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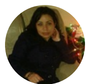
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ABSTRACT

Glycine betaine is a quaternary ammonium compound that accumulates in a large variety of species in response to different types of stress. Glycine betaine counteracts adverse effects caused by abiotic factors, preventing the denaturation and inactivation of proteins. Thus, its determination is important, particularly for scientists focused on relating structural, biochemical, physiological, and/or molecular responses to plant water status. In the current work, we optimized the periodide technique for the determination of glycine betaine levels. This modification permitted large numbers of samples taken from a chlorophyllic cell line of the grass *Bouteloua gracilis* to be analyzed. Growth kinetics were assessed using the chlorophyllic suspension to determine glycine betaine levels in control (no stress) cells and cells osmotically stressed with 14 or 21% polyethylene glycol 8000. After glycine extraction, different wavelengths and reading times were evaluated in a spectrophotometer to determine the optimal quantification conditions for this osmolyte. Optimal results were obtained when readings were taken at a wavelength of 290 nm at 48 h after dissolving glycine betaine crystals in dichloroethane. We expect this modification to provide a simple, rapid, reliable, and cheap method for glycine betaine determination in plant samples and cell suspension cultures.

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On a global scale, water shortage is the main ecological problem for food production in rain-fed agriculture. Consequently, a significant portion of genetic improvement efforts are aimed at obtaining plants with higher tolerance to water stress. Tolerance to water stress in plants is controlled by several genes that act in an additive manner [1,2].

A substantial increase in the cellular concentrations of osmotically active compounds, termed compatible solutes, has been observed in a vast number of organisms in response to salinity or drought stress [3–5]. Inorganic solutes such as K^+, Na^+, and Cl^- can also increase during osmotic stress, but Na^+ and Cl^- interfere with cellular activities and need to be compartmentalized to the vacuole [6]. For a solute to be compatible, a considerable increase in its concentration must not interfere with the normal metabolic functions of the cell. Some of the best-known osmolytes contain quaternary ammonium (glycine betaine), amino acids (proline, glycine, and taurine), polyols (glycerol, inositol, and sorbitol) and their derivatives (methyl-inositol), or sugars (mannitol, sorbitol, sucrose, and trehalose), among other compounds. How these compatible solutes protect cells against damage from osmotic stress is still a matter of debate [7,8]. An early hypothesis was that compatible solutes may help cells to conserve remnant water by biophysically functioning as water-attracting or water-conserving molecules, thereby maintaining cell turgor. A widespread hypothesis points to the interactions between elements of the ternary complex water–osmolytes–proteins as the underlying phenomenon [9], where osmolytes modulate biochemical reactions leading to the preferential exclusion mechanism, where the stabilizing solutes do not bind to proteins; on the contrary, they are excluded from a protein's hydration layer through conformational changes to fold up more

Abbreviations: GB, glycine betaine; PEG 8000, polyethylene glycol 8000; RWC, relative water content; SWC, soil water content; OD, optical density.

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compactly in order to reduce exposure to thermodynamically unfavorable interactions with the stabilizing solute [10]. A third hypothesis is that compatible solutes function as scavengers of reactive oxygen species [11].

Glycine betaine (GB) is an amphiphilic compound with a hydrophobic positive end and a hydrophilic negative end, and it is electrically neutral over a wide range of pH values [12]. This osmolyte is synthesized in several families of plants such as Chenopodiaceae, Amaranthaceae, Avicenniaceae, Convolvulaceae, Plumbaginaceae, Solanaceae, Leguminosae, Asteraceae, Malvaceae, Poaceae, Portulacaceae, and Caryophyllaceae. However, few families are able to accumulate quantities of this osmolyte sufficient to achieve an osmotic effect [13]. In higher plants, the enzymes that synthesize GB are found in the chloroplast stroma [14,15].

GB is the most effective compatible solute for the improvement of salinity and drought tolerance in higher plants [16]. By interacting with both hydrophilic and hydrophobic domains of macromolecules, this osmolyte is involved in reducing lipid peroxidation [17], neutralizing high NaCl concentrations [10], maintaining thylakoid membrane integrity [18], and stabilizing the structure of proteins from the damaging effects of abiotic factors such as drought, salinity, and freezing [12].

The properties of osmolytes are useful in agriculture, cell biology and biotechnology [10,17] because they are indicators of suboptimal environmental conditions. In particular, plant breeders interested in developing crops that are more tolerant of drought, salinity, and freezing require reliable, efficient, and affordable techniques for detecting osmolytes in a time-efficient, low-cost, and rapid manner. Experimental evidence shows that overexpression or incorporation of the biosynthesis of some osmolytes in the genome of some plants can result in increased tolerance to abiotic factors in plants [11].

Plant cell cultures are important systems for the study and isolation of genes related to water tolerance. Using these biological systems, several studies have been performed to analyze the physiological, molecular, and biochemical processes operating during saline stress [19], osmotic stress [20–24], and cold [25].

Cell cultures with high chlorophyll content, such as the chlorophyllic system studied in this work, offer additional advantages because certain enzymes of the plant's metabolism are located in chloroplasts [26,27]. Chloroplasts are important within the biotechnology of water stress [28,29] due to the confinement of certain compatible solutes (or enzymes involved in their biosynthesis) in these cellular compartments. For example, the osmoregulator glycine betaine is mainly located in chloroplasts [30], where it stabilizes the photosynthetic apparatus [31], and therefore the photosynthetic rate, during stressful conditions [32].

Determination of glycine betaine levels currently requires sophisticated and costly equipment such as a refraction index detector [33], a mass spectrometer [34,35], nuclear magnetic resonance spectroscopy [11], and pyrolitic instrumentation.

Grieve and Grattan [36], Stumpf [37], and Arakawa and coworkers [38] developed different methods for determining this compound using affordable equipment such as the spectrophotometer. Currently, the periodide method of Grieve and Grattan [36] is the method most widely used to precipitate quaternary ammonium compounds for glycine betaine determination. This technique, although successful in a large variety of species, is not efficient when applied to certain systems, such as the plant cell suspension culture analyzed here, because they contain large amounts of quaternary ammonium compounds and cause GB sedimentation, making this technique tedious and time-consuming.

The aim of this research was to optimize the periodide method for determining glycine betaine levels in recalcitrant samples such as chlorophyllic cell cultures of the grass *Bouteloua gracilis.*

Materials and methods

Chlorophyllic cell suspension growth kinetics

B. gracilis chlorophyllic cells were routinely cultivated in 125-ml flasks containing 25 ml of liquid MPC medium under optimal growth conditions [39]: continuous fluorescent light, 77 μmol s^{-1} m^{-2}, 33 ± 1 °C temperature, and shaking at 90 rpm. The MPC medium contained the basal salts and vitamins of MS medium [40], 2 mg L^{-1} 6-benzylaminopurine (BAP), 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 40 mg L^{-1} adenine, and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 before sterilization (120 °C/15 min).

Growth kinetics were assessed by culturing the chlorophyllic cells under either normal or hyperosmotic conditions. Osmotic treatment was achieved by adding polyethylene glycol 8000 (PEG 8000) to the basal MPC medium at a concentration of 14 or 21% PEG. An initial 8-day stock cell culture was used to inoculate 0.4 g FW cells into 120-ml flasks containing 25 ml of the different liquid media. The cells from the initial stock culture were considered the day 0 material in the growth kinetics. All cell growth kinetics were assessed under the same environmental conditions described before for the routine culture of the chlorophyllic cells. After initial sampling at day 0, cells were further collected at days 3, 6, 9, and 12 after inoculation of the 120 flasks (10 repetitions per treatment). Complete cell growth kinetics, including MPC, 14% PEG, and 21% PEG treatments, were repeated at least four times. All harvested cell material was lyophilized for further glycine betaine determinations.

Plants of tomato and wheat grown under greenhouse

To further test this technique, glycine betaine analyses were performed in monocot and dicot species. Tomato (*Solanum lycopersicum*), CID hybrid, and wheat (*Triticum aestivum*) cv. Tlaxcala seeds were sown in 1-L containers with peat moss (Sunshine) and then transplanted into 1-L vessels with sandy loam soil. Plants were grown under greenhouse conditions (minimum/maximum air temperature of 26/55 °C and relative humidity between 8 and 90% at mid-day during the experimental period). Irrigation was applied every other day, and the drought treatment began 40 days after planting. Plants were subjected to two water regimes: (i) optimal regime (control), where plants were constantly irrigated to maintain 21% of soil moisture content (field capacity of this soil was 16.1%), and (ii) drought treatment, without irrigation until the substrate reached permanent wilting point (PWP for this soil was 9.5%).

After treatments, in addition to determination of GB in leaves and roots, relative water content (RWC) and soil water content (SWC) were evaluated. SWC was estimated by the gravimetric method as recommended by Ortiz-Villanueva and Ortiz-Solorio [41], whereas the estimation of RWC was based on the methods described by Salisbury and Ross [42].

GB determination in chlorophyllic cell suspension

The concentration of glycine betaine was determined for all treatments and sampling dates using a modification of the method described by Grieve and Grattan [36], which is described below. H_2SO_4 (1.5 ml of 2N) was added to 1 mg lyophilized cells, and the mixture was heated up to 60 °C in an Eppendorf ThermoMixer C for

10 min; this step permits extraction of the quaternary ammonium compounds. To precipitate the cells and recover the supernatant, the mixture was centrifuged at 14,000 rpm for 10 min at room temperature. Because our material contained large amounts of glycine betaine, dilutions needed to be performed as follows: dilution 1:1 = 250 μl of supernatant +0 μl of H_2SO_4 2N (calibration curve) using a standard for the calibration curve (Sigma–Aldrich, St. Louis, MO, USA), dilution 1:2 = 125 μl of supernatant + 125 μl of H_2SO_4 2N (control samples), and dilution 1:4 = 62.5 μl of supernatant + 187.5 μl of H_2SO_4 2N (14 and 21% PEG-treated samples). Later, they were mixed and placed in a new tube containing 125 μl of sample + 50 μl of cold KI-I_2 (prepared with 15.7 g of iodine and 20 g of KI in 100 ml of sterilized water). The latter compound causes the precipitation of glycine betaine in the form of golden crystals.

After adding KI-I_2, the remaining steps were performed under darkness because light degrades the color of the samples. Then, the samples were stirred gently to prevent the mixture from reacting with remnants of KI-I_2 deposited on the lid of the Eppendorf tube, which can result in undesirable staining of the mixture. The tubes were stored at 0 to 4 °C for 16 h and then centrifuged at 14,000 rpm for 30 min at 0 °C. The supernatant was carefully eliminated so that only crystals of glycine betaine remained fixed to the walls and bottom of the tubes; samples should always be managed at low temperatures because glycine betaine crystals easily dissolve in the medium when the temperature is higher than 0 °C. Then the precipitate was diluted into 1.4 ml of 1,2-dichloroethane, and finally the absorbance was read using a spectrophotometer.

To determine the optimal wavelength and the adequate time for reading samples, the following tests were performed. First, the wavelengths 280, 290, 300, 310, 320, 330, 340, 350, 365, 400, 467, 500, 550, and 600 nm were evaluated for optimal determination of glycine betaine. After determining the optimal wavelength, the following times after dissolving the samples in 1,2-dichloroethane were tested: 24, 26, 28, 38, 40, 42, 44, 46, 48, 68, and 144 h.

GB determination in tomato and wheat plants

The same methodology, as described above for cell suspension, was used for GB determination in leaves and roots of plants grown under greenhouse. Lyophilized tissue (1 mg) was used to precipitate the tissue and recover the supernatant, and the mixture was centrifuged at 14,000 rpm for 25 min at room temperature (centrifugation was for 10 min in cell suspension); centrifugation time was increased in relation to cell suspension to ensure precipitation of tissue. Dilutions of 1:1, 1:2, and 1:4 were tested to determine the optimal one for these tissues; readings in the spectrophotometer were performed 2–2.5 h [36] and 48 h after dissolving the crystals in 1,2-dichloroethane.

Results and discussion

The calibration curve for glycine betaine was successful using the method described by Grieve and Grattan [36]; however, after applying the protocol developed by these authors to samples derived from *B. gracilis* chlorophyllic cells, the glycine betaine crystals were not diluted into the 1,2-dichloroethane (Fig. 1).

As shown in Fig. 1, glycine betaine completely dissolved in the control unstressed samples but not in cell samples treated with PEG 8000, which accumulated significant amounts of this osmolyte. The strategy used to solve this problem was to use the lowest amount of lyophilized cells (1 mg) and then perform dilutions with H_2SO_4 2N.

After the cells were weighed (1 mg), rather than shaking them in deionized water for 24 h (as indicated by Grieve and Grattan [36]), the samples were treated with 1.5 ml of H_2SO_4 2N and heated at 60 °C for 10 min; following this modification, the same results were achieved in less time.

Another important contribution to the original method was to perform the steps subsequent to the addition of KI-I_2 in the dark because it was clear that exposure of the samples to light altered their color and consequently reduced the readings in the spectrophotometer.

Likewise, samples with KI-I_2 must be shaken slowly and carefully to prevent the mixture from contacting the lid of the Eppendorf tube because the glycine betaine crystals diluted in 1,2-dichloroethane will react with KI-I_2 remaining on the lid, turning the original yellow color of the mixture into light pink, which reduces the reading in the spectrophotometer.

Fig. 1B and C shows glycine betaine crystals with some black spots that probably correspond to other quaternary ammonium compounds and free forms of periodide. Grieve and Grattan [36] mentioned that total quaternary ammonium compounds are precipitated as periodide complexes when an acidic pH is used.

Tests were performed to determine the optimal wavelength at which to read the glycine betaine in the spectrophotometer. The wavelengths evaluated ranged from 300 to 600 nm in 50-nm intervals. Instead of considering wavelengths of 350 and 450 nm, we initially evaluated the wavelengths 365 nm [36] and 467 nm [37]. When comparing the trends of the lines in Fig. 2A and B, the calibration curve shows that glycine betaine is best determined at wavelengths of 300–365 nm because the readings observed between 400 and 550 nm (Fig. 2A) are due not only to the concentration of glycine betaine but also to other quaternary ammonium compounds. Considering these results, a new wavelength sweep was performed from 280 to 365 nm in 10-nm intervals (Fig. 3).

Fig. 3 shows that when samples are read at a wavelength of 290 nm, there is a greater difference among the different points of the calibration curve, controls, and recovery; therefore, this wavelength was adequate for the glycine betaine determinations.

Grieve and Grattan [36] mentioned in their methodology that readings of glycine betaine in the spectrophotometer must be performed 2–2.5 h after dissolving the crystals in 1,2-dichloroethane. Figs. 2B, 4A and B show readings taken at different times. The values observed in the calibration curve did not change, although the OD value of the day 6 control cells (control cells, d6) increased with time, providing the highest concentration when the reading was performed 21 h after dissolving the glycine betaine crystals in 1,2-dichloroethane. This phenomenon may indicate that more time is required to dissolve the glycine betaine crystals in samples obtained from *B. gracilis* chlorophyllic cells. Therefore, tests were performed to determine the adequate time at which the samples must be read.

Fig. 5 shows again that the concentrations of glycine betaine obtained in the calibration curve do not vary with time, unlike the readings performed with the chlorophyllic cell samples. The adequate time to take the cell sample readings was within the 44- to 48-h range. After this time, the readings increased very slightly, although the value of the reagent blank also increased, and the values were decreasing gradually.

Once the previous parameters were optimized, the readings for the glycine betaine calibration curve and for the cell growth kinetics samples were performed at a wavelength of 290 nm and 48 h after dissolving the crystals in 1,2-dichloroethane. For the calibration curve, the concentrations considered were 150, 200, 250, and 300 μg ml^{-1} glycine betaine because the OD values obtained at these concentrations fall within the overall readings registered in all treatments (Fig. 6).

Greater concentrations of glycine betaine were observed in the cells that underwent osmotic stress due to gene expression generated by the high concentration of polyethylene glycol. The

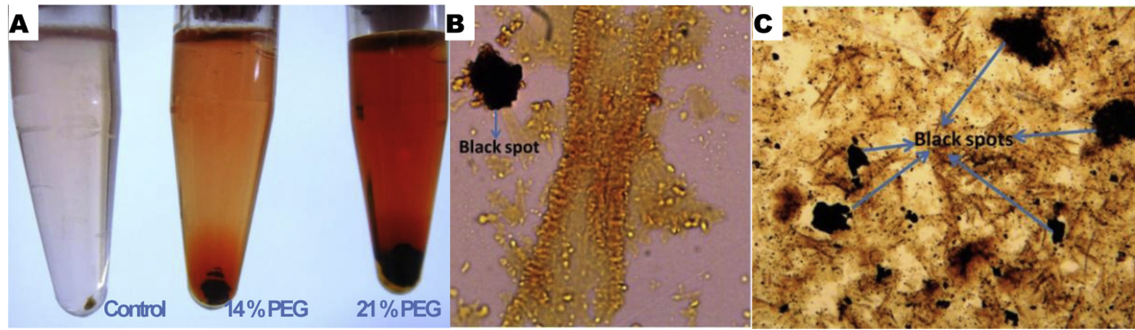

Fig. 1. Glycine betaine crystals and their sedimentation. (A) Sedimentation of glycine betaine in chlorophyllic cell growth cultures treated with 0, 14, or 21% PEG. (B) Glycine betaine crystals observed at 40 × magnification. (C) Glycine betaine crystals observed at 50 × magnification.

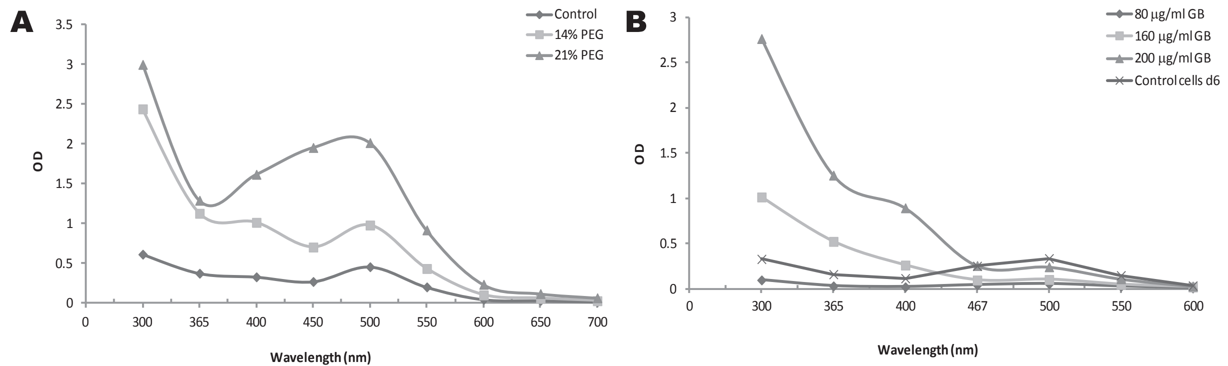

Fig. 2. Evaluation of the optimal wavelength for glycine betaine determination within the range of 300–600 nm. (A) Readings of the 0, 14, and 21% PEG treatments 2.5 h after dissolving the crystals in 1,2-dichloroethane. (B) Three-point reading of the calibration curve and the control treatment 2.5 h after dissolving the crystals.

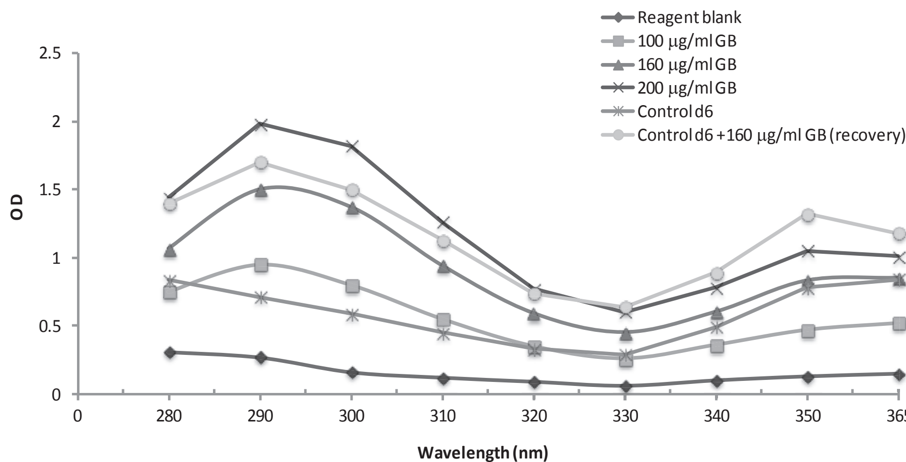

Fig. 3. Evaluation of the optimal wavelength for glycine betaine determination within the range of 280–365 nm.

highest concentration of glycine betaine (1413 µg/mg DW) was obtained in chlorophyllic cells treated with 21% PEG. However, glycine betaine peaked at day 3 in cells treated with 14 or 21% PEG (Fig. 7); this augmentation represents a 3-fold increase in relation to control cells.

RWC and SWC in plants grown under greenhouse were respectively 94.1 and 21.6% for the control treatment of wheat, 61.4 and 6.1% for the drought treatment of wheat, 83.4 and 21.0% for the control treatment of tomato, and 67.5 and 7.7% for the drought treatment of tomato.

Dilution 1:4 was optimal for this methodology in both wheat and tomato plants, and readings in the spectrophotometer taken 2–2.5 h after dissolving the crystals in 1,2-dichloroethane, as indicated by Grieve and Grattan [36], were the best ones.

Wheat and tomato plant tissue had less GB than *B. gracilis* chlorophyllic cells (Fig. 8). Wheat plants did not present differences between the control and drought treatments in concentration of GB, in agreement with previous reports [43] where 10 varieties of bread wheat (*T. aestivum*) did not differ statistically between the control and treatment of plants submitted to saline conditions; in contrast, differences in concentration of GB were observed between

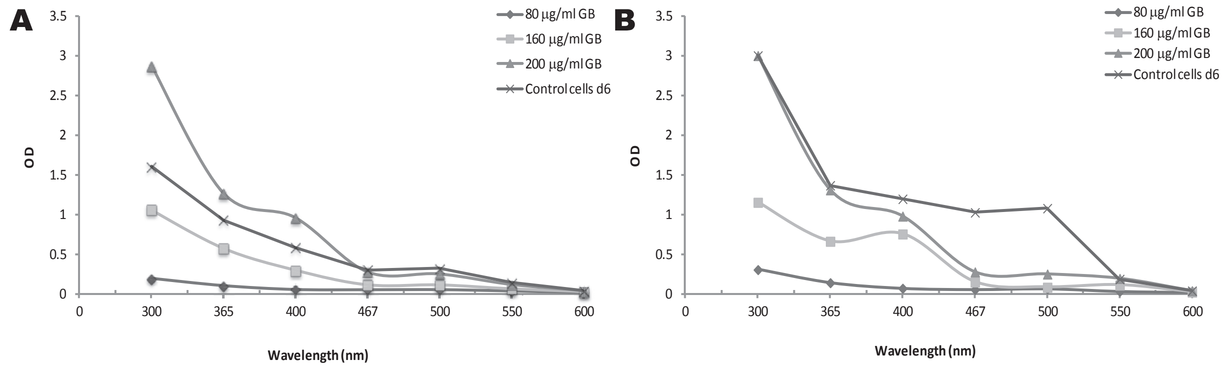

Fig. 4. Evaluation of optimal reading time for samples of glycine betaine derived from *B. gracilis* chlorophyllic cells. Three-point readings of the calibration curve and the control treatment 6 h (A) and 12 h (B) after dissolving the crystals in 1,2-dichloroethane are shown.

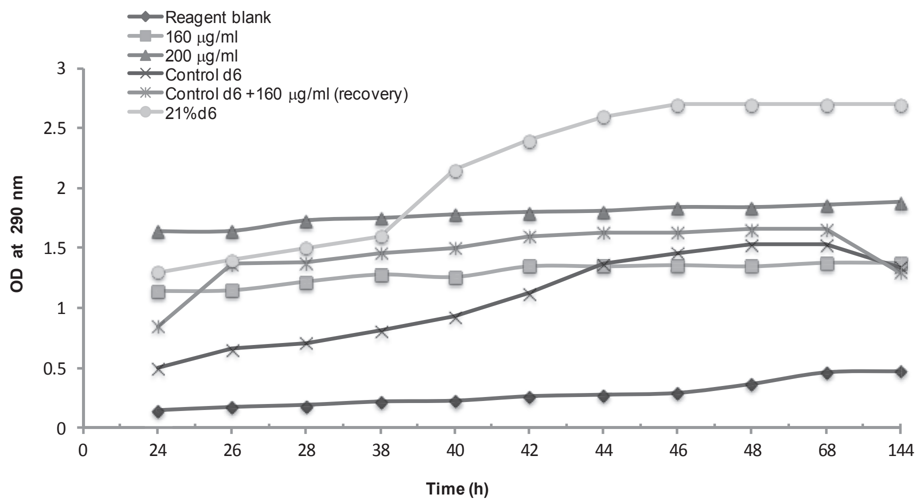

Fig. 5. Glycine betaine readings at 290-nm wavelength and different times after crystal dissolution in 1,2-dichloroethane.

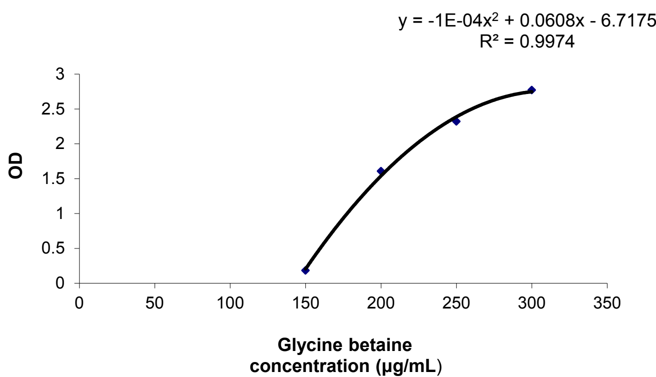

Fig. 6. Glycine betaine calibration curve obtained after optimization of wavelength and reading time after dissolution of glycine betaine crystals in 1,2-dichloroethane.

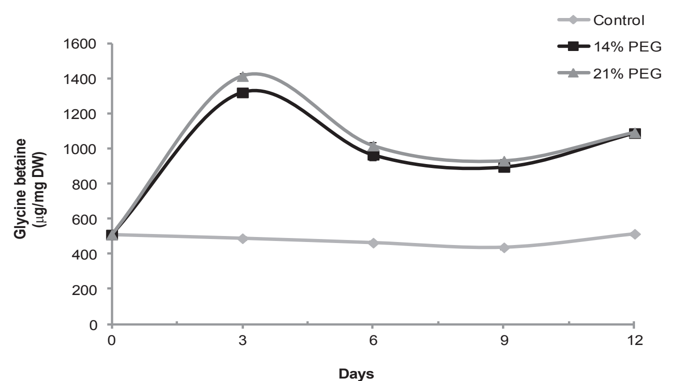

Fig. 7. Concentration of glycine betaine obtained after averaging the results of the four cell growth kinetics assessments performed for all osmotic treatments.

the control and drought treatment in both leaves and roots of tomato plants. The largest concentration of GB (745 μg/mg DW) was observed in leaves of tomato submitted to drought conditions.

Conclusions

The technique developed by Grieve and Grattan [36] was optimized to achieve fast and reliable spectrophotometric determinations of glycine betaine in samples containing high levels of quaternary ammonium compounds such as the chlorophyllic cells of the very drought-tolerant grass *B. gracilis*. Modifications to the original protocol included reading the samples at an optimal wavelength of 290 nm and allowing a time of 48 h after dissolving the glycine betaine crystals in 1,2-dichloroethane. After correction of the original protocol, the readings from cells treated with 14 or 21% polyethylene glycol were highest at day 3 of the cell culture,

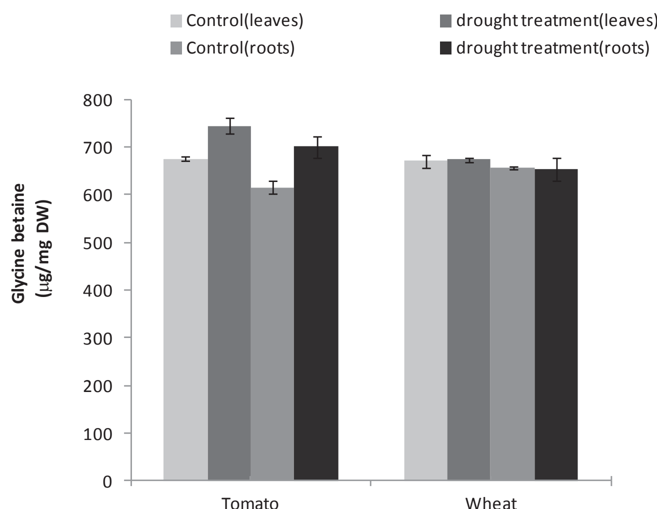

Fig. 8. Concentration of glycine betaine obtained in leaves and roots of tomato and wheat plants grown under greenhouse.

and the highest values of glycine betaine was obtained in cells treated with 21% PEG. The technique proved to be effective for determining GB content in systems different from cell suspension cultures such as leaf and root tissues of monocot and dicot species.

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