

Effect of tonicity and additives to the fixative on ultrastructure of mesophyllous cells in *Kandelia candel* (L.) Druce (Rhizophoraceae)

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(Received May 24, 1996; Accepted August 8, 1996)

Abstract. We investigated the effect of tonicity and additives to the fixative on the ultrastructure of *Kandelia candel*. Nonionic additives such as sucrose were better than ionic additives such as NaCl, KCl, Na₂SO₄ or K₂SO₄. Measurement of osmolality in the *K. candel* cells was carried out for adjusting the osmolality of the fixatives. We found that the hypertonic fixative and the washing buffer obtained by adding 0.15 M sucrose to the isotonic fixative and 0.1 M phosphate buffer yielded the best results; outer membranes of chloroplasts and mitochondria did not dilate or undergo plasmolysis. Varying the tonicity of the fixative with sucrose to a reasonable degree did not change the ultrastructural details within the chloroplasts and mitochondria. In contrast, hypertonic fixatives obtained by adding ionic additives caused significant swelling of thylakoid membranes in chloroplasts. This artifact was most pronounced when hypertonic fixatives containing NaCl or KCl were used. These observations suggest that high ionic fixatives disturb the ionic balance in thylakoid membranes.

Keywords: *Kandelia candel*; Mangrove; Osmotic potential; Salt; Sucrose; Tonicity.

Abbreviations: Ψ_s , Osmotic potential; **FB**, Fixative and washing buffer; **LSP**, 100 mM NaCl treated plant; **HSP**, 400 mM NaCl treated plant.

Introduction

Salinity has been known to affect the ultrastructure of plants, both glycophytes and halophytes. The swelling of organelles such as, chloroplasts, golgi bodies, mitochondria, and nuclei is probably the most obvious phenomenon found in plants growing in high salinities, which was suggested as a response to the changing internal environment of plant tissues (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968; Poljakoff-Mayber, 1975; Werker et al., 1983). Other studies found increasing formation of vesicles and myelin-like structures in the vacuoles of plants growing in high salinities, and these structures were thought to be the pinocytotic invagination of tonoplast, which allows plants to translocate salt ions from the cytoplasm into the vacuoles (Willert and Kramer, 1972; Kurkova and Balnokin, 1994).

However, most of the above studies using fixatives for electron microscopy did not mention the relationship between the tonicity of the fixative and the osmolality of the specimen. The effect of tonicity of fixatives on the ultrastructure has been demonstrated mostly in animal cells (Schultz and Karlsson, 1965; Maunsbach, 1966; Bone and Denton, 1971; Rasmussen, 1974); however, a few studies were carried out in plant systems (Fineran, 1971; Soikkeli,

1980). Although some of the studies mentioned above used either sucrose (Werker et al., 1983) or NaCl (Kurkova and Balnokin, 1994) in the fixative and buffer to maintain an osmolality equivalent to that of the culture medium of the plant cells, the effect of the tonicity of the fixative on the ultrastructure of the plant samples was not described.

Kandelia candel, a mangrove, like other halophytes (Flowers et al., 1977), were used to accumulate high salt ions to reduce the osmotic potential in tissues even growing in normal Hoagland's solution (Hwang and Chen, 1995). Moreover, the osmotic potential in root and leaves of *K. candel* was correlated with, and lower than, the osmotic potential of the saline culture solutions (Hwang and Chen, 1995). We noticed that the outer shape of organelles was affected tremendously by the tonicity of fixatives while we were studying the ultrastructure of *K. candel*. Therefore, the present investigation was carried out to determine how the ultrastructure of leaf cells in *K. candel* grown in varying NaCl salinities were influenced by the tonicity and different additives in the fixatives.

Materials and Methods

Plant

Mature propagules of *Kandelia candel* (L.) Druce, collected from mature trees growing along the Tamshui River, Taipei, Taiwan, (121°26'E, 25°9'N), were cultivated on sand in pots, partially submerged in nutrient solutions. The

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culture solution was a modified Hoagland's solution, with NaCl added at the beginning to form 100 and 400 mM NaCl solutions, which formed two treatments: 100 mM NaCl treated plant (LSP) and 400 mM NaCl treated plant (HSP). Details of the culture method were described by Hwang and Chen (1995). The osmotic potential (Ψ_s) of leaf tissues was measured immediately before fixation. The leaf sap of the tissue was extracted by the frozen-and-thawed method. The Ψ_s of leaf sap, fixative, and washing buffers were determined by a Wescor HR-33T Dew Point Microvoltmeter equipped with a C-52 Sample Chamber (Wescor, Logan, Utah, USA).

Fixation and Staining Methods

Leaf discs (7 mm diameter) of three-month-old seedlings were taken by a paper punch along the mid-rib at the central part of mature leaves and put immediately into an ice-cold fixative solution containing 1% glutaraldehyde, 1% acrolein, 1% formaldehyde, 0.1 M phosphate buffer, and an additive (see the "tonicity adjustment" section below) at pH 7.2. The material was then transported from the greenhouse to the laboratory within 10 min, and small segments were cut with a razor blade in the same fixative. These segments were degassed and fixed further under vacuum in the same fixative for 4 h at 4°C. They were then washed in tonicity-adjusted 0.1 M phosphate buffer 3 times (15 min each) and post-fixed in 1% OsO₄ in 0.1 M phosphate buffer for 1 h. After a few washings in buffer, the post-fixed segments were dehydrated in an acetone series and embedded in Spurr's resin (1969). Thin sections were cut with a diamond knife on a Reichert-Jung Ultracut-E ultramicrotome and stained with uranyl acetate and lead

citrate (Reynolds, 1963), and viewed and photographed with a Zeiss-109 transmission electron microscope.

Tonicity Adjustment

Since the osmosis of the cell is still active after the aldehyde fixation and is completely destroyed after postfixation with OsO₄ (Bone and Denton, 1971), the osmotic adjustment of fixation solutions was only conducted in pre-fixed glutaraldehyde fixative and the following washing buffer, and not in the post-fixed OsO₄ solution. Sucrose was used as additive in fixative and buffer solutions to form hypotonic, isotonic, hypertonic, and super-hypertonic conditions in accordance with the Ψ_s of the tissue (Table 1). The difference among tonicity treatments was arbitrarily chosen as a 0.15M sucrose difference, which was equivalent to ca. 5% (w/v) sucrose or 0.4 MPa in solution tonicity.

We also used NaCl as additive to replace sucrose in the tonicity experiment and found that the effect of the tonicity by NaCl was similar to that of sucrose with respect to the outer membrane of organelles. However, the ultrastructure of the chloroplast was greatly affected by all NaCl tonicity treatments. Therefore, we investigated further the effect of various ionic additives, i.e. NaCl, KCl, Na₂SO₄, and K₂SO₄, in hypertonic condition on the ultrastructure of chloroplasts (Table 2). In this second part of the experiment the phosphate buffer system in the fixative and washing buffer (FB) was replaced by cacodylate buffer (50 mM, pH=7.0), and the leaf discs were taken from two-month-old seedlings because of the time of the experiment. Since the ion content in leaves of seedlings increased with time during the first four months of growth (Hwang and

Table 1. The sucrose concentration and its measured osmotic potential, in parenthesis (MPa), in fixative and washing buffer. The concentration of sucrose in FB was adjusted to form different tonicity relative to the osmotic potential of the tested leaf tissues grown in 100 and 400 mM NaCl solutions. Phosphate buffer was used in this treatment. The ingredients of the fixative and washing buffer were described in the text.

	100 mM NaCl leaf (-2.3)		400 mM NaCl leaf (-2.7)	
	Fixative	Washing buffer	Fixative	Washing buffer
Hypotonic	0.25 M (-1.9)	0.35 M (-1.8)	0.48 M (-2.4)	0.55 M (-2.4)
Isotonic	0.40 M (-2.2)	0.50 M (-2.1)	0.60 M (-2.6)	0.67 M (-2.7)
Hypertonic	0.53 M (-2.5)	0.62 M (-2.6)	0.72 M (-3.0)	0.78 M (-3.0)
Super-Hypertonic	nt*	nt	0.97 M (-4.0)	0.98 M (-4.0)

*nt: no test.

Table 2. The concentration of ionic additives and its measured osmotic potential, in parenthesis (MPa), in FB. The concentration of the ionic additives in fixatives and washing buffers was adjusted to form a hypertonic solution relative to the osmotic potential of the tested leaf tissues grown in 100 and 400 mM NaCl solutions. Cacodylate buffer was used in this treatment. The ingredients of the fixative buffer were described in the text.

Ionic additive	100 mM NaCl leaf (-1.9)		400 mM NaCl leaf (-2.2)	
	Fixative	Washing buffer	Fixative	Washing buffer
NaCl	0.43 M (-2.2)	0.55 M (-2.2)	0.52 M (-2.7)	0.65 M (-2.7)
KCl	0.43 M (-2.2)	0.53 M (-2.2)	0.52 M (-2.6)	0.64 M (-2.6)
Na ₂ SO ₄	0.34 M (-2.2)	0.44 M (-2.4)	0.43 M (-2.6)	0.51 M (-2.7)
K ₂ SO ₄	0.33 M (-2.4)	0.44 M (-2.4)	0.43 M (-2.7)	0.51 M (-2.7)

Chen, 1995) the Ψ s of leaf tissues were different for the tested leaf tissues at different ages (compare the Ψ s of the leaves in Tables 1 and 2). The effect of the tonicity and additives to the fixative on the ultrastructure of mesophyllous cells in *K. candel* between these two ages were similar.

Results

The tonicity of the fixatives, adjusted by sucrose, affected mainly the shape of the outer membrane of mitochondria and chloroplasts in mesophyll cells of *Kandelia candel*. The responses were similar in 100 and 400 mM NaCl plants (Figures 1 and 2). No matter what salinity treatment was applied, hypotonic and isotonic fixatives caused swelling of the outer membrane of mitochondria and chloroplasts relative to the Ψ s of the fixed sample (Figure 1A–1B, Figure 2A–2B). Within chloroplasts, the one with starch grain was more sensitive to the tonicity of fixatives than the one without (Figure 1A–1B, Figure 2A–2B). Hypertonic fixatives gave the best appearance of organelles, in terms of the shape of the outer membrane of the chloroplasts and mitochondria (Figure 1C, Figure 2C–2D). However, compact chloroplasts, i.e. those with no extraspaces around the starch grains (Figure 2E–2F), and plasmolysis on plasmalemma could be found in the treatment of super-hypertonic fixative (Figure 2F).

Myelin-like and vesicle-like structures could be found in vacuoles of hypotonic and isotonic treatments (Figure 3) due to the expansion of outer membrane of chloroplasts and mitochondria. Figures 3A–3C and 3G–3I show the myelin-like structure in a vacuole caused by the swelling of outer membrane on a chloroplast in a series of sections, and most of the myelin-like structures in the vesicle originated from the thylakoid membrane. Figure 3D–3F shows a series of sections on a swollen mitochondrion, on which the vesicle could be mis-interpreted as a result of pinocytosis, invagination of tonoplast into vacuole (Figure 3D–3F), if the tonicity of the fixatives and the serial sections were not concerned with.

Once the sucrose was replaced by NaCl as additive, not only was the shape of the outer membrane of chloroplasts and mitochondria affected by the tonicity of the fixative, the ultrastructure of the chloroplasts, in the form of lumen swelling in thylakoid membranes, was affected as well (Figure 4A). Apparently, the ionic characteristic of NaCl has affected the nature of the thylakoid membranes in chloroplasts, but not in other organelles.

The thylakoid membrane swelling was a general effect in each of the two salinity treatments and by all ionic additives used in this investigation, i.e. NaCl, KCl, Na₂SO₄ and K₂SO₄. However, the degree of swelling was much more severe in most of the chloroplasts in the NaCl and KCl treatments (Figure 4B–4E) and only in some of the chloroplasts in the Na₂SO₄ and K₂SO₄ treatments (Figure 4F–4I). The swelling in the thylakoid membrane formed a wavy shape, and thus no grana structure in the chloroplast could be identified in the NaCl and KCl treatments (Fig-

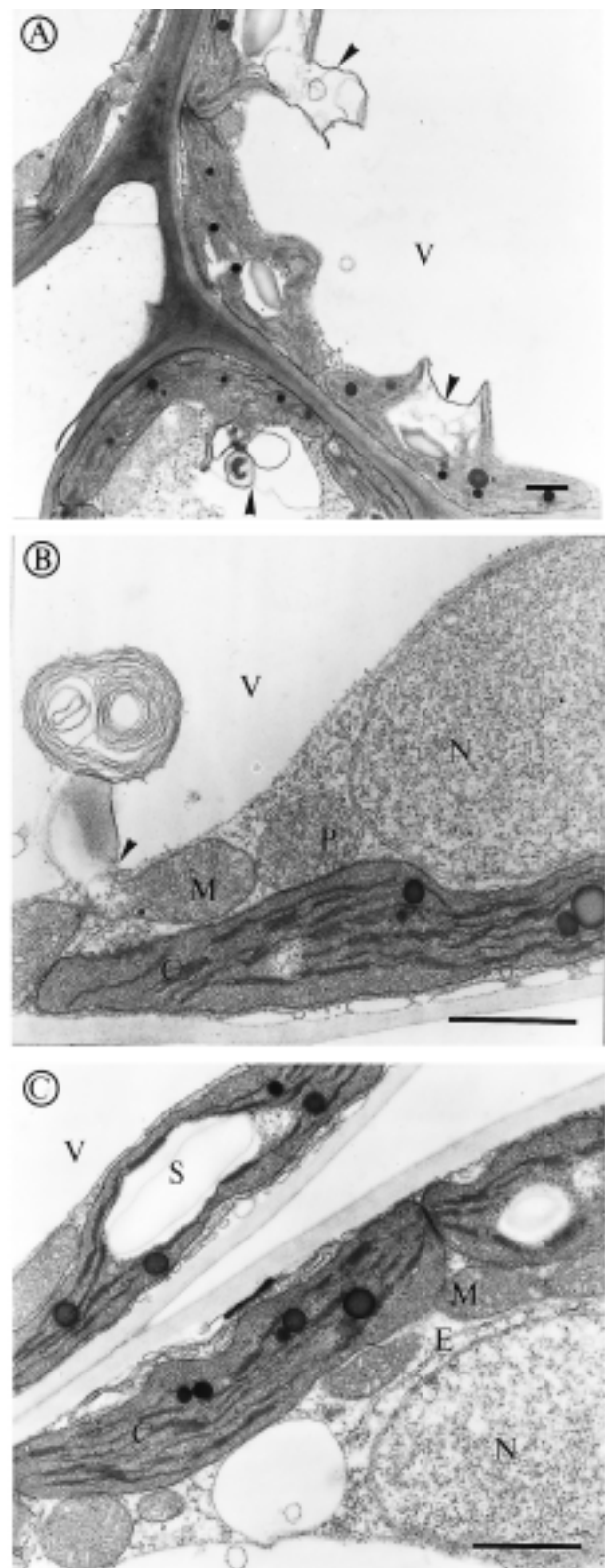


Figure 1. Effect of tonicity, adjusted by sucrose, on the ultrastructure of the palisade mesophyll in LSP. Leaf tissues were fixed in A, hypotonic; B, isotonic; C, hypertonic FB. Arrowheads represent areas of swelling on membranes of mitochondria and chloroplasts. C: chloroplast; E: endoplasmic reticulum; M: mitochondrion; N: nucleus; P: peroxisome; S: starch grain; V: vacuole; W: cell wall; Scale bar represents 1 μ m.

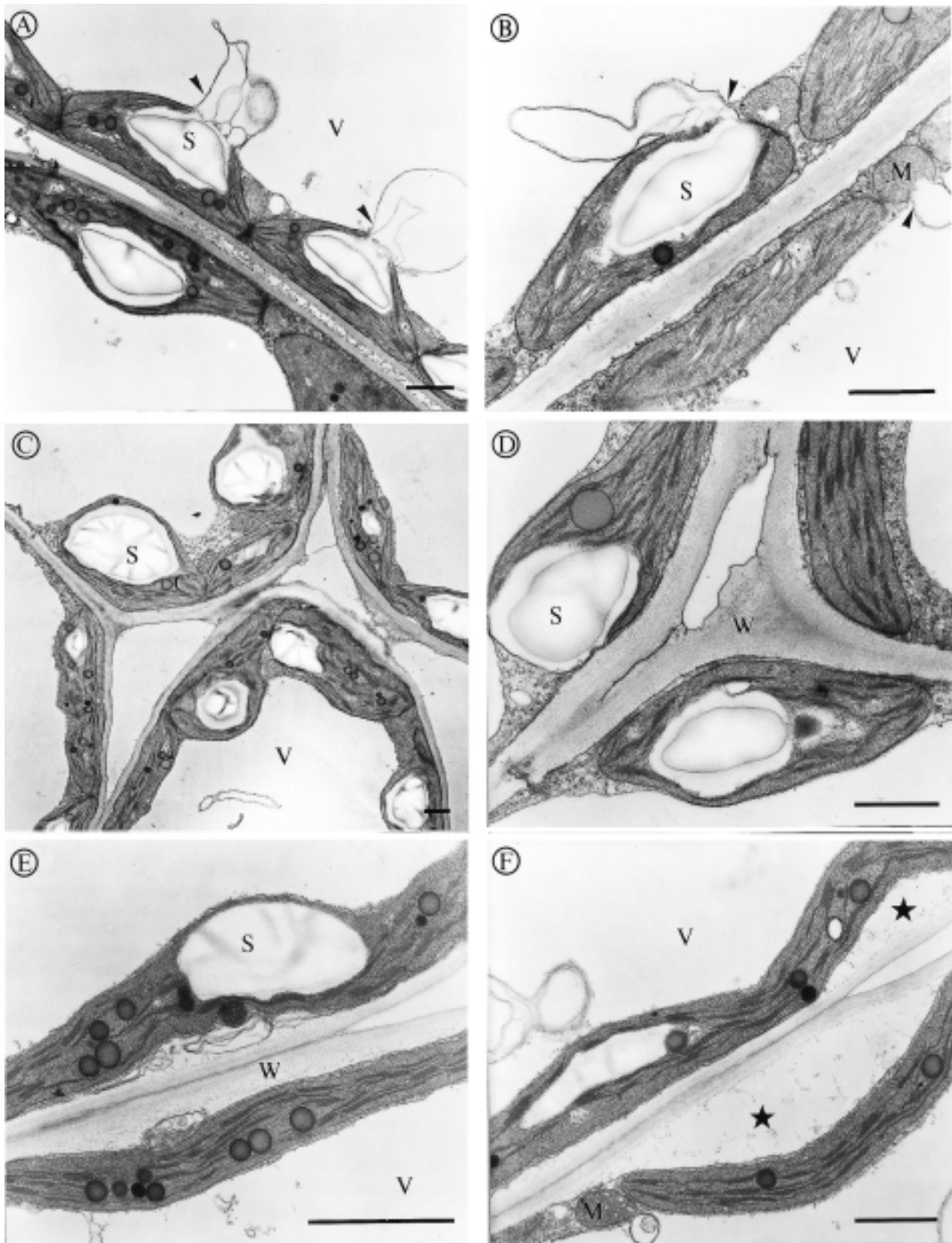


Figure 2. Effect of tonicity, adjusted by sucrose, on the ultrastructure of the palisade mesophyll in HSP. Leaf tissues were fixed in A, hypotonic; B, isotonic; C–D, hypertonic; E–F, super-hypertonic FB. Arrowheads in A and B represent areas of swelling on membranes of mitochondria and chloroplasts. Asterisks in F represent area of plasmolysis on plasmalemma. Notice that there is no extra space around the starch grain in chloroplast of E. C: chloroplast; M: mitochondrion; S: starch grain; V: vacuole; W: cell wall; Scale bar represents 1 μm .

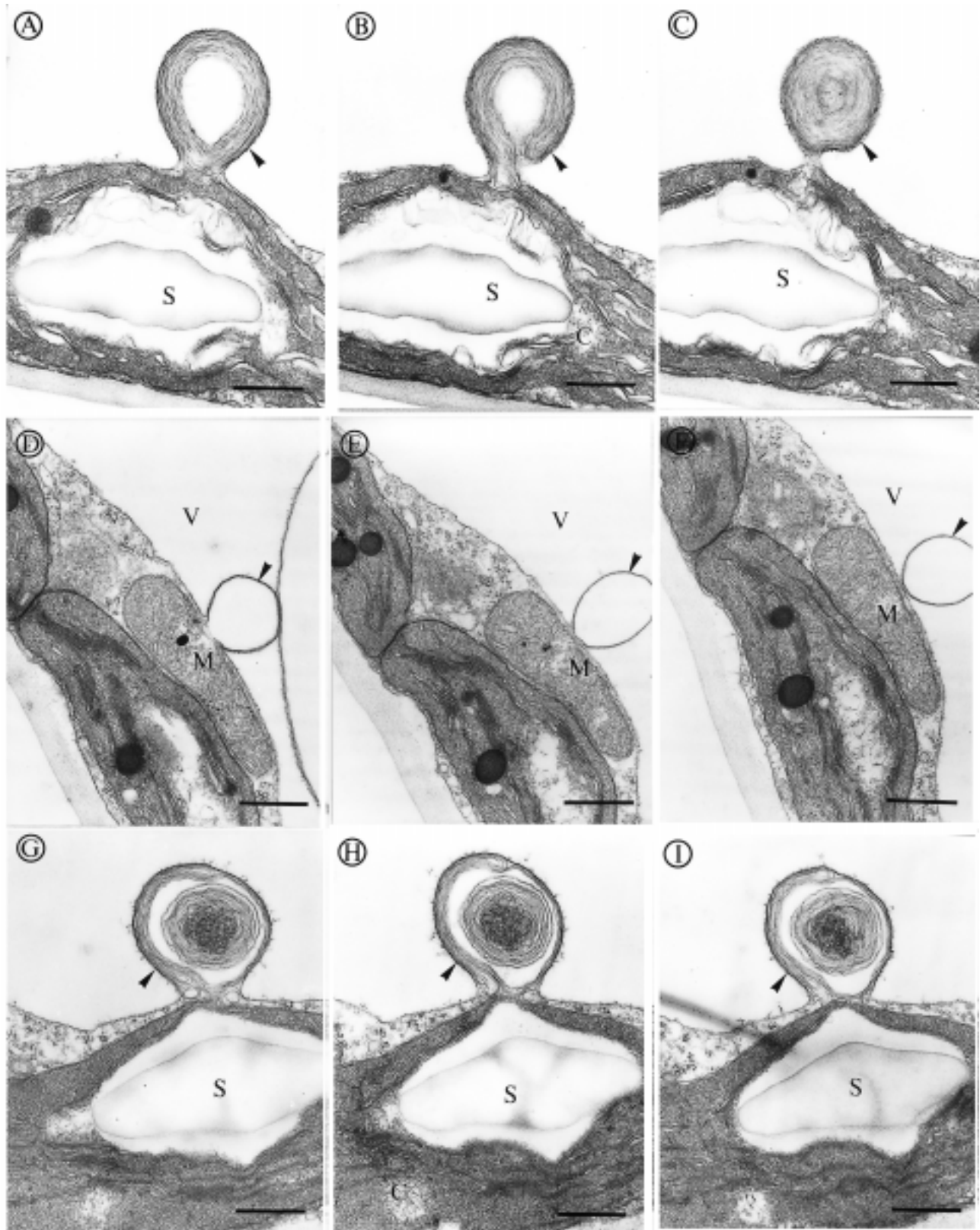


Figure 3. Serial sections on the swelling membrane. A–C, LSP in hypotonic FB, adjusted by sucrose. Myelin-like structure (arrowhead) originated from the tonoplast, chloroplast and thylakoid membranes of a swollen chloroplast in vacuole. D–F, LSP in isotonic FB, adjusted by sucrose. Pinocytosis-like vesicle (arrowhead) originated from a swelling mitochondria in vacuole. G–I, HSP in isotonic FB, adjusted by sucrose. Myelin-like structure (arrowhead) originated from the thylakoid membrane of a swelling chloroplast in vacuole. C: chloroplast; M: mitochondrion; S: starch grain; V: vacuole; W: cell wall; Scale bar represents 0.5 μm .

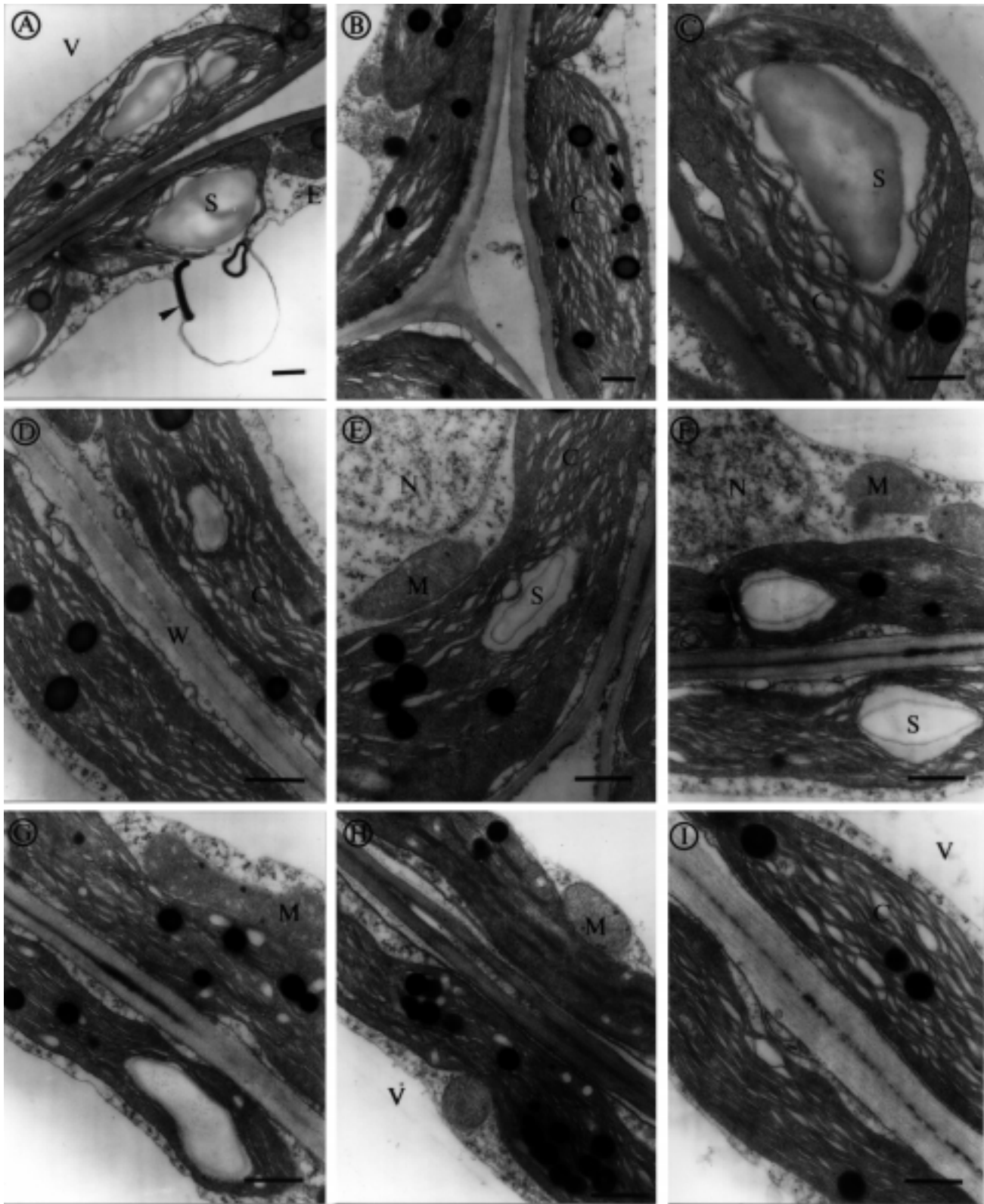


Figure 4. Effect of ionic additives on the swelling of thylakoid membranes in chloroplasts in palisade mesophyll of *K. candell*. A, HSP in NaCl-adjusted isotonic FB. Arrowhead represents area of swelling of chloroplast membrane. B, LSP in NaCl-adjusted hypertonic FB. C, HSP in NaCl-adjusted hypertonic FB. D, LSP in KCl-adjusted hypertonic FB. E, HSP in KCl-adjusted hypertonic FB. F, LSP in Na_2SO_4 -adjusted hypertonic FB. G, HSP in Na_2SO_4 -adjusted hypertonic FB. H, LSP in K_2SO_4 -adjusted hypertonic FB. I, HSP in K_2SO_4 -adjusted hypertonic FB. C: chloroplast; E: endoplasmic reticulum; M: mitochondrion; N: nucleus; S: starch grain; V: vacuole; W: cell wall; Scale bar represents 0.5 μm .

ure 4B–4E). Nevertheless, other organelles seemed unaffected by these ions. The difference in degree of swelling in thylakoid membranes indicated that Cl^- could be the key ion affecting the swelling in thylakoid membranes; however, the effect of other ions was not excluded.

Discussion

This study has demonstrated that the tissue Ψ_s of a halophyte, *Kandelia candel*, are important in determining the Ψ_s of the FB for accurate ultrastructural performance. Hypertonic conditions relative to the Ψ_s of the fixed tissues were best for the fixation (Figure 1C and Figure 2C–2D). The Ψ_s of the hypertonic condition were about 15% higher than those of the isotonic condition of the fixed samples (Tables 1 and 2). Hypo- and isotonic fixatives and buffers caused swelling of mitochondria and chloroplasts in leaf cells (Figures 1A–1B and 2A–2B), and super-hypertonic fixatives caused plasmolysis of plasmalemma (Figure 2F). The discrepancy between the Ψ_s of the FB and the fixed samples could be due to an underestimation of the fixed sample Ψ_s by the frozen-and-thawed method. Schroppe-Meier and Kaiser (1988) have suggested that the osmolality of expressed leaf sap was lower than cell sap osmolality by about 15% due to the lower salt concentration in the apoplastic water as compared with intracellular solution.

The effect of tonicity of fixatives on the ultrastructure has been demonstrated mostly in animal cells (Schultz and Karlsson, 1965; Maunsbach, 1966; Bone and Denton, 1971; Rasmussen, 1974); however, a few studies were carried out in plant systems (Fineran, 1971; Soikkeli, 1980). To our knowledge, no one has investigated the effect of tonicity of the FB on the ultrastructure of halophytes, although several reports have dealt with the effects of salinity (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968; Willert and Kramer, 1972; Poljakoff-Mayber, 1975; Werker et al., 1983; Kurkova and Balnokin, 1994). Since most halophytes accumulate a high concentration of ions in their tissues while growing in saline environments (Flowers et al., 1977), we suggested that the Ψ_s of the FB should be made hypertonic, 15% higher than that of the isotonic state, according to the Ψ_s of the fixed samples.

Without a correct tonicity in the FB has caused some morphological changes in ultrastructure of *K. candel*, for example: pinocytosis-like and myelin-like structures (Figure 3), which were similar to those found in some other studies (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968; Willert and Kramer, 1972; Kurkova and Balnokin, 1994). It is impossible to identify the possible causes of those morphological changes from this study because of different plant species, nevertheless, this study suggested that the osmotic relationship between the FB and the fixed samples is needed to be adjusted and serial sections are needed before making morphological conclusions.

It was an interesting coincidence that the swelling of thylakoid membranes in chloroplasts (Figure 4) caused by the addition of ionic additives in the FB was similar to

that of plants grown in high salinity treatments, e.g. *Atriplex halimus* (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968), *Mesembryanthemum crystallinum* (Willert and Kramer, 1972) and *Triticum aestivum* (Salama et al., 1994). It has been established that ionic conditions in the extraction buffers can control the stacking of thylakoid membranes in isolated chloroplasts (Barber, 1980) because of the surface electrical charges on thylakoid membranes (Barber, 1982). A high concentration of cations are required to maintain grana stacking in isolated chloroplasts (Burke et al., 1979), probably because of the negative charge on the thylakoid membrane surface (Barber, 1982). However, this study (Figure 4) showed that high ionic concentrations caused loose stacking of thylakoid membranes, contrary to what was found in previous in vitro studies. The much higher ionic concentrations used in this study (Table 2), compared to those in vitro studies (Burke et al., 1979), were probably the cause of the difference.

The swelling of the thylakoid membranes in chloroplasts was less pronounced in Na_2SO_4 and K_2SO_4 (Figure 4F–4I) than in NaCl and KCl (Figure 4B–4E), and was similar to those found in *Atriplex halimus*, in which the swelling was less pronounced in plants growing in Na_2SO_4 as compared with NaCl (Blumenthal-Goldschmidt and Poljakoff-Mayber, 1968). We could not explain the effect by attributing it to different ions on the thylakoid stacking; however, it seems that a high concentration of Cl^- ion in the NaCl and KCl treatments might have penetrated the chloroplasts and caused the repulsion of negative charges on the surface of the thylakoid membranes while the integrity of the outer membrane of the chloroplasts was modified by the fixative. Nevertheless, the effect of other ions is not excluded.

Acknowledgments. We thank three anonymous reviewers for their valuable criticism of the paper. This research was funded by the Institute of Botany, Academia Sinica, Taiwan, Republic of China.

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固定液中之滲透勢與添加物種類對水筆仔紅樹林葉肉細胞內之微細構造之影響

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本實驗為瞭解水筆仔微細構造樣品製備過程裡，固定液中滲透勢及添加物對其微細構造的影響。非離子性添加物如蔗糖較離子性添加物如 NaCl, KCl, Na₂SO₄ 或 K₂SO₄ 為理想。決定固定液的滲透勢前必須先測試水筆仔葉片的滲透勢。結果顯示等張固定液及等張磷酸緩衝液加上 0.15M 蔗糖，可得較理想的結果：即葉綠體與粒線體之外膜既不見膨脹亦不會萎縮。若以蔗糖改變固定液之滲透勢，只改變葉綠體與粒線體外膜之膨脹，並不會造成其內部微細構造之變化。然而，以離子性添加物增加固定液的滲透勢，會使葉綠體之類囊膜構造產生嚴重之膨脹。其中尤以 NaCl 和 KCl 為甚。這些離子性添加物可能影響類囊膜構造內部離子分布之平衡。

關鍵詞：水筆仔；紅樹林；滲透勢；鹽；蔗糖；張力。