Applied Microscopy
Korean Society of Microscopy
pISSN 2287-5123 · eISSN 2287-4445
http://dx.doi.org/10.9729/AM.2016.46.2.83
Regular Article

Structural Differentiation of the Connective Stalk in *Spirodela polyrhiza* (L.) Schleiden

InSun Kim*

Major in Biological Sciences, College of Natural Sciences, Keimyung University, Daegu 42601, Korea

Structural differentiation of the connective stalk in giant duckweed, *Spirodela polyrhiza*, was examined to reveal the anatomical and ultrastructural characteristics within reduced shoot. The study focuses primarily on structural features of the connective stalk (CT), which connect offspring to their mother fronds. Photoautotrophic offspring fronds remained connected by stalks to mother fronds in the reproductive pockets until separation. The CT originated from the meristematic region of the abaxial frond and joined the fronds laterally with two abscission layers. The most notable features of the CT were polymorphic mitochondria, random occurrences of fibrillar structures in intercellular spaces, and great variability in cell wall thickness. Vascular tissues in CTs were highly reduced, demonstrating only a central vascular strand. Grana with 2 to 4 thylakoids and starch grains were found in the chloroplasts. A chlorophyll assay indicated high chlorophyll concentrations in daughter fronds and low concentrations in CTs. The frond and CT, while physically connected to each other, functioned independently. Despite great reduction in *S. polyrhiza*, the CT has proven to be very efficient for separating offspring from the mother frond, which lends to its capacity for rapid vegetative reproduction. The ultrastructural aspects of CTs in *S. polyrhiza* were characterized for the first time in this study.

Key Words: *Spirodela polyrhiza*, Chlorophyll assay, Structural differentiation, Electron microscopy, Connective stalk

*Correspondence to:
Kim I,
Tel: +82-53-580-5305
Fax: +82-53-580-5305
E-mail: botany@kmu.ac.kr

Received June 11, 2016
Revised June 19, 2016
Accepted June 19, 2016

INTRODUCTION

Species of the genus *Spirodela* have been known for their small size, rapid growth, and relatively simple anatomy. The giant duckweed, *S. polyrhiza*, is a small, free-floating aquatic plant with rapid growth exhibiting the piling up of fronds in a short period of time as in *Lemna minor*. It consists only of connected fronds, leaf-like structures developed from fused stems and leaves (Kim, 2011, 2013; Kim & Kim, 2000; Kwak & Kim, 2008; Landolt, 1998; Lemon & Posluszny, 2000), with several roots on the lower surface of each frond (Kim, 2007).

Much attention has been given to the structural aspects of duckweeds, since a great reduction in plant organization has been well demonstrated within the small body. In morphology, the conspicuous frond is the most frequently focused organ in research, since offspring fronds, stalks connecting fronds and the root system all arise from it. With light microscopy, the nature of pigmentation (Formin et al., 1992), morphological patterns (Landolt, 1998), frond and turion anatomy (Kwak & Kim, 2008) and frond abscission (Witzum, 1974b) were studied in several *Spirodela* species. An elaborate ultrastructural feature has been shown within the *Lemna* root (Melaragno & Walsh, 1976; Walsh & Melaragno, 1976), but the study focused only on the development of the sieve-element excluding other components of the root. Echlin and colleagues (1982, 1992) included several scanning electron micrographs of *Lemna minor*, while comparing techniques involved in low temperature electron microscopy, no detailed cellular information was given. Formin et al. (1992) also revealed the granality of chloroplasts while surveying the

greening process of etiolated giant duckweed, *S. polyrhiza*. Previous studies (Landolt, 1998; Lemon & Posluszny, 2000) reported detailed structural information on *Spirodela* while describing relationships among taxa in the Lemnaceae. In particular, the comprehensive investigation performed on morphologically distinct shoot development and evolution of three genera, including *Spirodela*, of the family (Lemon & Posluszny, 2000) enabled us to gain a better understanding of the shoot architecture and the morphological and phylogenetic correlations suggested among duckweeds. However, almost no extensive investigations concerning the complete structural aspects of the connective stalk (CT) have been carried out using electron microscopy. Hence, the present study attempts a comparative analysis of the anatomy and ultrastructure of the CT prior to separation during the growth. This paper describes the morphology and ultrastructure of vegetative *S. polyrhiza* and considers the contribution of the structural aspects to function. The fronds, turions of dormant structures, and the root system were not included in the study. Ultrastructural differentiation of CT in this species was characterized for the first time.

MATERIALS AND METHODS

Plant Material

The experiments were carried out in plants of *Spirodela polyrhiza* collected from July through September from the pond located at Keimyung University (Daegu, Korea). Materials were transported from the collection sites to the laboratory in humidified plastic bags and processed immediately for the following experiments. Approximately 20 plants having two to three generations of offspring fronds were each used for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and several hundreds of plants were used for the chlorophyll assay experiment.

Electron Microscopy

For TEM, about 1 to 2 mm^2 tissue samples of fronds and 1 to 2 mm long CTs, from the distal end of daughter fronds, were used. They were fixed in 3% to 6% glutaraldehyde in 0.02 M phosphate buffer (pH 6.8~7.2) for 3 to 4 hours at room temperature and post-fixed in 2% osmium tetroxide at 4°C for 2 to 16 hours. Following three-time rinses in the same buffer, the materials were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin. Approximately 60 to 90 nm ultra-thin sections were made by Ultracut-S ultramicrotome (Leica, USA) using a diamond knife. The sections were mounted on 0.25% dichloroethane coated copper grids and stained with 2% aqueous uranyl acetate, followed by 1% lead citrate. The sections were examined and photographed with a Hitachi H-7100 TEM (Hitachi, Japan) operated at 75 kV. Tannins were localized in tissue fragments after being incubated in 10% $FeSO_4$ in 5% formalin for 48 hours, dehydrated, embedded in Spurr's resin and stained with erythrosine.

For SEM, samples of transversely dissected- and/or whole fronds and CTs were fixed and dehydrated as they were processed for TEM procedures. After dehydration, the tissues were substituted with isoamyl acetate 3 to 4 times and stored at 4°C. Following the substitution, the tissue samples were dried to critical point by Emitech K850 (Emitech Ltd., United Kingdom). They were sputter-coated with 20 to 30 nm platinum-palladium using Emitech K550X (Emitech Ltd.) and photographed on a Hitachi S-4200 SEM operated at 15 kV.

Chlorophyll Assay

To compare chlorophyll contents in fronds and CTs, daughter and mother fronds, approximately 2 to 3 mm and 7 to 8 mm in diameter, respectively, were collected for the estimation. The CTs were prepared as follows: CTs were isolated first, under a dissecting microscope, by carefully pulling daughter fronds away from mother fronds, and then excising them at stalk base. The tissue samples were extracted with N,N-dimethylformamide and estimated in 80% acetone spectrophotometrically. Pigment concentrations were calculated following the extinction coefficients proposed by Inskeep & Bloom (1985): Chlorophyll (mg/g)=17.90 A_{647}+8.08 $A_{664.5}$. Five replicates of each tissue extracts were assayed.

RESULTS AND DISCUSSION

In terms of structural development, giant duckweeds are considered to be poorly differentiated plants as they produce only small fronds, CTs, and roots as a mature plant. Fronds are reduced shoots that probably remain in an embryonic stage of development (Kwak & Kim, 2008; Landolt, 1998). In fact, fronds as the major photosynthetic organ that produce offspring vegetatively in the reproductive pocket several times during growth. The offspring become photoautotrophic fairly early while they remain in the mother frond. Several individual offspring in various stages of development were laterally attached to the mother frond by each CT (Fig. 1A). The CT was ca. 210 to 260 μm in diameter and elliptical in shape from the cross section (Fig. 1B). The CT possessed epidermis, cortex and vascular tissue, but no air chambers. The cells varied in size and organelles were diffused throughout the cytoplasm of actively functioning cortical cells (Fig. 1C). The cells were rich in mitochondria, rER and chloroplasts. Among the organelles, the mitochondria had the most distinct features, demonstrating a strangely elongated and highly polymorphic shape with well-developed cristae (Fig. 1D). Grana with 2 to 4 thylakoids and starch grains were found in the chloroplasts. Irregularly shaped chloroplasts were

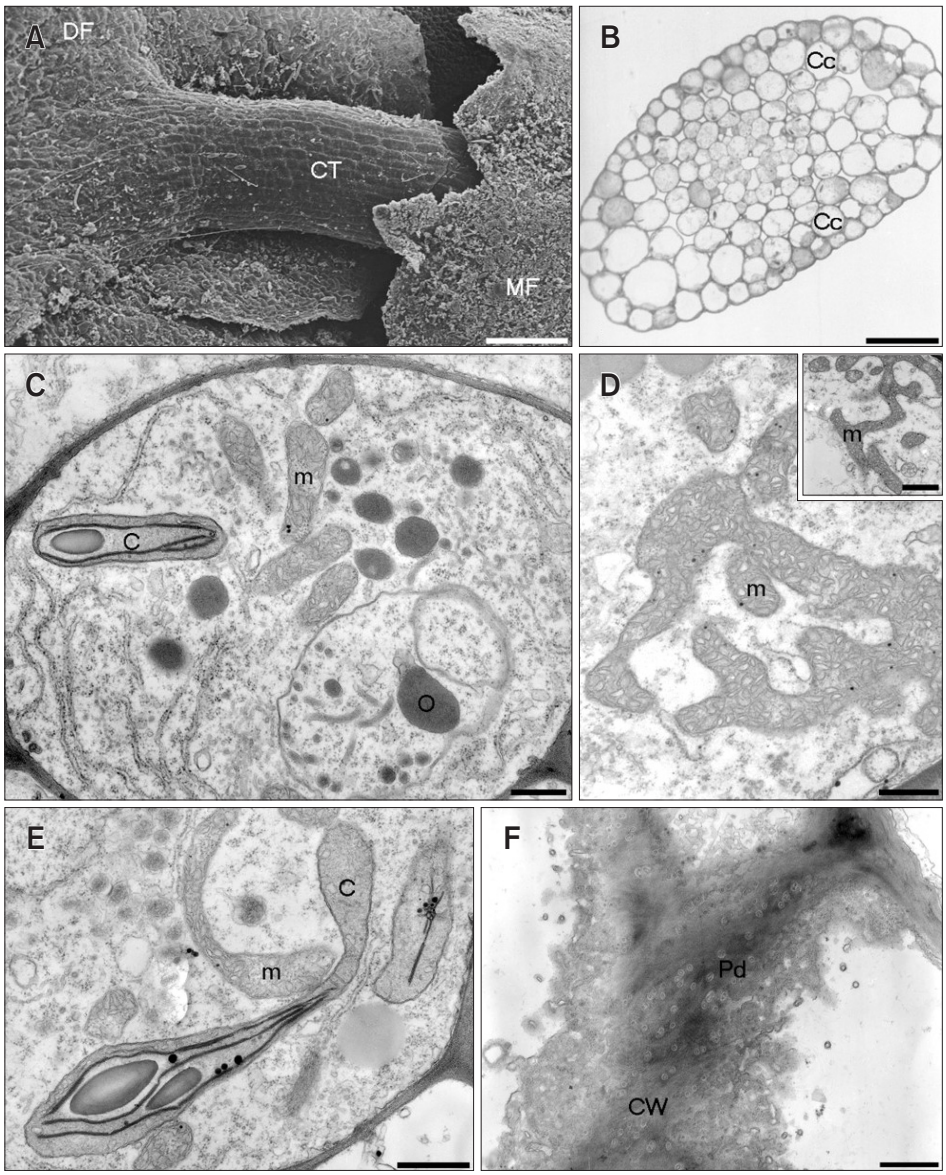

Fig. 1. Scanning electron microscopy (A) and transmission electron microscopy (B-F) images. Morphology and ultrastructure of a connective stalk (CT). (A) CT laterally connecting two fronds. Scale bar=300 μm. (B) Transverse section of a CT light micrograph. Scale bar=20 μm. (C) Cortical cell cytoplasm diffused with rER, chloroplasts, mitochondria, and electron-opaque globules (O). Scale bar=0.6 μm. (D) Polymorphic mitochondria with well-developed cristae. Scale bar=0.6 μm. Inset: Another irregularly shaped mitochondria. Scale bar=10 μm. (E) Irregularly shaped chloroplast found in cortical cell. Scale bar=0.8 μm. (F) Numerous plasmodesmata (Pd) between adjacent cells. Scale bar=1 μm. DF, daughter frond; MF, mother frond; Cc, cortical cell; C, chloroplast; m, mitochondria; CW, cell wall.

also observed (Fig. 1E) and plasmodesmata were numerous among neighboring cells (Fig. 1F).

After abscission of a daughter frond, the CT left a scar on detached region. The following characteristics were common to cells adjacent to detached scars: unevenly thickened walls (Fig. 2A) and the presence of an electron-dense cuticle on the epidermal surface. Some peripheral cells near the abscission layer developed partially thickened walls, while internally located cells had uniformly thin walls. In such cases, the thick portions of the wall were more than 10 times the width of the thin regions. While air chambers were well-established in fronds, yet none were detectable in CTs. An accumulation of electron dense substances was apparent in the cell walls delimiting intercellular spaces at the CT periphery. It was highly probable to consider that the intercellular spaces that formed from cell wall cleavage were lined with a continuous hydrophobic layer (Fig. 2B). In particular, some unusual columnar structures filled with fibrillar materials formed in some of the spaces (Fig. 2C) in random fashion. The cells neighboring this structure usually exhibited thick electron-dense walls.

The CT, unlike the fronds, only had one central vein which was continuous with one of the frond veins (Fig. 2D). This vein contained one tracheary and four sieve elements, where the tracheary element was completely surrounded by 7 to 10 parenchyma cells and sieve elements radially. Two abscission layers were distinguished at either end where separation between fronds is thought to occur. Prior to separation, cells at distal end of the stalk, divided repeatedly to form the abscission layer, which eventually thinned out with age (Fig.

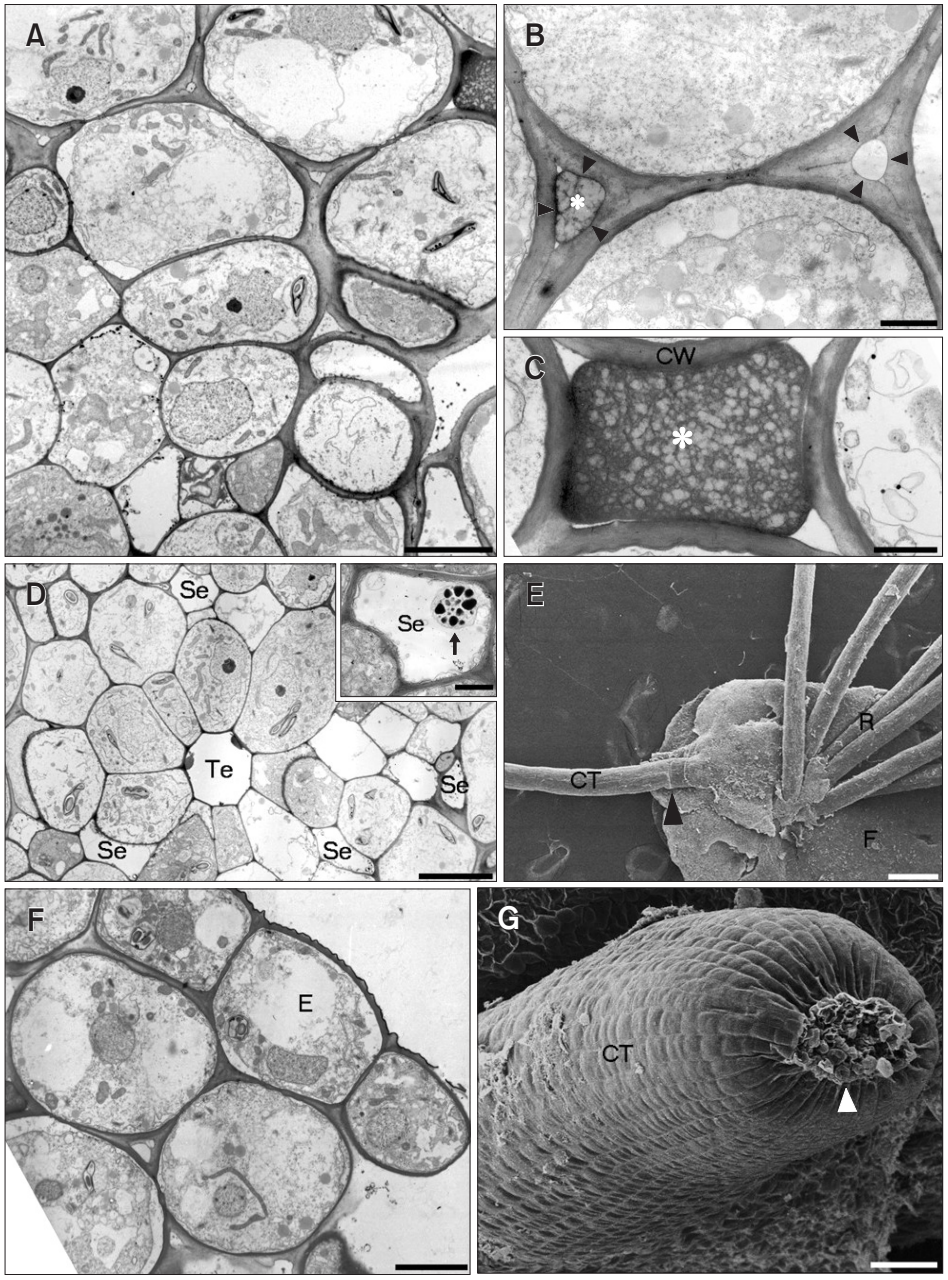

Fig. 2. Scanning electron microscopy (E, G) and transmission electron microscopy (A-D, F) images. Detailed connective stalk (CT) structure. (A) Irregularly thickened cell walls (CW) at the CT periphery. Scale bar=5 μm. (B) Two intercellular spaces demonstrating different stages of wall splitting (arrowheads). Accumulation of fibrillar materials in intercellular space is shown on the right (asterisk). Scale bar=500 nm. (C) Intercellular space filled with fibrillar materials (asterisk). Scale bar=3 μm. (D) Transverse section of a CT showing one tracheary element (Te) and four sieve elements (Se). Scale bar=7 μm. Inset: P-plastid (arrow) in sieve element. Scale bar=1 μm. (E) Abscission layer (arrowhead) at proximal end. Scale bar=500 μm. (F) Partial view of transverse section of CT prior to the abscission. Scale bar=5 μm. (G) CT scar (arrowhead) at distal end after abscission. Scale bar=70 μm. R, root; F, frond; E, epidermis.

2E). The small cells dissociated easily and only a weak vascular connection was maintained just before separation. Cells close to this layer exhibited much loose cytoplasm at this stage and eventually degraded (Fig. 2F). The CT which remained attached to the daughter frond left a scar at the distal end (Fig. 2G). Prominent subepidermal idioblasts (Fig. 2C) appeared to seal the stump of the CT that was still connected to the frond. The abscission layer, formed adjacent to these cells, isolated the offspring from the mother frond.

The offspring is always connected to the mother frond via a translucent stalk. Thus, the CT probably functions mechanically to maintain connection between the fronds as well as a channel to transport nutrients from the mother frond to the offspring (Kim, 2007, 2013; White & Wise, 1998). Cells filled with tanniferous materials were found in the CT as in fronds of *Spirodela* (Kim, 2013), and they are known to strongly absorb ultraviolet rays (Bonnet et al., 1996). This is reported to be consistent with some *Spirodela* where rapid frond senescence and/or rapid frond abscission occurs soon after exposure to ultraviolet radiation (Witzum, 1974a). In fact, a significant decrease in the growth rate was revealed in fronds of *S. polyrhiza* after being exposed to several hours of ultraviolet B range irradiation (Schwalbe et al., 1999). The presence of numerous idioblastic tanniferous cells beneath the

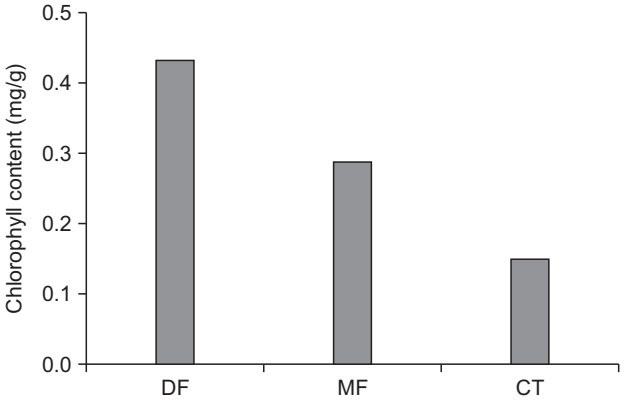

Fig. 3. Comparison of the chlorophyll assay of the examined fronds and connective stalk (CT). DF, daughter fronds; MF, mother frond.

abscission layer strongly suggests a correlation between frond abscission and the sealing of the CT in this species.

The epidermal cells, as well as the internal tissues of the entire plant even in the transparent CT, contain numerous chloroplasts, indicating the occurrence of photosynthesis throughout the plant body. Chlorophyll content in the CT, and young and mature fronds were measured (Fig. 3) to examine the CTs capacity to perform photosynthesis and also to compare their values to those of highly photosynthetic fronds. In general, the color of the frond can reflect chloroplast content and higher concentrations were measured in the fronds. However, the measured chlorophyll contents exhibited variability among different tissue types. The content was much higher in daughter fronds (0.432±0.101) than in mature fronds (0.289±0.081). However, the chlorophyll content in the translucent CT was 0.150±0.032.

A high concentration of chlorophyll in daughter fronds and the presence of poorly developed vascular tissue within the CT support the suggested mechanical function over the transporting one. However, in the case of *Wolffia*, where meristematic fronds do not contain a starch reserve, the possibility of the CT functioning as a transport for nutrients to the developing frond in the very earliest stages (White & Wise, 1998) cannot be ruled out. In a cross section of the CT, a particular lipophilic layer, lined with a hydrophobic layer, developed along the wall cleavage and eventually along the intercellular spaces. This layer may function as a diffusion barrier between the aqueous wall and the gaseous air space (Roland, 1978). The cells there remain exposed to the aerial condition and can maintain gaseous exchange with it. The intercellular spaces, although very reduced, most likely play some part in the buoyancy of thin CT. Moreover, peculiar structures filled with fibrillar materials were detected in random fashion among the air spaces at CT periphery in *S. polyrhiza*. Such structures may have provided support for the CT where no distinct supporting tissue was formed.

CONCLUSIONS

The anatomy and ultrastructure of CTs in *Spirodela polyrhiza* are considerably different from those of the frond and root. The CT, which had well-organized chloroplasts, thus having the ability to photosynthesize as also supported by the chlorophyll content, seemed to carry out a different strategy to fulfill its organic carbon demand. Since the photosynthetic ability in the submerged organ strictly depends on the availability of CO_2 entrapped in the intercellular spaces of its tissues (Rascio et al., 1991), whether this hypothesis can be applied in the same way to the free-floating CT and roots of *S. polyrhiza* remains to be elucidated.

In morphology, *S. polyrhiza* appears to be a poorly differentiated hydrophyte at maturity. However, the structural organization in the CT, with respect to cellular differentiation, was not as simple as it appeared to be. Characteristics such as an entirely chlorenchymatous plant body, well-established plasmodesmatal connection, rapid vegetative reproduction, offspring protection, aerenchyma formation, and effective abscission of the CT were clearly demonstrated in this *Spirodela* species. It is presumed that such reduction and differentiation of the plant body effectively contributes to the better adaptation of smaller plants to superficial aquatic environments during relatively short growing seasons, while also enabling rapid growth.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

The author thanks the Electron Microscopy Laboratory of Korea Basic Science Institutes, Daegu Branch, for use of their facilities and technical assistance.

REFERENCES

Bonnet S L, Steynberg J P, Bezuidenhoudt B C B, Saunders C M, and Ferreira D (1996) Structure and synthesis of phlobatannins related to bis-fisetinidol-epicatechin profisetinidin triflavanoids. *Phytochemistry* **43**, 253-263.

Echlin P, Lai C E, and Hayes T L (1982) Low-temperature X-ray microanalysis of the differentiating vascular tissue in root tips of

Lemna minor L. *J. Microsc.* **126**, 285-306.

Echlin P (1992) *Low-Temperature Microscopy and Analysis*, pp. 349-411, (Plenum Press, New York).

Formin A V, Ladygin G, and Semenova G A (1992) Accumulation of pigments and formation of chloroplast ultrastructure in the process of greening of etiolated duckweed plants. *Sov. Plant. Physiol.* **38**, 483-488.

Inskeep W P and Bloom P R (1985) Extinction coefficients of chlorophyll a and b in N,N-dimethylformamide and 80% acetone. *Plant Physiol.* **77**, 483-485.

Kim I (2007) Development of the root system in *Spirodela polyrhiza* (L.) Schleiden (Lemnaceae). *J. Plant Biol.* **50**, 540-547.

Kim I (2011) Features of plastids within *Spirodela polyrhiza*. *Korean J. Microsc.* **41**, 55-60.

Kim I (2013) Cellular features of the fronds and turions in *Spirodela polyrhiza*. *Appl. Microsc.* **43**, 140-145.

Kim K and Kim I (2000) Structural aspects of the reduced free-floating hydrophyte, *Spirodela polyrhiza*. *Korean J. Microsc.* **30**, 233-240.

Kwak M and Kim I (2008) Turion as dormant structure in *Spirodela polyrhiza*. *Korean J. Microsc.* **38**, 307-314.

Landolt E (1998) Anatomy of the Lemnaceae (duckweeds). In: *Extreme Adaptations in Angiospermous Hydrophytes,* eds. Landolt E, Jager-Zurn I, Schnell R A A, pp. 1-127, (Borntraeger, Berlin).

Lemon G D and Posluszny U (2000) Comparative shoot development and evolution in the Lemnaceae. *Int. J. Plant Sci.* **161**, 733-748.

Melaragno J E and Walsh M A (1976) Ultrastructural features of developing sieve elements in *Lemna minor* L. The protoplast. *Amer. J. Bot.* **63**, 1145-1157.

Rascio N, Mariani P, Tommasini E, Bodner M, and Larcher W (1991) Photosynthetic strategies in leaves and stems of *Egeria densa*. *Planta* **185**, 297-303.

Roland J C (1978) Cell wall differentiation and stages involved with intercellular gas space opening. *J. Cell Sci.* **32**, 325-336.

Schwalbe M, Teller S, Oelmuller R, and Appenroth K-J (1999) Influence of UVB irradiation on nitrate and ammonium assimilating enzymes in *Spirodela polyrhiza*. *Aquat. Bot.* **64**, 19-34.

Walsh M A and Melaragno J E (1976) Ultrastructural features of developing sieve elements in *Lemna minor* L.: sieve plate and sieve areas. *Amer. J. Bot.* **63**, 1174-1183.

White S L and Wise R R (1998) Anatomy and ultrastructure of *Wolffia columbiana* and *Wolffia borealis*, two nonvascular aquatic angiosperms. *Int. J. Plant Sci.* **159**, 297-304.

Witzum A (1974a) Ultraviolet irradiation and pigment cell idioblasts in *Spirodela oligorhiza* (Lemnaceae). *Amer. J. Bot.* **61**, 713-716.

Witzum A (1974b) Abscission and the axillary frond in *Spirodela oligorhiza* (Lemnaceae). *Amer. J. Bot.* **61**, 805-808.