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2,3,5-Triphenyltetrazolium reduction in the viability assay of *Ulva fasciata* (Chlorophyta) in response to salinity stress

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Abstract. The salinity tolerance in a marine green macroalga *Ulva fasciata* was assessed by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to water-insoluble red formazan. Thallus strips of 0.1 gram fresh weight were incubated in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.4) containing TTC, and the formazan extracted by 95% ethanol was measured at 530 nm to avoid pigment interference. The TTC reduction activity reached the maximal level 18 h after incubation in 0.8% TTC solution in the presence of 3% NaCl. Plants of different sizes had similar TTC reduction activity, but activity in the 3-cm-long base near the rhizoidal portion was lower than in the remaining parts ($p<0.01$). On exposure of whole plants to various salinities, ranging from 5‰ to 150‰, the reduction of TTC in the 2.5 cm tip (the distal end) of the largest thallus peaked in plants grown at 30‰ and was low at salinity above 120‰ or below 5‰. Both the net photosynthetic O_2 evolution rate in the 2.5 cm tip of the largest thallus and the specific growth rate (%/d) had similar trends. Compared to plants grown at 30‰, the relative change in TTC reduction activity is associated with the relative change in specific growth rate (r^2=0.90) and also with the relative change in net photosynthetic O_2 evolution rate (r^2=0.93). These results show that *Ulva fasciata* survives at salinities ranging from 15‰ to 90‰. Tracking relative changes in TTC reduction activity has potential as a tool for predicting salinity tolerance.

Keywords: Salinity stress; TTC; *Ulva fasciata*; Viability.

Introduction

It is established that 2,3,5-triphenyltetrazolium chloride (TTC), a water-soluble, colorless compound, can be reduced to water-insoluble red formazan by a variety of organisms. A general redox potential level, maintained by the operation of several physiologically active systems, is suggested to bring about the reduction of tetrazolium. The dehydrogenase systems are responsible for this reduction (Roberts, 1951). TTC reduction is used as a quantitative method in the evaluation of tissue viability. The intensity and extent of TTC staining are successfully employed to predict the germinability percentage of a sampled lot of seeds (Bennett and Loomis, 1949; Parker, 1953). In addition, TTC reduction has been widely used in the viability assay of plant tissues exposed to stressful conditions. By comparing the respiration rate, Parker (1953) suggested that one of the best methods in determining the viability of conifer leaves after various drying and freezing treatments is the in vivo reduction of TTC. TTC reduction has also been employed in evaluating cold injury in leaf discs and stem segments of English Ivy (*Hedera helix* L. var. Thorndale) (Steponkus and Lanphear, 1967) and in rice seedlings (Lee et al., 1997). In the suspension tissue culture of *Acer saccharum* and *Haplopappus gracilus*, there is a quantitative correspondence between the decrease in plating assay and in TTC assay when these cells are subjected to freezing stress and plasmolysis (Towill and Mazur, 1974). In the case of *Ulva rigida* C. Agardh, a green macroalga belonging to the Chlorophyta, TTC reduction was also used in the determination of protoplasts' viability (Badini et al., 1994).

Since salinity is variable in intertidal regions, marine macroalgae from intertidal habitats develop an ability to resist salinity change (Biebl, 1962; Kirst, 1990). Although the physiological and biochemical responses to altered salinity have been well studied in a large amount of marine algae, and several parameters such as survival, growth rate, photosynthesis and/or respiration have been commonly used in determining the tolerance of algae to varying salinity (Kirst, 1990); a quantitative method capable of quickly predicting their future performance is still needed.

Ulva is a macroscopic green alga which grows in the intertidal regions of rocky shores and estuaries. It usually suffers frequent fluctuations of salinity due to the interaction of tides, rainfall, and evaporation. This study was conducted to examine the activity of in vivo reduction of TTC (expressed as formazan levels per gram fresh weight) and its usefulness in monitoring the viability of *Ulva fasciata* Delile in response to salinity stress. Because *U. fasciata* is an alga with seawater as its growing medium, the impact of NaCl in the incubation solution on the in vivo reduction of TTC was evaluated. In addition, deter-

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gents such as Tween 20 are generally employed in the incubation solution to enhance the uptake of TTC by tissues in land plants (Steponkus and Lanphear, 1967; Towell and Mazur, 1974), so the effects of 0.1% (v/v) Tween 20 were determined. The difference in the activity of TTC reduction among plants, thalli or different parts of the same thallus was compared. The relationships between the TTC reduction activity and the specific growth rate, and between the TTC reduction activity and the net photosynthetic O_2 evolution rate, were compared in plants grown at salinities ranging from 5‰ to 150‰.

Materials and Methods

Plant Materials and Treatments

Ulva fasciata Delile (Chlorophyta) was collected in June 1998 from the high intertidal pools at Hsitzu Bay, Kaohsiung, Taiwan, Republic of China (ROC), and extensively washed with autoclaved natural seawater (35‰, pH 7.8). Plants with a similar size, ranging from 31~37.5 cm (the largest thallus), were selected, and approximately 1.5 gram fresh weight from each plant was pre-incubated in autoclaved natural seawater of 30‰ salinity (adjusted by NaCl and distilled water, pH 7.8) for 24 h at 25°C. As evaluated by net photosynthetic O_2 evolution rate, algae remained healthy for up to 4 days. On exposure to various salinities ranging from 5‰ to 150‰, a plant was cultured in a polycarbonate vessel (Magenta GA-7 vessel, Sigma, St. Louis, MO, USA) containing 300 mL seawater (pH 7.8) and enriched with full strength Provasoli nutrient solution (Provasoli, 1968) in a growth chamber (25°C) for 4 days. Inorganic carbon source was provided by applying $NaHCO_3$ to a final concentration of 3 mM. Salinity, ranging from 5‰ to 150‰, was adjusted by adding distilled water or NaCl. The photoperiod was 12 h : 12 h, and the photon irradiance was 300 μmol photon m^{-2} s^{-1} in the absence of algae, achieved by a mixture of cool-fluorescent and incandescent light. Seawater was changed every day.

2,3,5-Triphenyltetrazolium Chloride Reduction

The in vivo reduction of TTC was modified according to Steponkus and Lanphear (1967). Thalli were cut into 1 cm strips, immersed in the incubation solution (50 mM sodium phosphate, pH 7.4) containing various TTC concentrations, and incubated at 25°C in darkness. Since TTC reduction is sensitive to excessive oxygen (Altman, 1970), the incubation of TTC was carried out without shaking. The ratio of thallus fresh weight to incubation solution volume was 0.1 gram fresh weight : 2 mL. After two extractions by 95% ethanol (5 mL each time), the extracts were combined and made up to 10 mL. The formazan formed in green tissues was measured at 530 nm instead of 485 nm to avoid the interference of pigments such as chlorophyll (Steponkus and Lanphear, 1967). We also found that the ethanol extract from *U. fasciata* without TTC incubation had an absorbance at 485 nm. In the viability assay of *Ulva* protoplasts, the formazan was also determined at 520 nm (Badini et al., 1994).

Net Photosynthetic O_2 Evolution Rate and Specific Growth Rate

Net photosynthetic O_2 evolution rate was determined by measuring the amounts of released oxygen using a Clark-type oxygen electrode fitted with a DW3 chamber (Hansatech, Kings Lynn, Norfolk, England) thermostated at 25°C. A strip of 50 mg fresh weight (about 2.5 cm long) was cut from the tip, a distal end from the rhizoidal portion, and held with a nylon hook in a 20 mL square section reaction vessel filled with 15 mL artificial seawater (pH 7.8, 315 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 30 mM $MgSO_4$), incubated in darkness until there was a steady O_2 evolution. Photosynthetic O_2 evolution was then started by illumination. The photon irradiance was 300 μmol m^{-2} s^{-1} provided by low voltage Tungsten halogen lamps (12V, 50W, Sylvania, Japan). Light measurement was carried out with a LI-188B integrating radiometer using a spherical quantum sensor LI-193SA (LI-COR, Nebraska, USA). The O_2 electrode system was calibrated daily with sodium dithionite-treated artificial seawater, which was referred to the N_2 line, and O_2-saturated artificial seawater.

The initial fresh weight of each plant was determined as W_0. After growing in various salinities for 4 days, each plant was weighed as a value of W_4. The specific growth rate was expressed as a percentage of fresh weight increase per day ($\%/d = 100\times((\ln(W_4-W_0))/4\ d)$).

Statistical Analysis

The statistical analysis was performed using Duncan's multiple range test, $p<0.01$ (SAS for DOS, Version 6.03, SAS Ltd., NC, USA).

Results

Optimal Conditions for TTC Reduction

Based on the incubation in 0.8% TTC solution, the formazan was only detected in the presence of NaCl. The maximal level appeared at 3~4% NaCl (Figure 1A). The application of 0.1% Tween 20 did not affect the reduction of TTC (Figure 1B). Besides, it caused a leakage of formazan in the external incubation solution (Figure 1B). In the presence of 3% NaCl, the formazan amount increased with increasing TTC concentrations and reached a peak at 0.8% (Figure 2). Figure 3 shows that the formazan levels increased fast during the first 6 h of incubation in 0.8% TTC solution containing 3% NaCl and reached a plateau after 18 h. Therefore, an 18 h-incubation in 0.8% TTC solution containing 3% NaCl was used.

TTC Reduction Activity in Different Plant Parts

No significant difference was observed among either whole plants of different sizes (Figure 4A) or among thalli of different lengths (Figure 4B). Since *Ulva fasciata* has a slender and hard base proximally near the rhizoidal portion (approximately 3 cm long), a single thallus (12 cm) was divided into four equal segments of 3 cm each, and each segment was incubated individually to determine whether

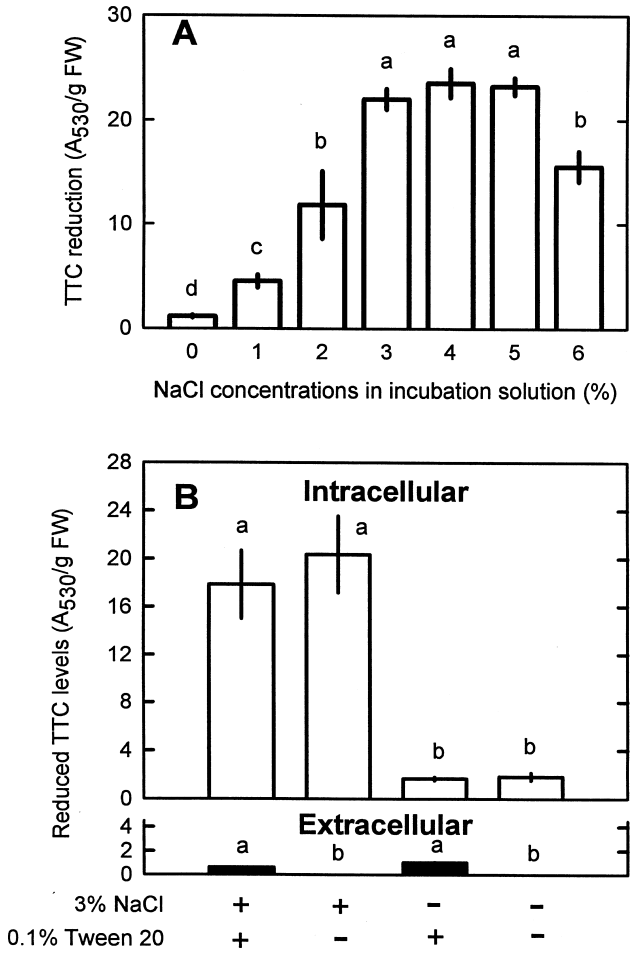

Figure 1. Effects of NaCl concentrations and Tween 20 in TTC (0.8%) incubation solution on TTC reduction activity. A, various NaCl concentrations; B, 3% NaCl and/or 0.1% Tween 20. Means ± S.E.M (n=3) are indicated, and different symbols represent a significant difference, $p<0.01$.

TTC reduction activity in the base was different from that in the remaining parts. As shown in Figure 4C, TTC reduction activity was low in the basal part compared to other segments ($p<0.01$) while activity in the other three segments was similar.

Relationship between TTC Reduction Activity and Salinity Tolerance

The specific growth rate was maximal at 30‰ and declined proportionately with the reduction or the increment in salinity (Figure 5A). By comparing with the 30‰ control, one can see that the relative change in specific growth rate is associated with the relative change in TTC reduction activity (r^2=0.90) (Figure 6A). Similarly, both the photosynthetic O_2 evolution rate (Figure 5B) and TTC reduction activity (Figure 5C) decreased with increasing or decreasing salinity. Based on the 30‰ control, the relative change in net photosynthetic O_2 evolution rate is also associated with the relative changes in TTC reduction activity (r^2=0.93) (Figure 6B).

Discussion

The present study shows that the incubation in 0.8% TTC solution containing 3% NaCl for 18 h in darkness maximizes the activity of TTC reduction in the marine macroalga *Ulva fasciata*. In terrestrial plants, the TTC viability assay is performed in the absence of NaCl. Incubation without NaCl is a stress condition of low salinity to marine algae and is not suitable for the in vivo TTC reduction in them. Since dehydrogenases, especially in the respiratory systems, are known to be responsible for TTC reduction (Towill and Mazur, 1975) and the respiration in *Ulva* is known to be inhibited by hyposaline conditions (Kirst, 1990), it is likely that *Ulva* immersed in TTC solution without NaCl lost its normal respiration activity, causing the dehydrogenase-mediated TTC reductionto decline.

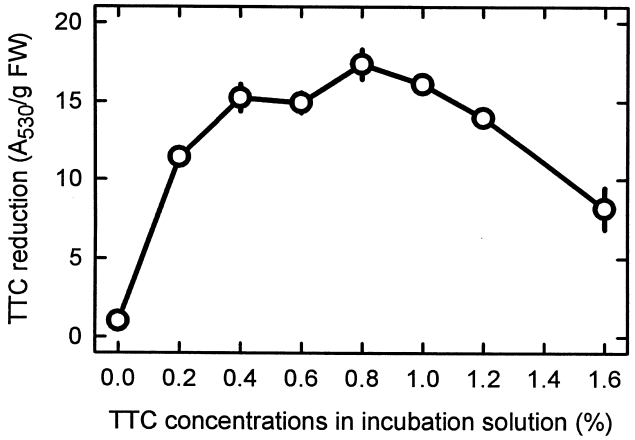

Figure 2. TTC reduction activity in response to various TTC concentrations in incubation solution. Means ± S.E.M (n=3) are indicated.

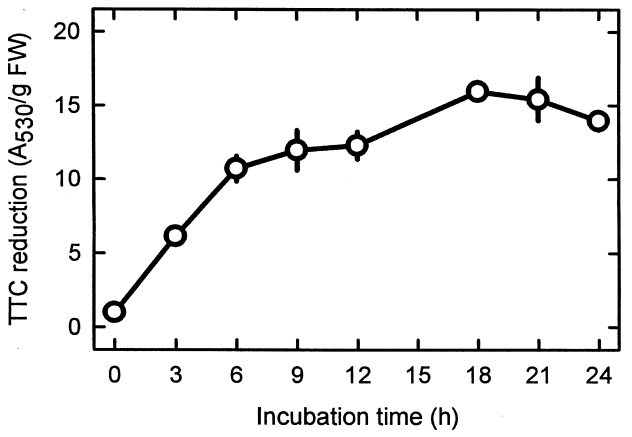

Figure 3. Time course changes in TTC reduction activity. The TTC incubation solution is 0.8% TTC and 3% NaCl. Means ± S.E.M (n=3) are indicated.

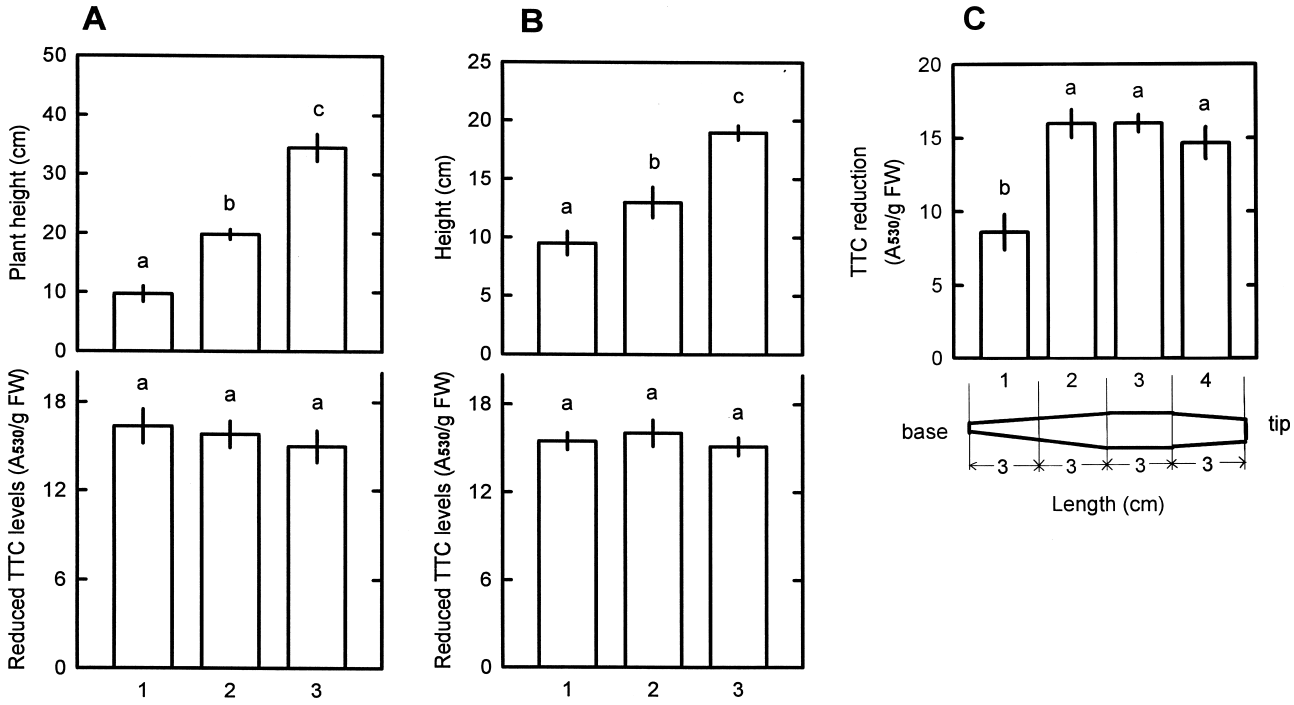

Figure 4. TTC reduction activities in different plant parts. A, whole plants of different heights; B, thalli of different lengths; C, different segments in a single thallus (3 cm long in each segment. Means ± S.E.M (n=3) are indicated, and different symbols represent a significant difference, $p<0.01$.

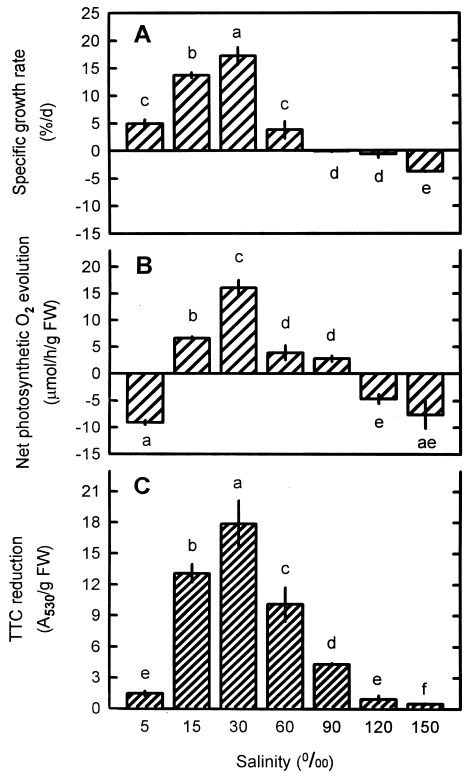

Figure 5. Changes in specific growth rate (A), net photosynthetic O_2 evolution rate (B) and TTC reduction activity (C) in response to various salinities for 4 days. Means ± S.E.M (n=3) are indicated, and different symbols represent a significant difference, $p<0.01$

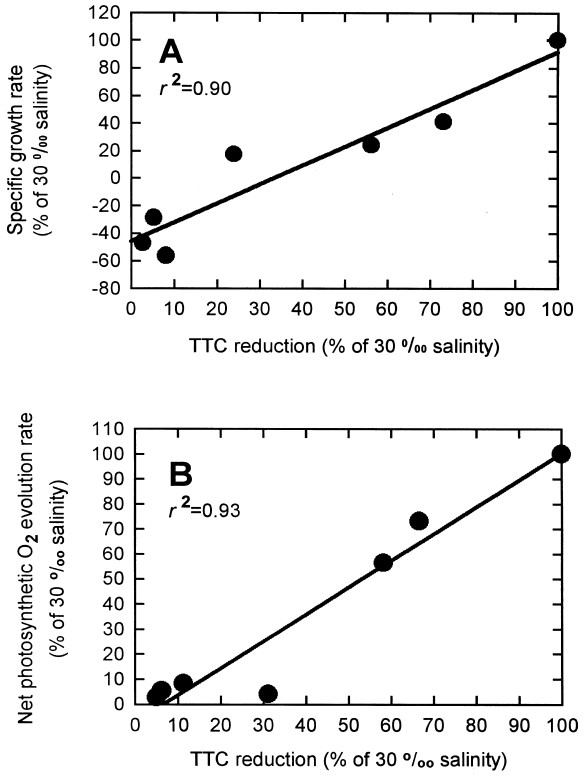

Figure 6. The relationship between the relative changes in specific growth rate and TTC reduction activity (A) and between the relative changes between net photosynthetic O_2 evolution rate and TTC reduction activity (B) in response to various salinities.

The requirement of optimal NaCl concentration for in vivo TTC reduction could be imagined for a marine macroalga living in the seawater medium.

The close association in the relative changes between TTC reduction activity and the specific growth rate (r^2 = 0.90), and between TTC reduction activity and the net photosynthetic O_2 evolution rate (r^2=0.93) suggests that relative TTC reduction activity could be used to determine the viability of *U. fasciata* exposed to salinity stress. As judged by the above physiological parameters, *U. fasciata* survives at salinities ranging from 15‰ to 90‰.

There is no significant difference in TTC reduction activity among thalli of different lengths or among whole plants of different heights. However, low TTC reduction activity in the basal part near the rhizoidal portion compared to the remaining parts ($p<0.01$) indicates that TTC reduction activity is not uniform throughout the thallus. This may result from physiological differences between the basal part (slender and hard) and the remaining part (wider and soft). It was found that the respiratory O_2 uptake rate in the basal and tip parts was 62.86 ± 9.30 and 98.07 ± 6.77 $\mu mol \cdot h^{-1} \cdot g^{-1}$ FW, respectively (data not shown). In addition, the net photosynthetic O_2 evolution rate in the basal part (175.73 ± 39.39 $\mu mol \cdot h^{-1} \cdot g^{-1}$ FW) was only 53.40% of that in the tip part (329.09 ± 28.74 $\mu mol \cdot h^{-1} \cdot g^{-1}$ FW), and TTC reduction activity in the basal part (10.48 ± 1.93 $A_{530} \cdot g^{-1}$ FW) was 56% of that in the tip part (18.37 ± 2.56 $A_{530} \cdot g^{-1}$ FW) (data not shown).

In addition to the TTC reduction method, the dyes neutral red, fluorescein diacetate, or Evans blue are used to assess the viability of protoplasts of green macroalgae such as *Entermorphora intestinalis* (Millner et al., 1979) and *U. fasciata* (Chen and Chen, 1991). Another dye, erythrosine, is also employed in the viability assay of other marine macroalgae (Saga et al., 1989), but these dye-binding methods should be observed under a light microscope.

In conclusion, a correlation between TTC reduction activity and tolerance to salinity stress is observed in *U. fasciata*. However, whether this is a general phenomenon has still to be clarified in other marine algae.

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利用 2,3,5-Triphenyltetrazolium 被還原能力評估裂片石蓴鹽度逆境下之存活能力

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本研究利用 2,3,5-Triphenyltetrazolium (TTC) 還原爲不可溶於水之紅色 formazan 能力評估裂片石蓴 (*Ulva fasciata* Delile) 的。0.1 克鮮重葉狀體置於 1.5 毫升含 TTC 之 50 mM 磷酸鈉緩衝液，還原後之formazan 以 95% 酒精萃取並在 530 nm 下測定吸光值以避免色素干擾。TTC 還原能力在含 3% NaCl 之 0.8% TTC 溶液培養 18 小時後達最大。TTC 還原能力在不同大小的裂片石蓴或葉狀體間並無差異，但葉狀體靠附著器之基部（約 3 公分）卻明顯較低 ($p<0.01$)。以最大葉狀體尖端 2.5 公分片段比較之，全株裂片石蓴在不同鹽度 (5~150‰) 下的 TTC 還原能力在 30‰ 達最大，在 120‰ 以上及 5‰ 以下最小。淨光合作用及比生長率在不同鹽度 (5~150‰) 下也有相似的趨勢。以 30‰ 爲基礎，相對 TTC 還原能力與相對淨光合作用 ($r^2 = 0.93$) 及相對比生長率 ($r^2 = 0.90$) 有相關。這些結果指出裂片石蓴可在 15~90‰ 間存活，而相對 TTC 還原能力可以作爲預測耐鹽性的工具。

關鍵詞：鹽度逆境；TTC；*Ulva fasciata*；存活力。