresh wt) inoculum (A) and a 2 g (fresh wt) inoculum, (B) of bromegrass tissue culture on the pH of 50 ml of Erickson's medium with and without ABA treatment (7.5×10^{-5} M). Error bar represents $LSD_{P=0.05}$ based on individual standard deviation ($n = 4$).

dium containing 2×10^{-3} M ethanol was not measured. However, ethanol remains in solution due to the low mol fraction of ethanol (20×10^{-3} M) in water (3.6×10^{-5} mol ethanol/mol water) which results in low vapor pressure of ethanol over water (13). This is confirmed by the measured biological activity of ethanol in the medium after autoclaving.

Ethanol (2.0×10^{-2} M) completely inhibited the effect of ABA in maintaining the freezing tolerance of alfalfa cell suspensions. Ethanol also inhibited the growth rate of alfalfa cells by 75% as compared to the controls. Ethanol at low concentrations in plants has been shown to decrease the survival of ice encased *Triticum aestivum* (2) and to break seed dormancy in *Avena fatua*. Biological activity of low concentrations of ethanol may be due to ethanol induced changes in membrane fluidity (16).

Alfalfa cells treated with ABA were inhibited in fresh weight accumulation by 33% after 5 d as compared to the controls. ABA has been previously demonstrated to inhibit growth (17). There was little evidence of growth inhibition by ABA for the first 3 d but, thereafter, inhibition became more pronounced. ABA had no significant effect on fresh weight accumulation of bromegrass cells until d 5. By d 7, there was a 32% reduction in the fresh weight of ABA treated cells as compared to the controls. However, if growth was expressed on a dry weight basis, ABA treated cells accumulated more dry matter than control cells by d 5. This coincided with the maximum increase in freezing tolerance of ABA induced cells.

The stage of growth of alfalfa cells had a pronounced effect on freezing tolerance. Upon transferring to a fresh medium, alfalfa cells could only tolerate −10°C, but by d 3 and 4, both control and ABA treated cells could tolerate −23°C. This occurred at the early stages of logarithmic growth. The development of freezing tolerance at 25°C in the absence of ABA has not been reported previously in tissue culture. Chen *et al.* (5) reported that the optimum time for cryopreservation of *Catharanthus roseus* L. was 2 to 3 d after subculture. Tepfer *et al.* (18) found that cells of winter hardy *Helianthus tuberosus* L. tubers are predominantly in G1, a resting stage of the cell cycle. These results suggest that one phase of the cell cycle may be more optimum for low temperature survival than other phases.

The pH of the cultural medium has a significant effect on nutrient (15) and ABA (10) uptake. A 6 g inoculum of bromegrass cells lowered the pH of the medium from 5.5 to 4.3 within 2 d; thereafter the pH increased to 6.7. A 2 g inoculum had a less pronounced effect on the pH of the cultural medium. A 2 and 6 g inoculum of bromegrass attained similar levels of freezing tolerance (−20 to −25°C) in response to ABA after 4 and 5 d. However, a 2 g inoculum after 7 d of ABA treatment could tolerate −60°C, whereas the 6 g inoculum could only tolerate −12°C. This may be due to the difference in pH of the medium or due to a faster depletion of nutrients by the large inoculum.

The pH of the medium has been shown to fluctuate during growing of cultures in B5 medium (6). Nutrient and hormone uptake is dependent on proton pumping mechanisms that acidify the growth medium (15). The uptake of nutrients, in turn, results in increases in extracellular pH (15). The compartmentation of nutrients and hormones is also regulated by pH (15). ABA is 80, 28, and 4% protonated at pH 4, 5, and 6, respectively (11). The acid form of ABA is believed to passively cross lipid bilayers, whereas the acetate form requires active transport mechanisms (10). Barring active transport or metabolism, ABA will concentrate as an acetate in the more alkaline compartments of the cell (10). The pH of the medium, thereby, governs the availability of ABA to internal compartments of cells. Also, ABA inhibits proton extrusion (12) and, thereby, may regulate its own uptake.

These results support the hypothesis that ABA plays a role in the adaptation to freezing. The full expression of hardness in tissue culture may be limited by the ratio of the inoculum to the culture medium, the presence of growth regulators, organic solvents such as ethanol, and the availability of nutrients within the medium. By adjusting culture technique we have increased the maximum level of hardiness of bromegrass cells to beyond −60°C as compared to −30°C as previously reported by Chen and Gusta (4).

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