

Respiratory enzyme activity and regulation of respiration pathway in seashore mallow (*Kosteletzkya virginica*) seedlings under waterlogging conditionsJian Zhou¹, Xueliang Tian¹, Lifang Qiao¹, Pei Qin^{2*}¹Henan institute of science and technology, 453003 Xinxiang, China²Halophyte Research Lab of Nanjing University, 210093 Nanjing, China

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Abstract

We determined the enzyme activities involved in the aerobic and anaerobic respiration of *Kosteletzkya virginica* on the 0th, 10th, 20th, and 30th d of waterlogging to understand its respiratory characteristics. We also determined the enzyme activities on the 10th and 20th d of recovery after water drainage. Non-waterlogged seedlings were regarded as control during this experiment. All waterlogged seedlings were averagely divided into two parts after the formation of adventitious roots. The first and second treatment groups contain seedlings with retained and removed adventitious roots, respectively. The activities of mitochondrial enzymes, including pyruvate dehydrogenase, isocitrate dehydrogenase, α -ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and cytochrome *c* oxidase, were lower in the two seedling groups than in the control. Furthermore, the adventitious root-removed seedlings exhibited the lowest mitochondrial enzyme activities during waterlogging. By contrast, an increase in the activity of fermentation enzymes, including alcoholic dehydrogenase (ADH), pyruvate decarboxylase (PDC), and lactate dehydrogenase (LDH), was observed in the two treatments compared with the control. However, the activities of ADH, PDC, and LDH were higher in the adventitious root-removed seedlings than those in the adventitious root-retained seedlings. Subsequently, all enzyme characteristics in waterlogged seedlings were restored gradually during the recovery period. Enzyme activity was analyzed to calculate the amount of released electrons. The contribution of the tricarboxylic acid (TCA) cycle to the survival of stressed plants decreased, whereas that of lactic and alcoholic metabolisms increased during waterlogging. The contributions of TCA (27.8%) and alcoholic fermentation (36.3%) were obviously different from their corresponding control (36.1% and 31.0%, $P \leq 0.05$) on the 30th d of waterlogging. The contribution rate of the TCA cycle in the adventitious root-retained seedlings was 6.7% and 5.9% higher ($P \leq 0.05$) than that in the adventitious root-removed seedlings during this experiment. In conclusion, adventitious roots can sustain aerobic respiration in the mitochondria to help *K. virginica* overcome waterlogging.

Keywords: *Kosteletzkya virginica*, Seedlings, Waterlogging, Respiratory enzyme**Abbreviations:** ADH_alcoholic dehydrogenase, Alt_alternative, AOX_Alt oxidase, CCO_cytochrome-C oxidase, Cyt_cytochrome, DCPIP_2,6-dichlorophenol-indophenol, IDH_isocitrate dehydrogenase, LDH_lactic dehydrogenase, MDH_malate dehydrogenase, PDC_pyruvate decarboxylase, PDH_pyruvate dehydrogenase, SDH_succinate dehydrogenase, TCA_tricarboxylic acid, UQ_ubiquinone, α -KDH_ α -Ketoglutarate dehydrogenase.**Introduction**

Soil water is a critical limiting factor in plant growth and distribution. Waterlogging is often caused by excessive rainfall or poor drainage in many regions around the world. Waterlogging can severely impair the morphological performance of terrestrial plants by inducing hypoxia in the plant rhizosphere (Armstrong et al., 1994; Blom, 1999; Jackson and Armstrong, 1999; Kozłowski, 1997; Vartapetian and Jackson, 1997). The function of mitochondrion, which plays an essential role in different plant metabolic activities, has been shown sensitive to adverse conditions (Hadži-Tašković Šukalović and Vuletić, 2001; Vassileva et al., 2009). This sensitivity is related to substrate oxidation and stress level (Flagella et al., 2006). In the absence of oxygen, mitochondrial activity is usually depressed by hypoxic stress (Sheng et al. 2008) because of the lack of a terminal electron acceptor (Polyakova and Vartapetian, 2003). Compared with those in the control plants, the respiration control and ADP/O in three varieties of winter wheat (*Triticum aestivum* L.) under drought stress decrease and then subsequently increase in recovery

period (Vassileva et al., 2009). The sensitivity or changes in the mitochondrial function is correlated with the damage or adverse effects of severe stress on plant respiration and electron transport chain (ETC). The mitochondrial ETC branches at the level of ubiquinone into a cytochrome (Cyt) pathway coupled to ATP synthesis and an alternative (Alt), non-phosphorylating pathway that channels electrons directly to oxygen through Alt oxidase (AOX) (Vassileva et al., 2009). Previous studies on Cyt or Alt pathway respiration (Ribas-Carbo et al., 2005; Clifton et al., 2005; Noguchi et al., 2005; Noguchi and Terashima, 2006; Vassileva et al., 2009) analyzed or compared their characteristics. Through reverse transcriptase-polymerase chain reaction, Clifton et al. (2005) reported that the amount of the AOX gene family in yeast under stress conditions showed > twofold increase compared with the control. In addition, they reported that Alt respiration pathway rapidly responded to stress treatment, whereas the Cyt respiratory chain was non-responsive. Vassileva et al. (2009) observed that both total and Cyt pathway respirations decrease significantly under

drought conditions, whereas Alt pathway respiration increases compared with well-watered plants in three varieties of winter wheat (*Triticum aestivum* L.). Similarly, the Alt pathway is up-regulated under water stress, whereas a corresponding decrease is observed in the activity of the Cyt pathway in soybean leaves (Ribas-Carbo et al., 2005). These studies focused on characterizing the two pathways involved in respiration and analyzing the major role of the Alt pathway in the overall mitochondrial ETC. In the Cyt pathway, electrons come from the mitochondrial NADH of the tricarboxylic acid (TCA) cycle and from the exogenous NAD(P)H of extramitochondrial metabolisms, such as the anaerobic fermentation pathway. However, quantification studies on the Cyt pathway for evaluation of the contributions of the TCA cycle and fermentation in the overall mitochondrial ETC, particularly under hypoxic conditions, are yet to be conducted. Some studies on the TCA cycle and fermentation have been conducted. However, these studies only focused on the sole aspect of respiration and did not comprehensively analyze both mechanisms. In germinating pea seeds (*Pisum sativum* L. cv. Bonneille) under heavy metal stress, the activities of four TCA cycle enzymes, namely, α -ketoglutarate dehydrogenase (α -KDH), isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH), were lower than those in the control (Bansal et al., 2002). Fox and Kennedy (1991) observed that the activities of TCA cycle enzymes in anaerobically germinated seedlings of barnyard grass (*Echinochloa phyllopogon* (Stapf) Koss) and rice (*Oryza sativa* L.) were lower than those in aerobically germinated seedlings. However, enzyme activities increased when the seedlings were transferred from anaerobic to aerobic conditions. Additionally, Hadži-Tašković Šukalović and Vuletić (2001) reported that ammonium deficiency can decrease the activities of TCA cycle enzymes in maize (*Zea mays* L.) roots. Compared with the TCA cycle, studies on the anaerobic fermentation of plant have been related to hypoxic stress. When oxygen is insufficient for respiration, plant root reacts with fermentative processes to produce ethanol and lactic acid (Sorrell, 1999). After lactic acid accumulation, cytoplasmic acidosis usually occurs, followed by lactate dehydrogenase (LDH) inhibition. As a result, the expression of the hemoglobin gene linked to the increased activity in alcohol dehydrogenase (ADH) is up-regulated (Silva-Cardenas et al., 2003). In addition, fermentation is switched to the production of ethanol instead of lactate (Armstrong and Drew, 2002). Mustroph and Albrecht (2003) reported the reactions of fermentation enzymes in three crops (rice, wheat, and maize). The enzyme activities in the crops increased significantly after 4 d of hypoxia treatment. The activity of ADH and pyruvate decarboxylase (PDC) showed a higher increase than LDH. Among the three crops, rice exhibited the slightest increase in each enzyme activity (Mustroph and Albrecht, 2003). Seashore mallow (*Kosteletzkya virginica* L. Presl) is a perennial herbaceous plant that was native to the brackish portions of the coastal tidal marshes of the mid-Atlantic and southeastern United States (Gallagher, 1985). Chinese scientists have studied *K. virginica* because it serves as a pioneer halophyte and a potential biodiesel plant species (Xu et al., 1996; Ruan et al., 2005). *K. virginica* was initially introduced to China in 1992 and transplanted on saline land along the coastal region of Jiangsu Province by the Halophyte Research Lab of Nanjing University, China. However, *K. virginica* often suffers from both drought and waterlogging stresses in North Jiangsu during the early drought period (March to May) and serious waterlogging from excessive rainfall during the rainy season (June and July). Interestingly, this plant species exhibits a strong tolerance to drought and waterlogging stress, as observed at the research

site, particularly in June to July 2007 when the Jinhai Farm of Dafeng County, North Jiangsu (research site) was waterlogged for approximately 40 d. Most introduced plants, including Chinese tamarisk (*Tamarix chinensis* L.) and Jerusalem artichoke (*Helianthus tuberosus*), died after this natural disaster, although they commonly exhibit higher tolerance to salt-alkali stress. However, the mallow survived this particular waterlogging and showed other distinct adaptation characteristics, such as the floating of adventitious roots in water. This study aims to investigate the damage induced by waterlogging and determine the role of *K. virginica* adventitious roots on mitochondrial function and respiration. We designed an experiment covering 50 d of waterlogging and another 20 d of recovery after drainage. Enzyme activities involved in the aerobic and anaerobic respirations of *K. virginica* during the experimental process were determined.

Results

Enzyme characteristics of TCA

On the 10th d of waterlogging, the effect of stress on the PDH activity of seashore mallow seedlings was not significant (Fig. 1-a). However, the difference between each treatment and the control increased with the duration of waterlogging. On 20th and 30th d, the PDH activity of the adventitious root-retained seedlings decreased by 22.7% and 11.4%, respectively, compared with the control. The activity of PDH was the lowest in the adventitious root-removed seedlings. No significant difference was observed in the PDH activity between the two treatments, but the PDH activity in the adventitious root-removed seedlings was lower than that in the control during waterlogging. After drainage, the PDH activity of the seedlings in the two treatments all restored gradually. On the 20th d of drainage, the PDH enzyme activity in the adventitious root-removed seedlings was 5.3% and 10.0% lower than that in the adventitious root-retained and controlled groups, respectively. However, no significant differences were observed among the three groups ($P > 0.05$) (Fig. 1a). The changes in IDH, α -KDH, SDH, MDH, and cytochrome *c* oxidase (CCO) activities in *K. virginica* seedlings were similar to those observed in PDH activity under the waterlogging stress. For the adventitious root-retained plants on the 10th, 20th, and 30th d of waterlogging, the respective IDH reduction data were 10.2%, 31.5%, and 30.2% of that in the control; α -KDH decreased to 8.9%, 17.0%, and 36.7%; and MDH were 7.7%, 12.0%, and 23.1%. SDH in the adventitious root-retained plants was 16.2% on the 30th d, and CCO decreased to 39.0% and 35.1% on the 20th and 30th d of waterlogging, respectively (Figs. 1a–1f). The enzyme activities in the treated seedlings restored gradually after drainage, and the difference between the treatment and control groups gradually decreased. On the 10th and 20th d of recovery period, the activity of IDH in the treated seedlings was 19.7% and 5.5% lower than that in the control. α -KDH, SDH, and CCO activities remained similar to that of IDH in the *K. virginica* seedlings. After 20 d of water drainage, no significant differences in enzyme activities were observed between the treated and controlled seedlings. The IDH, α -KDH, SDH, and CCO activities in the adventitious root-removed plants were significantly lower than those in the adventitious root-retained and control plants. However, the difference gradually decreased from the 20th d of waterlogging to the 10th d of recovery. On the 20th day of recovery, the IDH activity in the adventitious root-removed seedlings further restored to 80.2% and 85.4% of the control and adventitious root-retained seedlings, respectively. The restored level of α -KDH activities in the adventitious root-removed seedlings reached 91.4% and

Table 1. Potential contribution rate (%) of every respiratory pathway to survival of *K. virginica* seedlings under waterlogging conditions. Values carrying different letters are significantly different at $P \leq 0.05$, $n=3$.

Respiratory pathway	0 d	10 d	20 d	30 d	40 d	50 d
TCA cycle						
Control (%)	40.6 ^a	30.5 ^a	32.7 ^a	36.1 ^a	41.4 ^a	36.7 ^a
Waterlogging(+ adventitious root) (%)	40.6 ^a	28.4 ^a	24.4 ^b	27.8 ^b	32.5 ^b	36.8 ^a
Waterlogging(- adventitious root) (%)	-	-	17.7 ^c	21.9 ^c	27.2 ^c	29.2 ^b
Alcohol fermentation						
Control (%)	32.6 ^a	31.7 ^a	27.5 ^c	31.0 ^b	30.4 ^b	25.6 ^b
Waterlogging(+ adventitious root) (%)	32.6 ^a	32.4 ^a	32.9 ^b	36.3 ^a	34.9 ^a	27.4 ^b
Waterlogging(- adventitious root) (%)	-	-	37.8 ^a	37.2 ^a	34.4 ^a	31.6 ^a
Lactate fermentation						
Control (%)	26.8 ^a	37.8 ^a	39.8 ^b	32.9 ^b	28.2 ^c	37.7 ^{ab}
Waterlogging(+ adventitious root) (%)	26.8 ^a	39.2 ^a	42.6 ^{ab}	35.9 ^b	32.6 ^b	35.7 ^b
Waterlogging(- adventitious root) (%)	-	-	44.5 ^a	40.9 ^a	38.3 ^a	39.2 ^a

82.6% of the control and adventitious root-retained seedlings. The restored level of SDH activities was 94.6% of the control level, and changes in CCO activity exhibited the same trend. In addition, the lowest activity of MDH was observed in the adventitious root-removed seedlings during the experiment (Fig. 1-e). Furthermore, the MDH activity of the treated plants was significantly different ($P \leq 0.05$) from the control. Their difference reached almost 30% in the waterlogging and recovery period.

Enzyme characteristics of the alcohol fermentation pathway

The stress on the 10th and 20th d of waterlogging enhanced PDC activity in the adventitious root-retained seedlings. However, the increase was not significant ($P > 0.05$) (Fig. 2a). The PDC activity in the treated plants was significantly higher ($P \leq 0.05$) than that in the control (22.6% difference on the 30th d). After drainage, the activity of PDC in the adventitious root-retained seedlings reduced gradually (Fig. 2-a) but was still higher than that in the control, with a difference of 14.9% and 17.8%, respectively, on the 10th and 20th d of recovery. The PDC activity in the adventitious root-removed seedlings was significantly higher ($P \leq 0.05$) than that in the control. On the 20th d, the activity of PDC in the adventitious root-removed seedlings significantly enhanced compared with that in the adventitious root-retained seedlings ($P \leq 0.05$). However, only a slight difference was observed between them on the 30th d of waterlogging (Fig. 2-a). After drainage, the PDC enzyme activity in the adventitious root-removed seedlings was still higher than that in the adventitious root-retained seedlings. However, the difference decreased with the duration of the recovery period (e.g., 25.9% and 15.1% on the 10th and 20th d, respectively). On the 10th d of waterlogging, the ADH activity in the adventitious root-retained seedlings insignificantly decreased by 10.1% ($P > 0.05$) compared with that in the control. However, the ADH activity was distinctly higher ($P \leq 0.05$) than that in the control during the remaining time of waterlogging (Fig. 2b). For instance, 64.4% and 24.0% differences were observed on the 20th and 30th d, respectively. During recovery, the ADH enzyme activity in the treated seedlings gradually changed and was indistinctly higher by 7.4% ($P > 0.05$) than that in the control on the 20th d. The ADH activity in the adventitious root-removed seedlings was significantly higher ($P \leq 0.05$) than that in the control during the experimental period (Fig. 2b). Nevertheless, on the 20th d of waterlogging, the ADH activity in the waterlogged seedlings was slightly higher by 6.2% ($P > 0.05$) than that in the adventitious root-retained seedlings. Subsequently, greater

increases of 17.5% and 18.8% ($P \leq 0.05$) were observed in the adventitious root-removed seedlings, respectively, on the 30th d of waterlogging and on the 20th d of recovery (Fig. 2-b).

Enzyme characteristics of the lactate fermentation pathway

During waterlogging, the LDH activity in adventitious-root-retained seedlings was higher than that in the control. Subsequently, the difference between them increased with the duration of stress time (Fig. 3). For instance, the LDH activity in the waterlogged plants increased by 6.0%, 15.2%, and 15.3% on the 10th, 10th, and 30th d, respectively. During the recovery period, the LDH activity in this treatment decreased and was gradually close to that in the control, and finally accounted for 98.8% of the control. Additionally, the LDH activity in the adventitious root-removed seedlings was the highest during the experimental period and was significantly different with that in the adventitious root-retained seedlings and the control ($P \leq 0.05$). However, restoration was evident after drainage (Fig. 3). On the 20th d of recovery, only 8.2% and 9.5% of the enzyme activity was higher than that in the controlled and adventitious root-retained seedlings, respectively.

Discussion

Effect of waterlogging on the respiratory enzyme in *K. virginica* seedlings

At the late stage of waterlogging, the α -KDH, SDH, and IDH activities in the adventitious root-retained seedlings of seashore mallow reduced by 36.7%, 16.2%, and 30.2%, respectively. These data indicated that under waterlogging conditions, the reacting conditions of mitochondria were suited to the accumulation of α -ketoglutaric and isocitric acid but not to succinic acid in the waterlogged roots of *K. virginica*. Under anoxic conditions, *E. phyllopon* seedlings were treated with a pulse of acetate signed by ¹⁴C for 3 h. After a reaction period of 16 h, ¹⁴C combined with the succinic acid of TCA cycle (Rumpho and Kenedy, 1983). Obviously, succinic acid is not the end product of the reaction chain, and this metabolism must proceed under anoxic conditions. Kennedy et al. (1987) observed that the consumption of O₂ in the mitochondria increases when *E. phyllopon* seedlings are transferred from N₂ to air conditions. This observation suggests that ETC harmed by oxygen deficiency can still restore and adapt itself to adverse conditions when this stress is removed. The synthesis capacities of ATP and NAD, which originated from pyruvic acid through the TCA cycle, are seriously weakened in plant roots

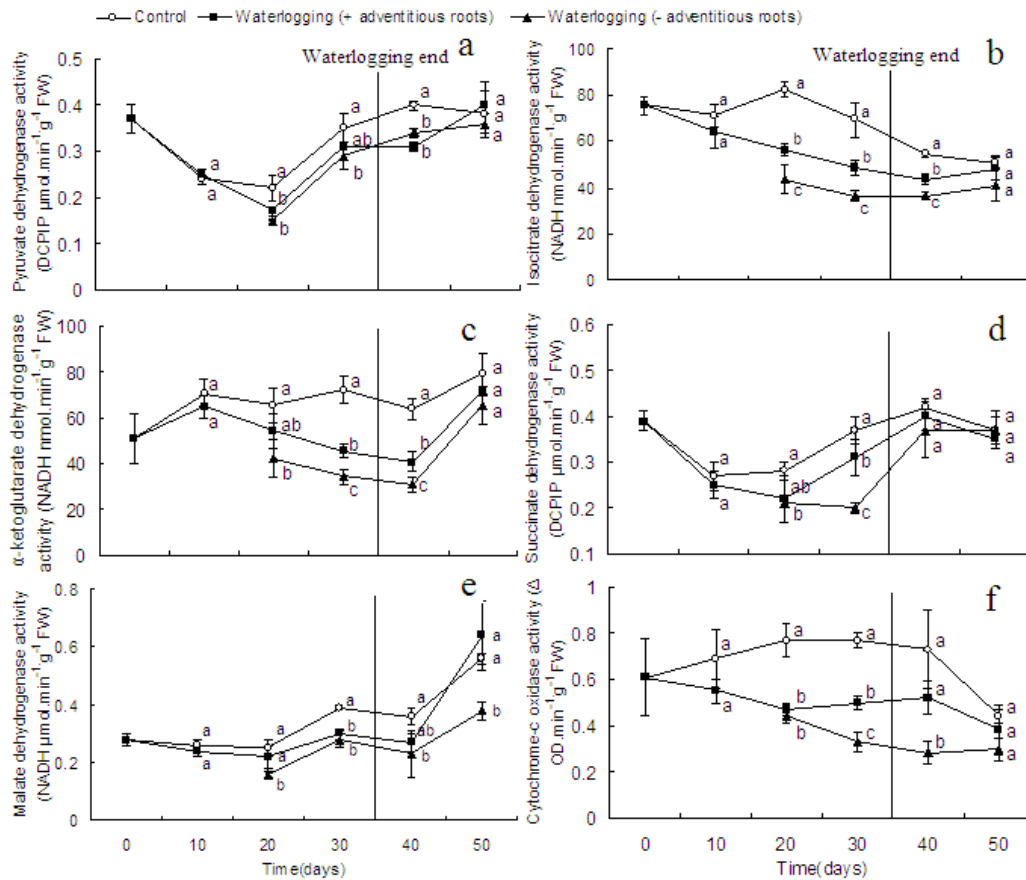


Fig 1. Mitochondrial enzyme activity of *K. virginica* seedlings on the 0, 10th, 20th and 30th of waterlogging, and on the 10th and 20th day of recovery after waterlogging, respectively. Vertical bar represents mean \pm SD of three replications. Values carrying different letters are significantly different at $P \leq 0.05$, $n=3$. In this figure, '+ adventitious root' and '- adventitious root' represents adventitious-root-retained and adventitious-root-removed seedlings, respectively, the same as the following.

under hypoxic stress (Buchanan et al., 2000;). Anaerobic fermentation plays an important role in a short-term adjustment period under hypoxic conditions to produce enough ATP and NAD to maintain normal cell functions (Buchanan et al., 2000). Pyruvic acid acts as the starting point of alcoholic and/or lactic fermentation with catalysis of the anaerobic respiratory enzyme (He, 2004). PDC acts as a significant factor in initiating alcohol metabolism. In the current study, PDC activity in waterlogged *K. virginica* seedlings (with adventitious roots) greatly increased with the duration of waterlogging. A slight increase was observed during the early and middle stages. However, a distinct increase accounted for 22.6% of the control during the late stage of waterlogging, which is similar to the PDC activity observed in flooded rice (Mustroph and Albrecht, 2003). In addition, Chen et al. (2007) found that waterlogging distinctly increases the ADH activity of cherry root tissue. On the 30th d of waterlogging, the ADH activity increased by 24.0% in the adventitious root-retained seedlings, and alcohol accumulated in the root tissue of *K. virginica*. Under hypoxic conditions, plant species survived this stress by two means: 1, sufferable alcohol concentration; 2, enough ATP for cell energy (Nilsen and Orcutt, 1996). Experimental data indicated that the

accumulated alcohol in the root tissue was lower than the tolerant threshold of seashore mallow and did not induce any fatal harm to *K. virginica* roots after 30 d of waterlogging. However, the experimental results or conclusions on the changes in LDH activities and relationships between the two fermentation pathways vary. Therefore, further studies should be conducted for general conclusions. The changes in LDH activity may be related to plant species. The LDH activity in barley gradually increases under hypoxic stress (Hoffman et al., 1986). However, Huang (1997) revealed that LDH activity is inhibited by waterlogging with decreasing cytoplasm pH. Conversely, the decrease can activate PDC activity and alcohol fermentation (Davies et al., 1980). In the present experiment, the LDH activity in the adventitious root-retained seedlings increased by 15.2% and 15.3% on the 20th and 30th d of waterlogging, respectively. Thus, the activities of PDC, ADH, and LDH in waterlogged roots significantly increased during the middle and late stages. However, a slight increase was observed during the early stage of stress. The varying characteristics of the particular enzymes indicate that activating the fermentation pathway may act as a positive adjustment to energy deficiencies (Buchanan et al., 2000) under

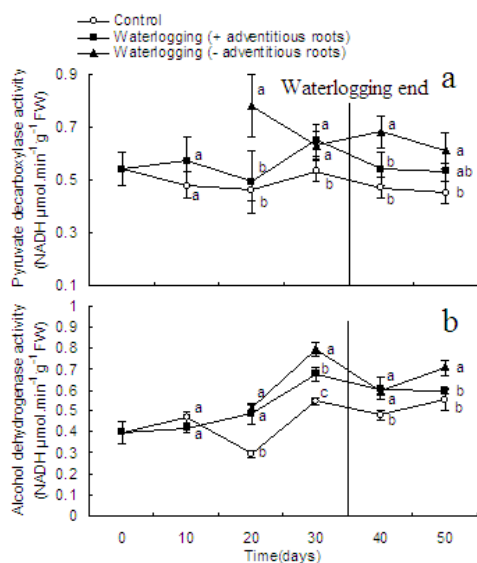


Fig 2. Enzyme activities of alcohol fermentation in *K. virginica* seedlings on the 0, 10th, 20th and 30th of waterlogging, and on the 10th and 20th day of recovery after waterlogging, respectively. Vertical bar represents mean \pm SD of three replications. Values carrying different letters are significantly different at $P \leq 0.05$, $n=3$.

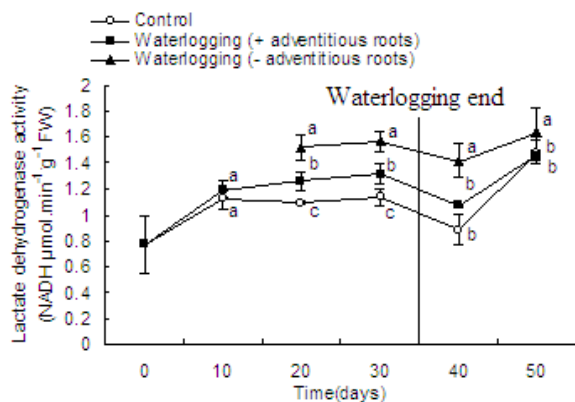


Fig 3. Enzyme activity of lactate fermentation in *K. virginica* seedlings on the 0, 10th, 20th and 30th of waterlogging, and on the 10th and 20th day of recovery after waterlogging, respectively. Vertical bar represents mean \pm SD of three replications. Values carrying different letters are significantly different at $P \leq 0.05$, $n=3$.

waterlogging, indicating that *K. virginica* plants exhibit strong tolerance to waterlogging.

Regulation of the respiratory pathway of *K. virginica* seedlings

Electron is released from NADH and transported by the respiratory chain in the TCA cycle, as well as alcoholic and lactic fermentation pathways (David and Michael, 2004). Combined with this oxidation, ATP is synthesized during the course of electron transfer (He, 2004). We can estimate the contribution rate of the amount of electrons in decomposing

organic materials or synthesizing ATP and evaluate the major role of each pathway in the survival of waterlogged plants under stress conditions by analyzing the amount of released electrons in one pathway. In the current research, the activities of respiratory enzyme were determined and expressed by synthesizing or decomposing the amount of NADH or 2,6-dichlorophenol-indophenol (DCPIP). Two electrons are released from a molecule of NADH or DCPIP in the reaction (He, 2004). The ratio can be calculated based on the amount of released electrons of one respiratory pathway in unit time as the number of released electrons to the total electrons in the respiratory chain. The contribution of this pathway to the survival of waterlogged seedlings is estimated by the calculated ratio. Table 1 shows that the ratio of the TCA pathway decreased gradually in the adventitious root-removed seedlings. Its contribution rate was 15.0% and 14.2% lower than that in the control on the 20th and 30th d of waterlogging, respectively. However, the contribution rate of alcohol fermentation enhanced by 10.3% and 6.2%, and the contribution rate of lactic fermentation increased by 4.7% and 8.0%, respectively. The aerobic respiration of *K. virginica* roots under waterlogging conditions was restrained. However, anaerobic respiration was promoted to compensate for the oxygen deficiency and to provide enough energy for the survival of damaged plants (Zhao, 2003). Alcohol metabolism decreased with the duration of waterlogging, whereas lactate fermentation activity increased gradually. The promoted lactic acid metabolism can partly replace alcoholic fermentation and decrease the alcohol accumulation and its toxicity in plant cells (Li and Wang, 2002). Thus, it can be regarded as the adjustment mechanism of *K. virginica* seedlings to waterlogging.

Alleviation of adventitious roots to respiratory damage of waterlogged *K. virginica* seedlings

The activities of mitochondrial enzymes in adventitious root-retained seedlings during waterlogging were higher than those in the adventitious root-removed seedlings. A significant difference in the activities of IDH, α -KDH, SDH, and CCO was found between the two treatments. Data indicated that the mitochondrial function in the adventitious root-removed seedlings exhibited a more serious damage than that in the adventitious root-retained seedlings. The contribution rate of the TCA pathway was higher, whereas that of the fermentation pathway was lower in the adventitious root-retained seedlings than in the adventitious root-removed seedlings (Table 1). The adventitious roots of *K. virginica* can possibly sustain aerobic respiration in the mitochondria, increase the utilization of organic carbon, and alleviate damage to stressed plants to help them overcome waterlogging stress.

Materials and methods

Experimental materials and design

The experimental materials, including seeds and cultivating soil, were obtained from Jinhai Farm, one of the introducing sites of *K. virginica*. The selected and full seeds were soaked in 95% H_2SO_4 for 30 min to destroy the hard seed capsule and improve seed germination. The seeds were then soaked for 24 h in water. The treated seeds were sown in individual pots (8 cm diameter, 8 cm deep, with five seeds per pot) filled with saline soil from Jinhai Farm. The seedlings were irrigated with 1/4 strength Hogland solution every 7 d after germination. Subsequently, the pots were placed in nine white plastic tanks (12 cm deep, 36 cm long, 26.5 cm wide, with eight pots per tank). The roots in

the three pots (three replicates) were sampled to measure the respiratory enzyme activities (TCA cycle, alcoholic and lactic fermentation) when the seedlings were 60 d old. After the first measurement, the seedlings in 6 tanks distributed into two groups (three tanks as three replicates per group) were waterlogged. The water level was maintained at approximately 5 cm above the soil surface and then drained after 30 d. The remaining three tanks were used as the control. After the formation of adventitious roots, the roots above the soil surface were removed in one group and retained in another group. The roots of the seedlings, including those in the treatment and control groups, were sampled to measure the same indices on the 10th, 20th, and 30th d after waterlogging and on the 10th and 20th d after drainage. The controlled plants were irrigated with 500 mL of water per tank every 2 d.

Extraction of crude enzyme in the TCA cycle

Crude enzymes in the mitochondria were extracted using the methods described in a previous study (Fox and Kennedy, 1991). The roots (1.0 g to 2.0 g) were sliced and then ground using a mortar and pestle in 10 mL extraction medium containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.5), 10 mM β -mercaptoethanol, and 5% (w/v) polyvinylpyrrolidone at 4 °C. The extraction buffer also contained 0.5% digitonin to obtain the optimal activity of α -KDH. The homogenates were filtered through four layers of muslin and centrifuged at 14,500 g for 20 min. The supernatant was stored as crude enzyme at 4 °C.

Measurement of enzyme activity in aerobic respiration

All the enzyme activities were determined colorimetrically. PDH activity was determined using a previously described method (Nemeria et al., 1969) with some modifications. IDH activity was estimated based on the increased NADH (Hirai and Ueno, 1977). α -KDH activity was determined using the methods described by Pettit et al. (1973) with a slight modification. SDH activity was estimated by a decrease in DCPIP (Singer, 1973). MDH activity was determined using the methods described by Sere (1969) with some modifications. CCO activity was measured according to *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (Pan et al., 2002) with some modifications.

Extraction of crude enzyme in fermentation

Crude enzymes in fermentation were isolated using previously described methods (Mustroph and Albrecht, 2003). The roots (0.2 g to 0.3 g) were cut into slices and ground using a mortar and pestle in 5 mL extraction medium containing 5 mM MgCl₂, 5 mM β -mercaptoethanol, 15% (w/v) glycerol, 1 mM ethylenediamine tetraacetic acid, 1 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, and 0.1 mM phenylmethanesulfonyl fluoride at 0 °C ice bath. The homogenates were centrifuged at 12,000 g for 20 min. The supernatant was stored as crude enzyme at 4 °C.

Measurement of enzyme activity in anaerobic respiration

ADH activity was measured using the method described by Waters et al. (1991) with a slight modification. PDC activity was measured based on a decrease in NADH (Waters et al., 1991). LDH activity was determined based on the methods described by Bergmeyer et al. (1983) with some modifications.

Statistical analysis

Duncan's multiple comparison test was conducted using SPSS 11.5; $P \leq 0.05$ was considered to indicate statistical significance.

Conclusions

Waterlogging stress exhibited harmful effects on the mitochondrial function of *K. virginica* seedlings. The enzyme activities were lower than those in the control in the TCA cycle, whereas the activities of fermentative enzymes increased significantly. The contribution rate of the TCA pathway decreased gradually in the adventitious root-removed seedlings based on the analysis of the amount of released electrons in the respiratory pathway. However, the alcoholic and lactic acid fermentation rates enhanced in *K. virginica* seedlings. Subsequently, the respiratory enzyme activities and contribution rates of the respiratory pathway restored gradually after drainage.

Acknowledgements

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