Ultrastructure changes of seedlings of *Kosteletzkya virginica* under waterlogging conditions

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Abstract

The changes in ultrastructure of leaf cell in waterlogged *Kosteletzkya virginica* seedlings were observed by transmission electron microscope. During waterlogging, the chloroplasts turned into a round shape and their volume was gradually reduced. Furthermore, the lamellae of thylakoids swelled, and the inclusions in chloroplast decreased. The shape of starch grains slightly changed, but their number and volume decreased, and they even disappeared in the end. On the other hand, plastoglobules enlarged and their amount enhanced gradually. The membrane and inner cristea of mitochondria gradually became unclear, while the mitochondria firstly enlarged but disappeared at the end. Similar to chloroplast, cell nucleus dwindled gradually, concentrated and tended to a round shape. Additionally, the annulate lamellae and multivesicular body occurred on the 20th day of experiment, and the cell wall distorted and twisted at the late stage of waterlogging. Consequently, these changes of organelles are the typical characteristics of the complete cell disintegration or death for *Kosteletzkya virginica* under long-term waterlogging.

Additional key words: chloroplast, halophyte, mitochondria, nucleus, plastoglobules, starch grains, thylakoids.

Introduction

Adverse environmental conditions not only affect plant growth or physiological characteristics (*e.g*. Kumutha *et al*. 2009, Roychowdhury *et al*. 2009, Sairam *et al*. 2009), but also cause ultrastructural changes or disintegration of cell organelles (Käfirenlampi and Houpis 1986, Sutinen 1987, Saastamoinen and Holopainen 1989, Sutinen *et al*. 1990, Wulff and Käirenlampi 1996). Wulff and Käirenlampi (1996) observed the increased vacuole, enlarged and darkened plastoglobules and accumulated lipid bodies in Scots pine and Norway spruce needles suffered from fluoride. Wi *et al*. (2005) found that chloroplast was more sensitive to γ-irradiation than other cell organelles, and parts of the mitochondria and endoplasmic reticulum were structurally altered in *Arabidopsis*. Accumulation of heavy metal also harmed cell ultrastructure, *e.g.*, cadmium treatment induces swelling and merging of grana thylakoids, and even a destruction of the whole plastid apparatus in the leaves of *Myrophyllun spicatum* (Stoyanova and Tchakalova 1997). In addition, extreme sensitivity of chloroplasts to salinity was reported (Li and Ong 1997, Barhoumi and Djebali 2007). Palomäiki *et al*. (1994) detected the ultrastructural changes of Scots pine and Norway spruce needles under drought and waterlogging, however, as for starch grain number, an increase under waterlogging and a decrease under drought were found in both plants. However, in these studies, none of them is concerned with the complete disintegration process of ultrastructure under environmental stress.

Kosteletzkya virginica (L.) Presl is a perennial herbaceous halophyte of the family *Malvaceae*. In May 2007, after an approximately 40 days of waterlogging, most plant species were dead, whereas *K. virginica* survived with altered morphology on Golden-Sea Farm (Jiangsu Province, China). Therefore, the aim of this project was to characterize the ultrastructural changes and the typical adaptation mechanisms of *K. virginica* seedlings during waterlogging.

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Materials and methods

After germination of *Kosteletzkya virginica* (L.) Presl seeds in mixed medium (*Perlite* + *Vermiculite*; 1:1), seedlings were irrigated with 1/4 strength Hogland solution. Afterwards, 45-d-old seedlings were transplanted to pots filled with salt soil (electrical conductivity 2.77 dS m^{-1}) from Golden-Sea Farm. Then pots were placed in white plastic tanks (12 cm deep, 36 cm long, 26.5 cm wide). Pots were divided into 6 groups: 5 waterlogging treatments plus 1 control. After a 45-d adjusting in salt soil, 6 leaves in each group of seedlings (from upper part of plant; 1 leaf per pot) were sampled, and immediately soaked in the 0.4 % glutaraldehyde solution diluted by 0.2 M phosphate buffer (pH 7.2). Subsequently, these samples were treated in laboratory. After the first sampling, the seedlings were waterlogged to a level of approximately 5 cm above the soil surface. Then 6 sampled leaves from upper part of plants were in one group (1 leaf per pot) on the 10^{th}, 20^{th}, 30^{th}, 40^{th} and 50^{th} days of waterlogging and treated in the same way as mentioned above. In course of experiment, the controls and the treatments were all irrigated with 1/2 strength Hogland solution every week.

In laboratory, the slices (about 1 mm wide and 2 mm long) crossed by a lateral vein, were cut from the sampled leaves, and each measurement consisted of six slices. The samples were prefixed for about 20 h in 0.4 % glutaraldehyde fixative, subsequently washed in 0.1 M phosphate buffer and postfixed in 1 % OsO_4 solution, which was diluted by 0.2 M phosphate buffer (pH 7.2), for 6 h at 6 °C. Then they were dehydrated in a graded acetone series, saturated in mixture of acetone and *Epon 812* (1:1 and then 1:2, v/v, 30 min) and subsequently in pure *Epon 812* (2 h). The blocks were polymerized for 1 d at 30 °C, 1 d at 40 °C and 3 d at 60 °C, and finally cut into ultrathin-sections with ultramicrotome *LKB-V* (*LKB*, Bromma, Sweden). The sections for electron microscopy were stained with uranyl acetate and lead citrate. The status of different organelles and cytoplasm in mesophyll cell tissues were observed and photographed with electron transmission microscope *Hitachi H-600* (Tokyo, Japan). The length and width of cell organelles were measured with software *NIS-Elements BR 2.30*.

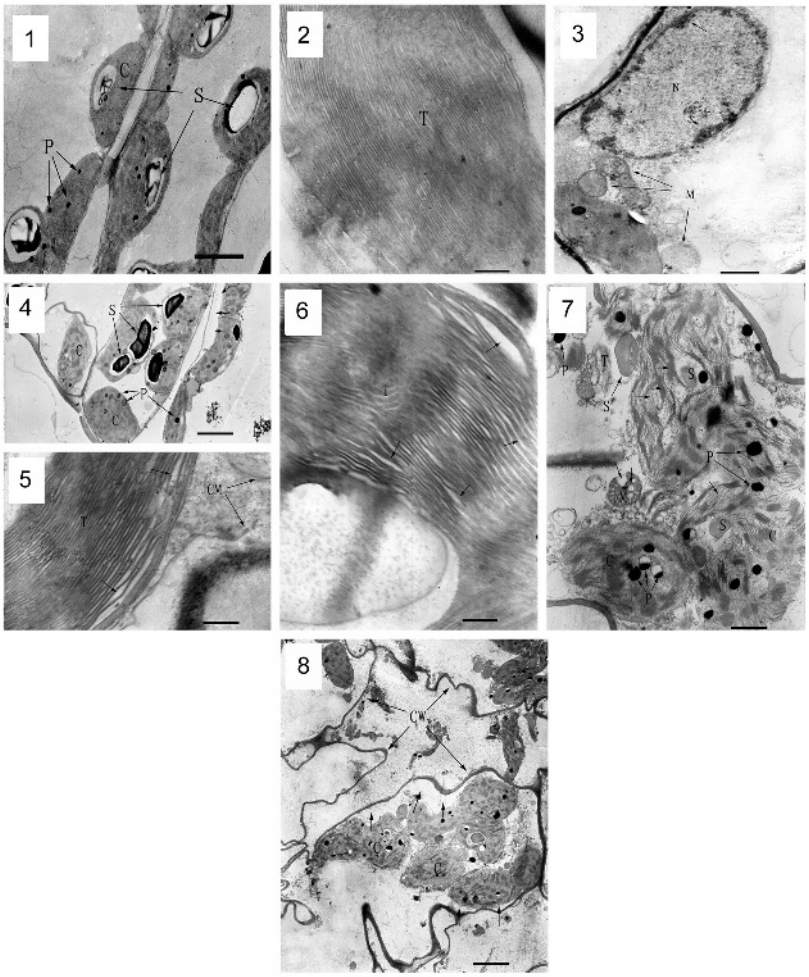
One-way analysis of variance was conducted with *SPSS 11.5* (*SPPS Inc*., Chicago, USA)

Results

Before waterlogging, chloroplasts clung to cell walls and most of them contained starch grains and plastoglobules. Outer membrane of chloroplasts was clear, and thylakoid lamellae were regularly and clearly arranged in chloroplasts (Figs. 1,2). During waterlogging, most chloroplasts clung to the cell walls, and contained starch grains and plastoglobules. However, a slight plasmolysis of protoplast occurred (Figs. 3,4), the outer membrane of chloroplast bulged in some area, and some of thylakoid lamellae swelled on the 10^{th} day (Fig. 5). Subsequently,

Table 1 Effects of waterlogging on characteristics of chloroplasts, starch grains, plastoglobules, mitochondria and cell nucleus in *K. virginica* seedlings on the 0^{th}, 10^{th}, 20^{th}, 30^{th}, 40^{th} and 50^{th} day of waterlogging. Means ± SD, n = 7 - 17, values carrying different letters are significantly different at $P \leq 0.05$ (* ratio = chloroplasts containing starch grains/all chloroplasts).

Organelle	Parameter	0^{th}	10^{th}	20^{th}	30^{th}	40^{th}	50^{th}
Chloroplasts	length [μm]	13.70±2.50^{a}	11.29±1.80^{b}	11.07±2.40^{b}	10.53±1.80^{b}	3.00±0.53^{c}	2.45±0.26^{c}
	width [μm]	6.03±1.06^{a}	5.98±1.17^{a}	5.63±1.95^{a}	5.76±2.11^{a}	2.52±0.39^{b}	1.99±0.30^{b}
	W/L	0.46±0.12^{b}	0.54±0.15^{b}	0.50±0.11^{b}	0.56±0.21^{b}	0.85±0.10^{a}	0.81±0.07^{a}
Starch grains	length [μm]	4.40±0.88^{a}	3.22±0.71^{b}	2.16±0.60^{c}	2.11±0.76^{c}	-	-
	width [μm]	2.27±0.55^{a}	1.14±0.32^{b}	1.21±0.29^{b}	1.30±0.41^{b}	-	-
	W/L	0.53±0.14^{a}	0.52±0.10^{a}	0.57±0.04^{a}	0.62±0.10^{a}	-	-
	number [section^{-1}]	0.84±0.60^{a}	1.06±0.80^{a}	0.56±0.73^{ab}	0.38±0.51^{b}	-	-
	ratio [%]*	73.68	72.20	44.44	38.46	-	-
Plastoglobules	diameter [μm]	0.63±0.10^{bc}	0.84±0.23^{a}	0.72±0.20^{ab}	0.86±0.24^{a}	0.86±0.18^{a}	0.59±0.22^{c}
	number [section^{-1}]	3.33±1.83^{a}	2.95±1.66^{a}	3.80±1.49^{a}	3.75±1.84^{a}	6.07±1.69^{b}	7.14±1.46^{b}
Mitochondria	length [μm]	1.06±0.11^{b}	1.26±0.25^{b}	1.75±0.68^{a}	1.73±0.41^{a}	-	-
	width [μm]	0.75±0.11^{c}	1.14±0.24^{b}	1.57±0.52^{a}	1.51±0.37^{a}	-	-
	W/L	0.71±0.08^{b}	0.91±0.08^{a}	0.91±0.06^{a}	0.87±0.08^{a}	-	-
Nucleus	length [μm]	8.67±1.11^{a}	8.99±0.96^{a}	9.62±1.88^{a}	8.69±0.29^{a}	4.70±1.66^{b}	3.43±1.34^{b}
	width [μm]	5.20±0.13^{a}	5.11±0.45^{a}	4.89±0.30^{a}	4.97±0.38^{a}	4.19±1.43^{ab}	2.86±1.15^{b}
	W/L	0.61±0.06^{b}	0.58±0.11^{b}	0.52±0.09^{b}	0.57±0.04^{b}	0.89±0.06^{a}	0.84±0.10^{a}



Figs. 1 to 8. Electron micrographs of organelles in mesophyll cell of *K virginica* seedlings before waterlogging (Figs. 1,2) and at early (Figs. 3,4,5) and middle (Figs. 6,7,8) stages. Fig. 1 - chloroplasts, starch grains and plastoglobules (*bar* = 8 μm). Fig. 2 - chloroplast composed of regular and clear thylakoid lamellae (*bar* = 0.5 μm). Fig. 3 - darkening of cell nucleus membrane (*arrows*) and normal mitochondria after 10 d of waterlogging (*bar* = 2 μm). Fig. 4 - slight plasmolysis of protoplast and large plastoglobules after 10 d of waterlogging (*bar* = 8 μm). Fig. 5 - slight swelling of thylakoid lamellae (*arrows*) and disintegration of chloroplast outer membrane after 10 d of waterlogging (*bar* = 0.5 μm). Fig. 6 - swelling and curling of thylakoid lamellae in a mesophyll cell of palisade parenchyma after 20 d of waterlogging (*arrows*; *bar* = 0.5 μm). Fig. 7 - disappearance of chloroplasts membrane, released organelles in cytoplasm, swelling of mitochondrial (*arrows*) and distortion of thylakoid lamellae (*arrows*) after 30 d of waterlogging (*bar* = 2.5 μm). Fig. 8 - Distortion and serious separation of protoplast (*arrows*) from cell wall, and the twist of cell wall in mesophyll cells of palisade parenchyma after 30 d of waterlogging (*bar* = 8 μm). C - chloroplast, CW - cell wall, CM - outer membrane of chloroplast, M - mitochondrion, N - nucleus, P - plastoglobule, S - starch grain, T - thylakoid lamellae.

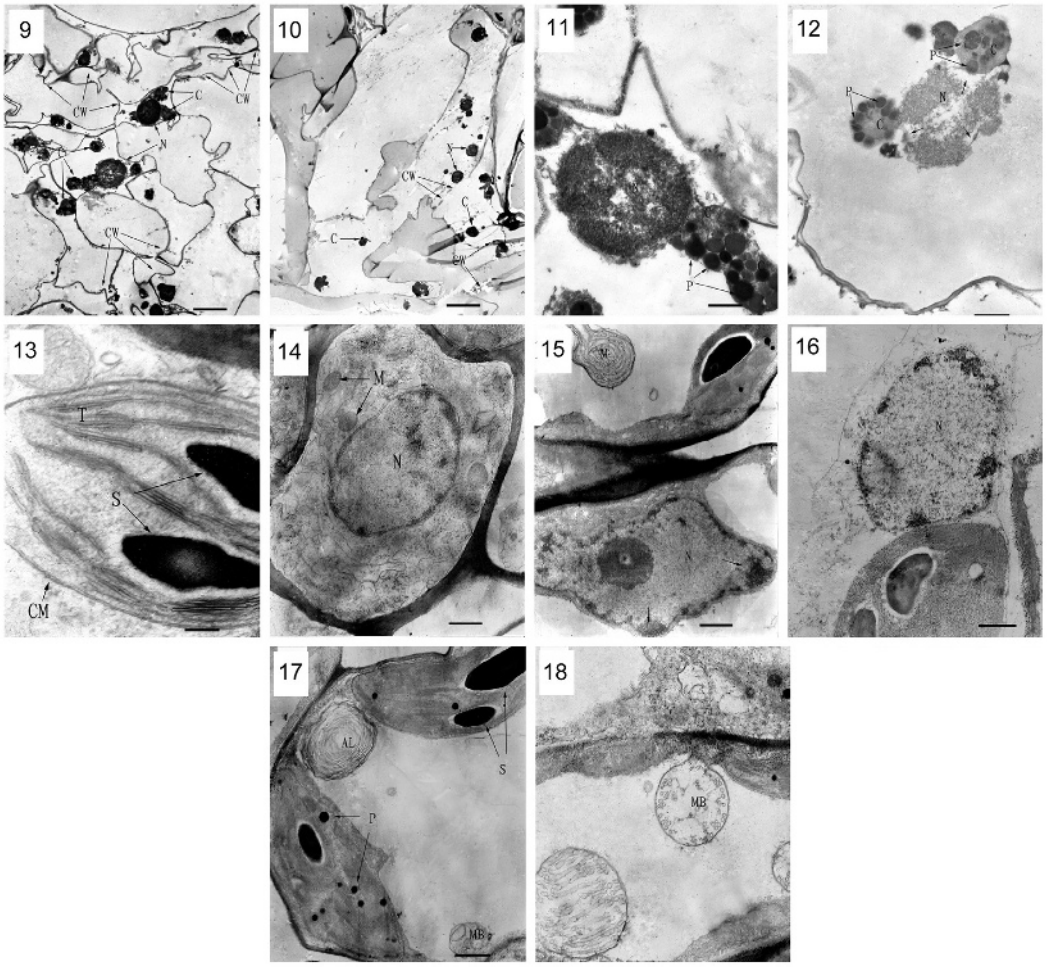
the thylakoid lamellae disturbed and swelled further in chloroplasts (Fig. 6) on day 20. On the 30th day of stress, most outer membranes of chloroplasts disappeared (Fig. 7), protoplast obviously pulled away from the cell wall, and a few starch grains appeared in abnormal chloroplasts (Fig. 8). Furthermore, thylakoid lamellae were seriously disorganized and curled, and some of them disseminated in cytoplasm with the other inclusions (Fig. 7). After 40 and 50 d of waterlogging, the cells were near to death or already dead, and species and amount of cell organelles decreased obviously (Figs. 9,10). The chloroplasts disintegrated and contained no inclusions except the enlarged plastoglobules (Figs. 11,12).

During waterlogging, the shape of chloroplast changed (Table 1). The normal section looked like a kidney, whose ratio of width/length was 0.46. During 30 d of waterlogging, the length and width change indistinctly, and then ratios of them were insignificantly different from the control (Table 1). The section of chloroplast retained a kidney-like shape on day 10 and 20, and, afterwards, became misshapen on day 30 of stress. On the 40th and 50th day of waterlogging, the length and width decreased significantly, and the ratio of width/length was 0.85 and 0.81, respectively, which indicated that the chloroplast sections looked close to a round.

In controlled seedlings, most chloroplasts (73.7 %) contained starch grains. The sections of starch grains retained a kidney-like shape with width/length ratio 0.53. During 30 d of waterlogging, the section length and width decreased significantly (Figs. 1,4,13), but the width/length

ratio was stable and insignificantly different from the control (Table 1). The percentage of chloroplasts containing grains changed slightly on the 10th day, but decreased to 38.6 % after 30 d of stress. On the 40th and 50th day of waterlogging, the starch grains were decomposed, and subsequently disappeared (Figs. 9,10,11,12).

Before waterlogging, a few plastoglobules existed in chloroplasts, approximately 3.33 grains per chloroplast section, and the section diameter was only about 0.63 μm (Fig. 1). During waterlogging, this diameter increased significantly on the 10th day, then stabilized, and finally reduced at the end of stress period (Table 1, Figs. 7,9). As for amount, in comparison with the control, it changed slightly from the 10th to 30th day of waterlogging, and then increased significantly on the 40th and 50th day of waterlogging (Table 1). In addition, most plastoglobules were opaque and black before stress (Fig. 1). However, after waterlogging, transparency was increased gradually. On the 10th day, some of them showed semi-transparency and were grey (Fig. 4). Half-moon plastoglobules possessing transparency in half area and opacity in the other, were observed on 30th day. At the late stage of experiment, most plastoglobules showed semi-transparency, and the rest were opaque and black (Figs. 11,12).



Figs. 9 to 18. Electron micrographs of organelles in mesophyll cell of *K virginica* seedlings at the later stages of waterlogging (Figs 9 to 12), and some special organelles (Figs. 13 to 18). Fig. 9 - twist of cell wall, decrease of organelle species and disintegration of chloroplasts after 40 d of waterlogging (*bar* = 8 μm). Fig. 10 - ruptured cell and broken cell wall after 50 d of waterlogging (*bar* = 8 μm). Fig. 11 - concentrated cell nucleus, and disintegrated chloroplasts after 40 d of waterlogging (*bar* = 2 μm). Fig. 12 - destroyed cell nucleus (*short arrows*) and disintegrated chloroplasts after 50 d of waterlogging (*bar* = 2 μm). Fig. 13 - normal chloroplast before waterlogging (bar = 0.5 μm). Fig. 14 - cell nucleus and some mitochondria before waterlogging (*bar* = 2 μm). Fig. 15 - bulged and destroyed outer-membrane and dark inner-cristeae in mitochondria; dark nucleus membrane (*short arrows*) and distorted cell nucleus on the 20th day of stress (*bar* = 2 μm). Fig. 16 - disappearance of most parts of nuclear membrane after 30 d of waterlogging (*bar*= 2 μm). Fig. 17 - annulate lamellae and multivesicular body on day 20 of stress (*bar* = 1.67 μm). Fig. 18 - annulate lamellae and multivesicular body on day 20 of stress (*bar* = 1.33 μm). C - chloroplast, CW - cell wall, CM - chloroplasts envelope, M - mitochondrion, N - nucleus, S - starch grain, T - thylakoid lamellae, P - plastoglobule, AL - annulate lamellae, MB - multivesicular body.

In the course of waterlogging, mitochondria gradually swelled and finally disintegrated (Table 1). In the control, outer membrane and cristae were much clearer, and the mitochondrial section displayed an ellipse shape (Fig. 14) with width/length ratio 0.71. On the 10th day, the outer membrane and cristae were clearly visible (Fig. 3), but after 20 d, the outer membrane bulged and got unclear in some area, whereas the cristae also obscured and curled (Fig. 15). On the 30th day, the inner cristae swelled, and some bubble-like structures formed in the mitochondria (Fig. 7). Length, width and width/length ratio of section significantly increased compared to the control, which showed a change from ellipse to round shape (Table 1). At the late stage of waterlogging, the mitochondria decomposed and disappeared in mesophyll cells (Figs. 9,10).

Before waterlogging, outer membrane of cell nucleus was clear and complete, and chromatin was distributed equally. The section of cell nuclear looked like an ellipse, whose width/length ratio was 0.61 (Fig. 14, Table 1). On the 10th day of waterlogging, the chromatin was uniform and the outer membrane was basically clear, but slightly darkened in some area, which denoted electron dense heterochromatin under membranes (Fig. 3). After 20 d, the cell nucleus distorted, but chromatin remained uniform. However, the outer membrane was unclear, got damaged and even disappeared in some area (Fig. 15). On the 30th day, outer membrane mostly disappeared, and the chromatin was not uniform, indicating its decomposition (Fig. 16). In succession, the outer membrane decomposed and disappeared, the chromatin seriously disintegrated, and the cell nucleus was concentrated at late stage (Figs. 11, 12). The length, width and width/length ratio of the section changed only slightly compared to the control during 30 d of stress. Subsequently, length and width significantly decreased, whereas the ratio increased to 0.89 and 0.84, which showed a change from ellipse to round shape (Table 1).

During waterlogging, annulate lamellae and multi-vesicular body only formed on the 20th day in the cytoplasm and clung to the cell wall. They consisted of many concentric lamellae and vesicles (Figs. 17,18).

In the controls, the cell wall stuck by the cytoplasm was smooth (Fig. 1). On the 10th and 20th day of waterlogging, even if slight plasmolysis occurred, the cell wall was smooth basically (Fig. 4). Further, the cell wall began to distort with the visible plasmolysis on the 30th day (Fig. 8), then seriously twisted (Fig. 9), and broke into fragments (Fig. 10), which caused the cracking of the cells at late stage of waterlogging.

Discussion

Under waterlogging, the reduced size of chloroplast was induced in *K. virginica* seedlings, and mitochondria, thylakoid lamellae and plastoglobules also changed. The similar changes have been reported in some other stressed plants (Wample and Davis 1983, Eleftheriou and Tsekos 1991, Palomäki *et al*. 1994, Wulff and Käirenlampi 1996, Wi and Chung 2005, Barhoumi and Djebali 2007). Swelling and curling of thylakoid lamellae might be partly related to nutrient deficiency under waterlogging conditions (Vignolio *et al.* 1999). However, the increase and enlargement of plastoglobules, should resulted from the accumulation of lipids, which were released from the degrading thylakoids (Kessler and Vidi 2007). The variable lipid composition might cause the translucence and half-moon in plastoglobules (Goodwin and Mercer 1983). In addition, it is not certain that darkening and swelling of mitochondria resulted from either environmental oxygen deficiency or due to enzymes appearing during cell death processes caused by long-term waterlogging (Wei *et al*. 2000).

In this study, the size and amount of starch grains decreased, the grains disintegrated and disappeared finally. However, the large starch grains accumulated in waterlogged Scots pine and Norway spruce seedlings (Palomäki *et al*. 1994). This special change in waterlegged *K. virginica* seedlings might be due to reduction of photosynthesis, which can not provide enough glucose for synthesizing starch in chloroplasts. After 10 and 30 d of waterlogging, the photosynthesis capacity according to chlorophyll fluorescence parameter decreased (unpublished data from Halophyte Research Laboratory, Nanjing University). The other possibility is increased need of glucose to resist waterlogging. Under waterlogging, plants obtain energy mainly from anaerobic respirations, whereas the aerobic respiration (Krebs cycle) is restrained gradually because of oxygen deficiency (Visser *et al*. 2003, Liu and Li 2007). Additionally, it is well known that anaerobic respiration has a lower efficiency in usage of glucose than the aerobic one. On 10th and 30th day of waterlogging, the aerobic respiration decreased, whereas alcoholic and lactic fermentation increased (unpublished data form Halophyte Research Laboratory, Nanjing University). The decreasing glucose supply and increasing glucose consumption may promote the starch grains to decompose into glucose during waterlogging.

An interesting observation in this experiment was annulate lamellae in the leaf mesophyll cell of 20th day of waterlogging. This cell organelle is only a temporary structure derived from endoplasmic reticulum in plant cells (Wu *et al*. 1998), and forms at special situations as cell division, cell multiplication or environmental stress (Shen and Ma 1989). Its function is not clear, but it thought to be the autophagy (Wu *et al*. 1998), which can disintegrate the aging or damaged organelles, and then provide products for constructing new organelles in the cell. Actually, serious stress causes cell necrosis in higher plants, but the less severe stress could induce the programmed cell death (PCD) acting as an active adjusting method under adverse environment (Pan *et al*. 2002).

Furthermore, in the course of PCD, autophagy occurs and prevents intact cells from the initial death (Patel *et al*. 2006). So this emergence of annulate lamellae indicated that PCD might have been induced by waterlogging for *K. virginica* seedlings, and the annulate lamellae should be digesting the damaged organelles to rebuild the cell system and to increase the survival capacity of these seedlings. So the annulate lamellae may be an active adjustment of *K. virginica* seedling under waterlogging. Moreover, the stress threshold of PCD might be 20 d under waterlogging. But a series of further experiments are needed to prove this presumption in succession.

The other typical adaptation was that the formation of multivesicular body, which also emerged only on the 20th day of waterlogging. Multivesicular body is regarded as an organelle originated from Golgi appratus (Segui and Staehelin 2006), and can transport materials by exocytosis, for example, hemicellulose and pectin to construct cell wall (Xu *et al*. 2008). This formation of multivesicular body might play a role in repairing the damaged parts of cell walls in the waterlogged seedlings. Similar to annulate lamellae, the absence of multivesicular body observed on the other days of waterlogging, may be caused by different damage extent. So the formation of multivesicular body could be considered as another adjustment mechanism in *K. virginica* under waterlogging conditions.

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