Jap. J. Phycol. (Sôrui) 32: 300-306. December 10, 1984

Contribution of glycerol to osmoregulation in *Dunaliella tertiolecta* under magnesium hypertonicity

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FUJII, S., YAMAMOTO, R. and TAKADA, H. 1984. Contribution of glycerol to osmoregulation in *Dunaliella tertiolecta* under magnesium hypertonicity. Jap. J. Phycol. 32: 300-306.

The intracellular concentration of glycerol as an osmotic substance was sufficient to osmotically balance the external concentration of NaCl. Glycerol was the main osmoticum for osmotic adjustment in cells of *D. tertiolecta* cultured in a hypertonic medium with $MgSO_4$ and/or Na_2SO_4 as well as NaCl. Glycerol formation seemed to depend on the osmotic pressure in the medium, irrespective of the kind of cation or anion present. However, contents of amino acids, reducing sugars and potassium ions in the cells did not change in response to the increase in the osmotic concentrations of the media.

Key Index Words: Dunaliella; *glycerol*; *magnesium hypertonicity*; *osmoregulation*.

Unicellular green algae of the genus *Dunaliella* show outstanding adaptability and tolerance towards a very wide range of salinities from seawater to saturated salt solutions. Therefore, *Dunaliella* cells must have the osmoregulatory mechanisms to maintain suitable osmotic pressure against environmental pressure. To maintain osmotic pressure in a cell, various osmotica, which are active as osmotic substances, should be needed. The glycerol content in *Dunaliella* cells cultured in NaCl media of various concentrations has been reported to increase linearly with increasing extracellular NaCl concentration (BEN-AMOTZ and AVRON 1973 and 1981, FRANK and WEGMANN 1974). The osmotic pressure supported by intracellular glycerol in cells cultured over a wide range of NaCl concentrations has not been well examined. Thus, it is not clear whether or not glycerol is the main osmoticum in the osmoregulation of *Dunaliella*. To elucidate the role of the glycerol, the osmotic pressures derived from the intracellular glycerol content in the cells should be estimated and compared with the osmotic pressure of the medium.

Previously, we reported that *D. tertiolecta* could grow in $MgSO_4$-hypertonic medium as well as in NaCl-hypertonic medium but not in a hypertonic medium with $MgCl_2$ or $Mg(NO_3)_2$ (FUJII *et al.* 1983). In this study, we first checked whether the cells of *D. tertiolecta* cultured in a hypertonic medium with a salt other than NaCl could produce glycerol. We also estimated the osmotic pressures derived from the intracellular glycerol content in the cells and compared them with the osmotic pressures of the medium to evaluate the contribution of intracellular glycerol to osmoregulation.

Materials and Methods

Material: Cells of the green alga *Dunaliella tertiolecta*, LB 999, were cultured in a basal medium with a hypertonic concentration of $MgSO_4$, NaCl or Na_2SO_4 as described previously (FUJII *et al.* 1983). In all cultures, the initial cell number was 10^4 cells per m*l* of medium. Although the cultures were not axenic, no bacteria were observed microscopically. The cell number was determined using a microscope with a haemacytometer.

Determinations of glycerol, amino acids, reducing sugars and potassium ion: To obtain the cell extract, the cells were spun down by centrifugation at 3000 r.p.m. for 10 min resuspended in the same volume of distilled water and heated at 100°C for 5 min to inactivate the enzymes. The cell suspension was then treated with a sonicator (Tomy Seiko Co., Ltd., Model UR-200P) at 5°C for 3 min and centrifuged at 15000 r.p.m. for 10 min.

The glycerol concentration in the extract was determined enzymatically using the Biochemical Test Combination "glycerol and neutral fat" (Toyobo Co., Ltd.). A 0.02 m*l* portion of the extract was added to 3 m*l* of the enzyme solution. After the mixture had been incubated for 15 min at 37°C, the optical density was measured at 545 nm at room temperature (Hitachi, Ltd., spectrophotometer Model 220A).

The amino acids in the extract were measured according to the method of GARREL *et al.* (1972), and calculated as the L-leucine equivalent. Ninhydrin reagent contained 0.4 g ninhydrin, 80 m*l* 95% ethanol, 1 g $CdCl_2$ and 5 m*l* acetic acid in 20 m*l* water. The extract of 0.2 m*l* was incubated with 2.5 m*l* the ninhydrin reagent at 80°C for 10 min, and the optical density was measured at 506 nm.

The reducing sugars in the extract were measured by the method of Somogyi and Nelson (SOMOGYI 1952). The extract of 1 m*l* was mixed with 1 m*l* of a copper solution, heated in boiling water for 10 min, and cooled quickly. After addition of 1 m*l* the Nelson reagent, the mixture was made up to 25 m*l* with distilled water, its optical density at 500 nm was measured, and the glucose equivalent was calculated.

To measure the amount of potassium ion in the extract, 10 m*l* of it was mixed with 40 m*l* of distilled water and 5 m*l* of the ionic strength adjustor. Then the K^+ content in the sample was measured electronically with an ion meter (Toa Electronics Ltd., Model IM-20E).

Determination of osmotic pressure: The cells of *D. tertiolecta* cultured in a medium with one of the three salts, were photographed through a microscope. Their mean cell volume was calculated assuming that all cells were ellipsoidal. The osmotic pressure of the medium after removal of the cells by centrifugation was measured using a vapor pressure osmometer (Wescor Model 5100C).

Results

a) Growth of *D. tertiolecta* in hypertonic media containing various salts.

Growth experiments of *D. tertiolecta* cultured in hypertonic media with different salts showed that no growth occurred in $MgCl_2$- and $Mg(NO_3)_2$-hypertonic media, as previously reported (FUJII *et al.* 1983), but good growth occurred in NaCl-, Na_2SO_4-, $NaNO_3$- and $MgSO_4$-hypertonic media (Table 1).

b) Effect of salts on contents of var-

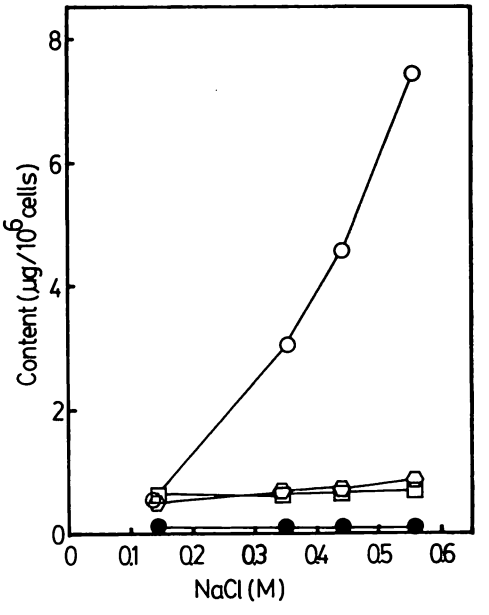

Fig. 1. Contents of various osmotica in *Dunaliella* cells maintained at different NaCl concentrations. Algae were cultured for 4 days in NaCl media of the indicated concentrations, and then contents of various osmotica in its cell extract were analyzed. Data represent the averages of three experiments. (○) glycerol; (□) amino acids; (○) reducing sugars; (●) K^+.

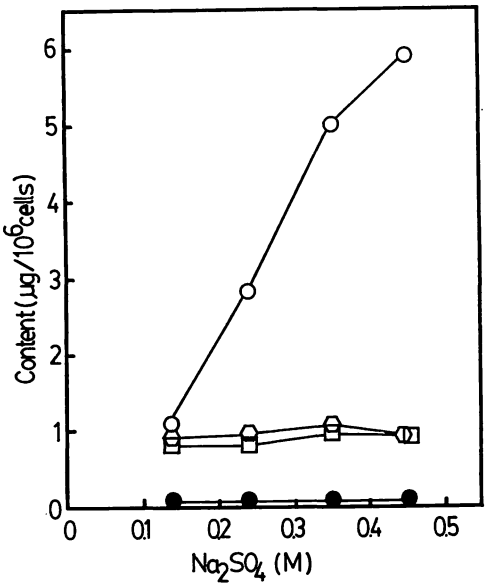

Fig. 2. Contents of various osmotica in *Dunaliella* cells maintained at different Na_2SO_4 concentrations. Algae were cultured for 4 days in Na_2SO_4 media of the indicated concentrations and then contents of various osmotica in its cell extract were analyzed. Data represent the averages of three experiments. (○) glycerol; (□) amino acids; (○) reducing sugars; (●) K^+.

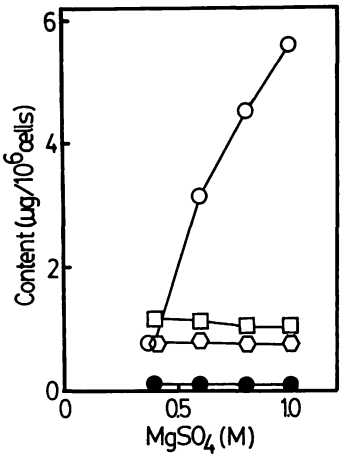

Fig. 3. Contents of various osmotica in *Dunaliella* cells maintained at different $MgSO_4$ concentrations. Algae were cultured for 4 days in $MgSO_4$ media of the indicated concentrations and then contents of various osmotica in its cell extract were analyzed. Data represent the averages of three experiments. (○) glycerol; (□) amino acids; (○) reducing sugars; (●) K^+.

ious osmotica in cells.

D. tertiolecta cells were cultured in a hypertonic medium with NaCl, Na_2SO_4 or $MgSO_4$ and contents of intracellular glycerol, reducing sugars, amino acids and K^+ per 10^6 cells were determined. As shown in Figs. 1, 2 and 3, the intracellular glycerol increased in approximately a linear relationship to the external salt concentration, while amino acids, reducing sugars and K^+ content of cells were almost constant in spite of the increasing salt concentration. Thus, glycerol seems to be a major, and probably the sole, osmoregulating agent in the cells. Proof of this requires that the osmotic pressure due to intracellular glycerol and that of ambient solution are determined.

c) Osmotic pressures of NaCl, Na_2SO_4, $MgSO_4$ and glycerol solutions.

Since the osmotic pressure of an electrolyte depends on its extent of dissociation, the osmotic pressure of each medium with NaCl. Na_2SO_4 or $MgSO_4$ was measured with the vapor pressure osmometer (Fig. 4). A linear relationship was found between the osmotic pressure and the concentration of each salt.

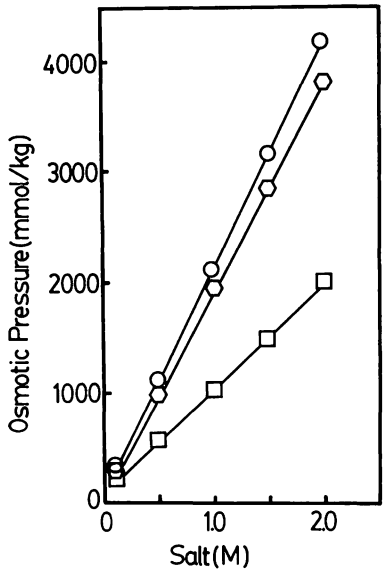

Fig. 4. Osmotic pressures of media with NaCl, Na_2SO_4 or $MgSO_4$. The osmotic pressure was measured with a vapor pressure osmometer at room temperature. (○) NaCl; (○) Na_2SO_4; (□) $MgSO_4$.

At the same concentration, the highest osmotic pressure was obtained from Na_2SO_4 and the lowest one from $MgSO_4$. The medium with 0.5 M NaCl was approximately isotonic with that of 0.45 M Na_2SO_4 or 0.94 M $MgSO_4$.

To estimate the osmotic pressure due to intracellular glycerol, that of glycerol aqueous solution at various concentrations was measured with the vapor pressure osmometer (Fig. 5). The concentration of glycerol up to 1 M was virtually equal to the osmotic pressure. In the following experiments, the values of osmotic pressure thus obtained were used.

d) Changes in the mean cell volume of cells cultured in NaCl-, Na_2SO_4- and $MgSO_4$-hypertonic media.

As the intracellular concentrations of glycerol were estimated by dividing the intracellular content of glycerol by the cell volumes, we determined the cell volume. Figure 6 shows the changes in the cell volume of *D. tertiolecta* cultured in media of different osmotic pressures containing NaCl, Na_2SO_4 or $MgSO_4$.

e) Contribution of glycerol to osmotic adjustment.

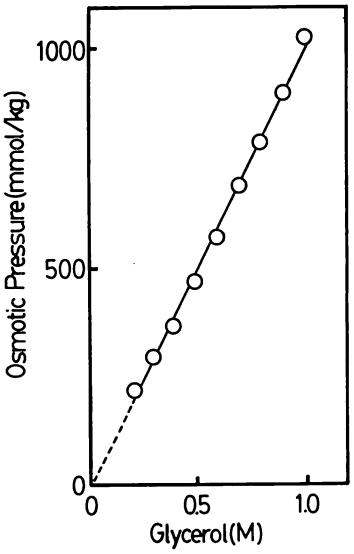

Fig. 5. Osmotic pressures of glycerol aqueous solutions of different concentrations. The osmotic pressure was measured with a vapor pressure osmometer at room temperature.

To elucidate the contribution of intracellular glycerol to osmotic adjustment, the osmotic pressures arising from the concentrations of

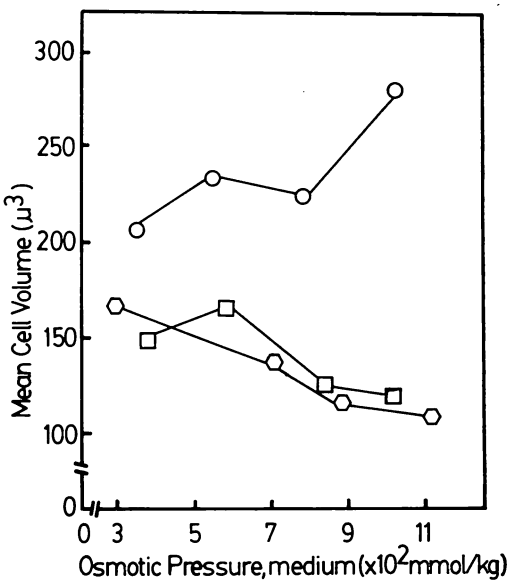

Fig. 6. Mean cell volumes of *D. tertiolecta* cells cultured for 4 days in media of different osmotic pressures containing NaCl, Na_2SO_4 or $MgSO_4$. The cells were photographed through a microscope, and then the mean cell volume was calculated, assuming that the cells had ellipsoidal shapes. (○) NaCl; (□) Na_2SO_4; (○) $MgSO_4$.

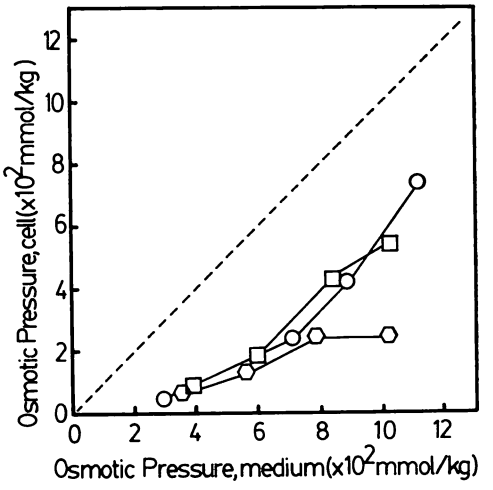

Fig. 7. Contribution of glycerol to osmotic adjustment estimated on the basis of the data shown in Fig. 5. The dotted line represents the osmotic pressure of intracellular glycerol which is equal to that of the culture medium. (○) NaCl; (□) Na_2SO_4; (○) $MgSO_4$.

intracellular glycerol were obtained on the basis of the data in shown in Fig. 5 and compared with the osmotic pressures of the culture medium. The estimated osmotic pressure due to intracellular glycerol was plotted against the osmotic pressure of the culture medium, as shown in Fig. 7, where the dotted line represents the osmotic pressure of intracellular glycerol equal to that of culture medium.

f) Contribution of glycerol to osmoregulation in cells cultured in media with higher concentrations of NaCl.

As seen in Figs. 1, 2 and 3, only intracellular glycerol increased in relation to the concentrations of salts in media. However, the contribution of intracellular glycerol was not 100% as shown in Fig. 7, indicating that other soluble components contribute as osmotica.

The contents of various components in cells cultured in media with concentrations of NaCl higher than those in Fig. 1 were measured by the methods described above, because the solubilities of Na_2SO_4 and $MgSO_4$ were not larger that that of NaCl. The results are shown in Fig. 8. The changes in

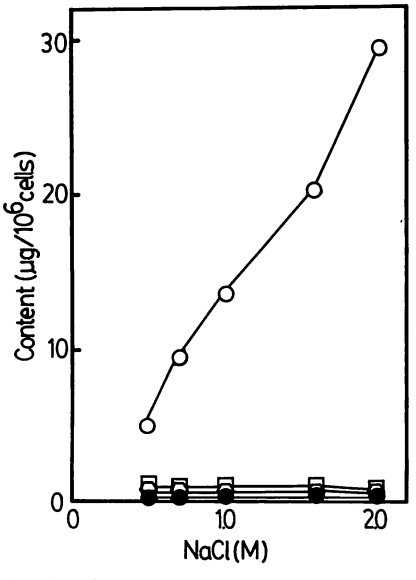

Fig. 8. Contents of various osmotica in *Dunalielia* cells cultured for 4 days in medium with higher concentrations of NaCl than those in Fig. 1. Data represent the averages of three experiments. (○) glycerol; (□) amino acids; (◇) reducing sugars; (●) K^+.

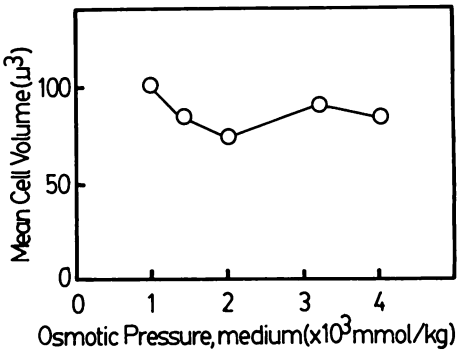

Fig. 9. Mean cell volume of *Dunaliella tertiolecta* cells cultured for 4 days in NaCl media of different osmotic pressures. The cells were photographed through a microscope, and then the mean cell volume was calculated, assuming that the cells had ellipsoidal shapes.

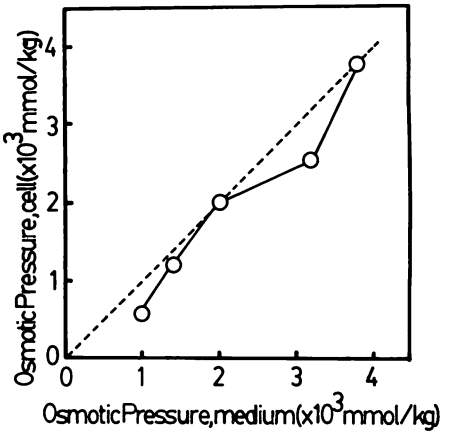

Fig. 10. Contribution of glycerol to osmotic adjustment estimated on the basis of the data in Fig. 5. The dotted line represents the osmotic pressure of intracellular glycerol which is equal to that of the culture medium.

Table 1. Effect of hypertonicity with various salts on the growth of *Dunaliella tertiolecta*.

NaCl	$NaNO_3$	Na_2SO_4	$MgCl_2$	$Mg(NO_3)_2$	$MgSO_4$
++	++	++	−	−	++

D. tertiolecta was cultured for 4 days in 0.5 M NaCl, 0.5 M $NaNO_3$ 0.45 M Na_2SO_4, 0.35 M $MgCl_2$, 0.36 M $Mg(NO_3)_2$ or 0.94 M $MgSO_4$ containing the basal culture medium. The initial cell number was 10^4 per ml (++ : good growth; − : no growth).

cell volume with increasing NaCl concentration were measured in the same manner (Fig. 9) and the contribution of glycerol to osmoregulation was also evaluated (Fig. 10).

Discussion

Little has been reported on the effects of salt, except for NaCl, on the growth of *Dunaliella* and on osmoregulation in *Dunaliella* cells cultured in a hypertonic medium of salt other than NaCl. Previously, we reported that *D. tertiolecta* could grow in $MgSO_4$-hypertonic medium as well as NaCl-hypertonic medium (FUJII *et al.* 1983). Good growth was also observed in Na_2SO_4- or $NaNO_3$-hypertonic medium, but not in $MgCl_2$- or $Mg(NO_3)_2$-hypertonic medium. Therefore, when Mg salt was used, the growth pattern differed from that of Na salt.

For this strain to grow in a hypertonic medium of salt other than NaCl, osmoregulatory mechanisms are necessary to maintain suitable osmotic pressure within the organisms. As shown in Figs. 1, 2 and 3, glycerol may be produced within the cells to counterbalance the osmotic pressures of the medium with Na_2SO_4 or $MgSO_4$ as with NaCl. According to WEGMANN (1971), *D. tertiolecta* can grow in a hypertonic medium with sucrose or 2-deoxy-D-glucose as well as NaCl, with the cells producing glycerol to maintain the osmotic pressure within them. From these results, he suggested that this glycerol formation occurred in response to osmotic pressure rather than ionic strength. We also ascertained that the changes in glycerol were caused by changes in the osmotic pressure in the medium with $MgSO_4$ or Na_2SO_4 as well as NaCl as shown in Figs. 1, 2 and 3, respectively. This means that glycerol formation depends on the osmotic pressures in a medium, irrespective of the kind of cation or anion present.

Figure 7 shows the contribution of intracellular glycerol to osmotic adjustment of *D. tertiolecta* cultured in a medium with NaCl, Na_2SO_4 or $MgSO_4$. The osmotic pressure in cells derived from the intracellular glycerol content increased with an increase in the osmotic pressure of the medium. These results indicate that glycerol plays the leading role as the major osmoticum responsible for osmoregulation of *D. tertiolecta*, irrespective of the kind of salt. However, the extent of contribution by the intracellular glycerol was not 100% as shown in Fig. 7. Especially in the case of the $MgSO_4$-hypertonic medium, its contribution was lower than in that in the NaCl- or Na_2SO_4-hypertonic medium. This may be due to differences in the cell volume, because the cell volume of *D. tertiolecta* cultured in $MgSO_4$-hypertonic medium was about twice that in the NaCl- or Na_2SO_4-medium, although the glycerol contents per 10^6 cells were almost same in all cases. Study of the instantaneous shrinkage of *D. maria* cells transferred to hypertonic media containing copper showed that the shrinkage becomes smaller, the initial rate of volume readjustment becomes faster and the new steady-state volume progressively increases as the copper concentrations become higher (RIISGARD 1979, RIISGARD *et al.* 1980). Our result may indicate that Mg-ion hypertonicity has a different effect on the volume regulation of *D. tertiolecta* compared with Na-ion hypertonicity.

Other soluble components, that is amino acids, reducing sugars and cations, also had roles as osmotic agents. In fact, as pointed out by GINZBURG *et al.* (1983), glycerol was clearly not the only osmotic agent within the cells. And as reported by GIMMLER and SCHERLING (1978), Na or K cation in *D. parva* may have been partially responsible for the compensation of the external osmotic pressure. However, the K^+ content in *D. tertiolecta* did not increase in proportion to the salt concentration increase in the medium as shown in Figs. 1, 2 and 3. This difference may be due to a difference in species.

We evaluated the contribution of intracellular glycerol to the osmotic adjustment in cells cultured in media with higher NaCl concentrations than those as shown in Fig. 1. As seen in Fig. 10, the sum of the intracellular concentration of glycerol in cells

cultured in the medium with an osmotic pressure greater than 1000 mmol/kg (equivalent to 0.5 M NaCl medium) is sufficient to balance the external NaCl concentration. These results also show that intracellular glycerol is the major osmoticum and other soluble components are minor osmotic agents.

We evaluated the contribution of intracellular glycerol to the osmotic adjustment, assuming that the intracellular glycerol is homogeneously distributed within a *D. tertiolecta* cell. However, no definite evidence exists to verify this assumption. If glycerol is localized within an organelle, for example, a vacuole, the content of intracellular glycerol may be sufficient to the compensate for the external osmotic pressure.

Acknowledgement

The authors wish to express their thanks to Dr. S. MANTANI of Tezukayama College for his advice.

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藤井修平・山本良一・高田英夫：マグネシウム塩高張環境下における *Dunaliella tertiolecta* の浸透圧調節に対するグリセロールの寄与

D. tertiolecta の硫酸マグネシウム高張環境下における浸透圧調節を調べた。その結果，グリセロールのみが浸透圧調節物質として寄与していることを確かめた。塩化ナトリウムまたは硫酸ナトリウムの高張環境下についても同様に調べたところ，硫酸マグネシウム高張下の場合と同じ程度に，グリセロールが浸透圧調節物質として寄与していることがわかった。このことより，*D. tertiolecta* における浸透圧調節物質としてのグリセロール合成は，塩の種類ではなく，培地の浸透圧に依存していると考えられる。(631 奈良市学園南3丁目 帝塚山短期大学)