

## Response of *Taxodium distichum* Root Organic Acids and NSC Metabolism to Water Level Changes in the Three Gorges Water-Level Fluctuation Zone: Postprint

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**Date:** 2018-05-18T00:00:00+00:00

### Abstract

To investigate the response characteristics of root organic acids and non-structural carbohydrates (NSC) in *Taxodium distichum* under water-level fluctuation in the Three Gorges Reservoir water-level fluctuation zone, the well-adapted woody plant *Taxodium distichum* was used as the study subject. Three treatments were established in the in-situ environment of the water-level fluctuation zone: control-SS (altitude 175 m, no flooding during the experimental period), MS (altitude 170 m, moderate flooding stress), and DS (altitude 165 m, deep flooding stress). Samples were collected at altitudes of 170 m and 165 m during water recession, and changes in root organic acids and NSC were measured and analyzed. The results showed: (1) Flooding in the reservoir area had no significant effect on the basal diameter of *Taxodium distichum*, and only the DS group significantly inhibited height growth, indicating that *Taxodium distichum* growth could respond relatively actively to flooding in the reservoir area. (2) The effects of reservoir flooding on organic acids in lateral roots and total roots were consistent, with organic acid metabolism in lateral roots being superior to that in main roots. Compared with the SS group, root organic acid content increased in the MS group and decreased in the DS group; except for significant changes in tartaric acid, malic acid, and citric acid in some roots, no significant changes were observed in other organic acids. (3) Different intensities of flooding in the reservoir area had varying effects on root NSC of *Taxodium distichum*. Compared with the SS group, the MS group showed no significant change in soluble sugars, but significant increases in starch and NSC content; however, total root NSC showed no significant difference before and after flooding. In stark contrast, the DS group significantly reduced soluble sugars and NSC content, with no significant effect

on starch, and total root NSC after flooding was significantly lower than before flooding. (4) Correlation analysis indicated that oxalic acid, malic acid, and citric acid in main roots and total roots, as well as shikimic acid in lateral roots and total roots, showed significant correlations with starch and NSC ( $P < 0.05$ ). The research results demonstrate that under flooding stress in the Three Gorges Reservoir water-level fluctuation zone, root organic acid and NSC metabolism in *Taxodium distichum* are closely linked. By maintaining certain root starch content and preserving normal organic acid metabolism levels, the plants can better adapt to the habitat of the Three Gorges Reservoir water-level fluctuation zone.

## Full Text

## Preamble

ACTA ECOLOGICA SINICA ChinaXiv Partner Journal

Vol. 38, No. 9, May 2018

DOI: 10.5846/stxb201706091053

## Responses of Root Organic Acids and Non-Structural Carbohydrate Metabolism of *Taxodium distichum* to Water-Level Changes in the Hydro-Fluctuation Belt of the Three Gorges Reservoir

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## Abstract

Along the Three Gorges Dam reservoir on the Yangtze River in China, water levels fluctuate annually between 145–175 m, resulting in severe ecological problems such as vegetation degradation and decreased biodiversity. In recent years, many efforts have been made to restore vegetation in water-level-fluctuating zones. While some flood-tolerant species have been selected through pot experiments, only a few survived after being planted in the hydro-fluctuation zones, bringing new challenges to vegetation restoration. Chronic submergence is one of the major factors limiting plant survival in the Three Gorges Reservoir.

Previous studies showed that root metabolism of organic acids and non-structural carbohydrates (NSC) plays an important role in plant responses to flooding. However, it remains unclear how root metabolism of organic acids and NSC in adaptable plants responds to water-level changes in the hydro-fluctuation belt of the Three Gorges Reservoir. In this study, *Taxodium distichum*, a flood-tolerant plant, was planted at three elevations in the reservoir riparian region of Zhong County, Chongqing: control-SS (175 m above sea level,

abbreviated as ASL), prolonged moderate submergence-MS (170 m ASL), and prolonged deep submergence-DS (165 m ASL). Organic acid and NSC content were measured and analyzed after collection during de-submergence of MS and DS.

The results showed that: (1) There was no significant difference in the base diameter of *T. distichum*, and height was inhibited only by DS, indicating that the height and base diameter of *T. distichum* could positively respond to water-level changes in the riparian zone. (2) The organic acids in lateral roots showed a trend consistent with that of the total root system. Root organic acid content was higher in MS and lower in DS compared to SS. Except for tartaric acid, malic acid, and citric acid in some roots, there were no significant differences in the remaining organic acids. (3) Soluble sugar did not significantly change, whereas starch and NSC content was significantly higher in MS than in SS. However, the NSC content of the total root did not change after being flooded. In contrast, the content of soluble sugars and NSC was significantly lower, while starch content did not differ significantly in DS from that in SS. On the other hand, the NSC in total roots reduced to some extent after flooding. (4) Correlation analysis showed that the content of starch and NSC was negatively correlated with the oxalic acid content of taproots and total roots, whereas NSC displayed a strong and positive correlation with the concentration of malic acid, citric acid of the taproot and total root, and shikimic acid of the lateral and total root.

The results indicated that there was a close relationship in root metabolism between organic acids and NSC of *T. distichum*. With metabolic levels similar to those in the control, *T. distichum* could adapt well to the water-level-fluctuating zones of the Three Gorges Reservoir by maintaining sufficient starch and normal organic acid metabolism levels.

**Keywords:** Three Gorges Reservoir; hydro-fluctuation belt; *Taxodium distichum*; organic acid; NSC

## Introduction

After the construction of the Three Gorges Project, the water level management scheme causes annual fluctuations between 145-175 m in altitude, creating a vertical hydro-fluctuation belt with alternating aquatic and terrestrial environments. Most native plants cannot adapt to periodic submergence and have disappeared, leading to vegetation degradation, soil erosion, reduced ecological service functions, and other environmental problems. To address these issues, previous studies screened flood-tolerant plants through indoor simulation experiments, but only some survived when applied to vegetation restoration in the hydro-fluctuation belt, presenting new challenges. Studying the flooding tolerance mechanisms of suitable plants in situ in the hydro-fluctuation belt is crucial, as the results can provide effective theoretical guidance for vegetation restoration and management.

*Taxodium distichum* is a deciduous tree of the Taxodiaceae family and an excel-

lent candidate species for vegetation restoration in the hydro-fluctuation belt. Studies have shown that different ages of *T. distichum* respond similarly to flooding. Scholars have investigated its growth characteristics, photosynthetic physiology, nutrient element changes and distribution, and secondary metabolism under water and salt stress, but less attention has been paid to its material metabolism under flooding stress. Under the reverse-season, high-intensity flooding stress in the Three Gorges hydro-fluctuation belt, plant root metabolic pathways are closely related to flooding tolerance.

Root primary metabolite non-structural carbohydrates (NSC) are energy sources that ensure plant life activities in flooded habitats. Plant secondary metabolism plays an important role in stress defense, and root secondary metabolite organic acids can enhance enzyme activity, reduce toxicity, and improve flooding tolerance. Previous studies on organic acids and NSC responses to flooding have mainly focused on indoor simulation experiments and content changes. Since plant organic acid metabolism is derived from primary metabolism and the two are closely related, research on their responses to large-scale water level changes in the Three Gorges hydro-fluctuation belt is lacking. However, their response strategies to the in situ environment are crucial for vegetation restoration and management. This study, based on the in situ environment of the Three Gorges Reservoir hydro-fluctuation belt and using *T. distichum* as the subject, aims to clarify the response strategies of root organic acids and NSC metabolism to provide a theoretical basis for ecological restoration.

## 1 Materials and Methods

### 1.1 Experimental Materials and Design

In September 2015, 45 two-year-old potted *T. distichum* seedlings with uniform growth were selected. Each pot (12.5 kg) was filled with purple soil from the Three Gorges hydro-fluctuation belt (mixed in a 2:1:1 ratio). The seedlings were placed at three different elevations in the Ruxi River hydro-fluctuation belt in Zhong County, Chongqing (30°25 55.47 N, 108°09 59.18 E): control group at 175 m (SS), moderate submergence group at 170 m (MS), and deep submergence group at 165 m (DS). The experimental area has a subtropical monsoon climate with annual average temperature of 18.2°C, annual precipitation of 1200 mm, total solar radiation of  $83.7 \times 4.18$  kJ/cm<sup>2</sup>, frost-free period of 341 days, and sunshine duration of 1327.5 hours. According to the water level fluctuation pattern of the Three Gorges hydro-fluctuation belt, the submergence time and depth for each treatment are shown in .

The experiment began on September 15, 2015 (impoundment period) and ended on April 16, 2016 (recession period). Initial samples were collected on September 15, 2015. The first batch of plant samples was collected on February 17, 2016 (170 m and 175 m elevations), and the second batch on April 16, 2016 (165 m, 170 m, and 175 m elevations). Plant height and base diameter were measured

with a vernier caliper and height gauge. The survival rate of experimental seedlings was 100% during the trial period.

Experimental treatment levels in the Three Gorges Reservoir hydro-fluctuation belt

Treatment group	Elevation (m)	Maximum flooding depth (m)	Flooding time (d)
SS (control)	175	0	0
MS (moderate)	170	5	180
DS (deep)	165	10	210

## 1.2 Determination of Root Metabolites

After collection, taproots and lateral roots were separated, immediately placed in ice boxes, and transported to the laboratory. Samples were washed with tap water and deionized water, then killed in an oven at 105°C for 15 minutes, and dried to constant weight at 70°C. Dried samples were ground to <0.2 mm using an MM400 ball mill.

**1.2.1 Determination of Root Organic Acids** Based on preliminary experiments and previous studies, organic acids were determined using a Shimadzu high-performance liquid chromatograph (HPLC) system with an Agilent 1100 diode array detector. A Sepax Sapphire C18 column (4.6 × 250 mm, 5 μm) was used with 20 mmol/L KH<sub>2</sub>PO<sub>4</sub> (pH = 2.5) and methanol as the aqueous and organic phases of the mobile phase, respectively. Flow rate was 0.9 mL/min; detection wavelength was 210 nm; column temperature was 30°C; injection volume was 20 μL. Oxalic acid standards were provided by Chengdu Preferred Biotechnology Co., Ltd. (98%); shikimic acid standards were provided by TCI Chemical Industry Development Co., Ltd. (99.5%); chromatographically pure KH<sub>2</sub>PO<sub>4</sub> was provided by Chengdu Kelong Chemical Reagent Factory; and chromatographically pure methanol was provided by Chengdu Kelong Chemical Reagent Factory.

For sample preparation, 0.1 g of *T. distichum* powder was accurately weighed, mixed with 10 mL of ultrapure water, ultrasonically extracted for 10 minutes, and centrifuged at 8000 r/min. The supernatant was filtered through a 0.45 μm syringe filter (Millipore) to obtain the test solution. Organic acid content in taproots and lateral roots was expressed as mg per gram of dry weight (mg/g, DW). Total root organic acid content was calculated as the average content of the whole root system composed of taproots and lateral roots.

**1.2.2 Determination of Root NSC** In this study, plant NSC = soluble sugars + starch. For soluble sugar determination, 0.01 g of *T. distichum* powder was accurately weighed, mixed with 10 mL of 80% ethanol, extracted in a water bath at 80°C for 40 minutes, and centrifuged at 7000 r/min. The supernatant

was collected, and the residue was extracted twice more. The combined supernatants were diluted to 50 mL. Soluble sugar content was determined using the anthrone colorimetric method at 625 nm with a UV-Vis spectrophotometer (UV-2550, Japan).

For starch determination, starch was hydrolyzed into soluble sugars with acid and then measured using the same anthrone method. Soluble sugar and starch contents were expressed as mg per gram of dry weight of taproots and lateral roots (mg/g, DW), and total root content was calculated for the whole root system.

### 1.3 Data Analysis

SPSS 22.0 and Origin 8.5 were used for statistical analysis and graphing. Paired-sample t-tests were used to analyze significant differences between pre-flooding initial values and post-flooding treatment values. Independent-sample t-tests were used to analyze the effects of different flooding treatments on root organic acids and NSC. Pearson correlation coefficient was used to evaluate correlations between root metabolites.

## 2 Results

### 2.1 Changes in *Taxodium distichum* Growth

The effects of in situ flooding in the Three Gorges hydro-fluctuation belt on *T. distichum* growth are shown in . Except for the second batch of SS group, which showed significantly higher plant height than the initial value, no significant differences were observed in other groups. Plant height in the DS group was significantly lower than in the SS group ( $P < 0.05$ ), indicating that flooding inhibited height growth. Compared with initial values, base diameter increased under flooding but showed no significant differences among treatment groups.

Growth characteristics of *T. distichum* under water level changes in the Three Gorges Reservoir

Sampling	Treatment group	Initial value	Treatment value
First sample	SS (175 m)	69.100 ± 0.590 A	78.250 ± 0.303 Aa
	MS (170 m)	69.100 ± 0.590 A	69.250 ± 0.197 Aa
Second sample	SS (175 m)	69.100 ± 0.590 A	83.350 ± 0.106 Ba
	MS (170 m)	69.100 ± 0.590 A	69.675 ± 0.256 Ab
	DS (165 m)	69.100 ± 0.590 A	69.675 ± 0.256 Ab

Note: Values are means ± SE (n = 5). Different uppercase letters in the same row indicate significant differences between pre-flooding and post-flooding values ( $P < 0.05$ ). Different lowercase letters in the same column indicate significant differences among treatments in the same batch ( $P < 0.05$ ).

## 2.2 Changes in Root Organic Acid Content

Different flooding intensities had varying effects on organic acid content in taproots, lateral roots, and total roots of *T. distichum*. In the first batch, MS flooding increased oxalic, malic, and citric acid contents in taproots, with malic and citric acids reaching significant levels. Lateral root tartaric acid increased, while oxalic acid decreased. Total root tartaric and malic acid contents showed non-significant upward trends under MS stress. In contrast, DS significantly decreased lateral root and total root citric acid contents, with total root organic acid content showing consistent changes with lateral roots, though not reaching significant levels.

In the second batch, MS taproot tartaric and citric acids showed non-significant decreases, while lateral root and total root citric acids were significantly lower than in SS, with tartaric and citric acids reaching significant levels. Under in situ flooding stress, total root organic acid contents in both MS and DS groups were lower than in SS, with DS group showing significantly lower total root tartaric and citric acids ( $P < 0.05$ ). Lateral root organic acid metabolism appeared superior to taproot metabolism.

Organic acid content in *T. distichum* roots in the Three Gorges Reservoir hydro-fluctuation belt

Sampling	Root type	Treatment	Oxalic acid	Tartaric acid	Shikimic acid	Malic acid	Citric acid
First sample	Taproots	SS (175 m)	0.926 ± 0.114 a	4.531 ± 0.697 a	4.172 ± 0.418 a	1.722 ± 0.287 a	6.598 ± 0.161 a
		MS (170 m)	1.006 ± 0.465 a	6.309 ± 0.518 a	5.018 ± 0.505 a	4.833 ± 0.598 b	8.677 ± 0.529 b
	Lateral roots	SS (175 m)	2.239 ± 0.572 a	7.270 ± 0.656 a	5.085 ± 0.347 a	5.630 ± 0.872 a	5.430 ± 0.756 a
		MS (170 m)	0.737 ± 0.155 a	8.072 ± 0.734 a	6.568 ± 0.512 a	5.726 ± 0.446 a	1.674 ± 0.125 b
	Total roots	SS (175 m)	1.583 ± 0.297 a	5.901 ± 0.601 a	4.629 ± 0.567 a	3.676 ± 0.459 a	6.014 ± 0.373 a
		MS (170 m)	0.872 ± 0.236 a	7.190 ± 0.406 a	5.793 ± 0.540 a	5.279 ± 0.599 a	5.176 ± 0.423 a
Second sample	Taproots	SS (175 m)	3.570 ± 0.512 a	4.227 ± 0.338 a	4.377 ± 0.480 a	1.649 ± 0.175 a	1.417 ± 0.104 a

Root Samplingtype	Treatment	Oxalic acid	Tartaric acid	Shikimic acid	Malic acid	Citric acid
	DS (165 m)	4.326 ± 0.318 a	3.724 ± 0.452 a	2.375 ± 0.301 a	1.310 ± 0.384 a	1.130 ± 0.161 a
	Lateral roots (175 m)	2.554 ± 0.218 a	6.844 ± 0.702 a	1.488 ± 0.368 a	4.550 ± 0.727 a	4.763 ± 0.680 a
	DS (165 m)	1.173 ± 0.409 a	4.787 ± 0.274 b	1.961 ± 0.450 a	2.478 ± 0.473 a	1.351 ± 0.102 b
	Total roots (175 m)	3.062 ± 0.611 a	5.535 ± 0.395 a	2.932 ± 0.753 a	3.100 ± 0.793 a	3.090 ± 0.673 a
	DS (165 m)	2.750 ± 0.297 a	4.256 ± 0.317 b	2.168 ± 0.221 a	1.893 ± 0.320 a	1.241 ± 0.081 b

Note: Values are means ± SE (n = 5). Different lowercase letters in the same column indicate significant differences among treatments for the same root type and batch (P < 0.05).

### 2.3 Changes in Root Soluble Sugar

The effects of in situ flooding on root soluble sugar content are shown in [Figure 1: see original paper]. In the first batch, except for lateral root soluble sugar being significantly lower than the initial value, other root soluble sugar contents in MS group showed no significant changes compared to initial values. In the second batch, soluble sugar contents in taproots and total roots of DS group were significantly lower than in SS group and initial values (P < 0.05).

[Figure 1: see original paper] Soluble sugar content in *T. distichum* roots under different flooding conditions

### 2.4 Changes in Root Starch

The effects of flooding on root starch content are shown in [Figure 2: see original paper]. In the first batch, starch contents in taproots, lateral roots, and total roots of MS group were significantly higher than in SS group (P < 0.01), but showed no significant difference from initial values. In the second batch, starch contents in taproots and total roots of DS group were significantly lower than initial values (P < 0.05), but showed no significant difference from SS group.

[Figure 2: see original paper] Starch content in *T. distichum* roots under different flooding conditions

## 2.5 Changes in Root NSC

The effects of in situ flooding on root NSC content are shown in [Figure 3: see original paper]. In the first batch, NSC contents in taproots, lateral roots, and total roots of MS group were significantly higher than in SS group ( $P < 0.01$ ), consistent with starch changes. Except for lateral root NSC being significantly lower than the initial value, other treatments showed no significant differences from initial values. In the second batch, NSC contents in taproots and total roots of DS group were significantly lower than in SS group and initial values ( $P < 0.01$ ), similar to soluble sugar changes.

[Figure 3: see original paper] NSC content in *T. distichum* roots under different flooding conditions

## 2.6 Correlation Analysis of Root Organic Acids and NSC

Correlation analysis revealed different relationships between organic acids and NSC in taproots, lateral roots, and total roots. Soluble sugar showed weaker correlations with organic acids, with no significant correlations between lateral root soluble sugar and any organic acids. However, taproot soluble sugar showed significant negative correlations with taproot oxalic acid and tartaric acid ( $P < 0.05$ ).

Starch and NSC showed significant correlations with organic acids. Taproots and total roots oxalic acid were significantly negatively correlated with starch and NSC ( $P < 0.05$ ). Malic acid, citric acid in taproots and total roots, and shikimic acid in lateral roots and total roots were significantly positively correlated with NSC ( $P < 0.05$ ). Total root shikimic acid was significantly negatively correlated with starch and NSC.

Correlations between organic acid and NSC content in *T. distichum* roots

## 3 Discussion and Conclusion

After the Three Gorges Reservoir began operation, the 145-175 m water level fluctuation caused prominent ecological problems in the reservoir area, drawing considerable attention to vegetation restoration in the hydro-fluctuation belt. *Taxodium distichum*, a Taxodiaceae tree with knee-shaped pneumatophores, is an excellent candidate species for hydro-fluctuation belt restoration. This study shows that after experiencing off-season flooding in the hydro-fluctuation belt, *T. distichum* maintained 100% survival rate, though DS significantly inhibited height growth. This suggests that *T. distichum* can actively respond to reservoir flooding, possibly related to aerenchyma formation in the stem base that helps tolerate oxygen-deficient conditions.

Flooding is a primary factor limiting plant growth in hydro-fluctuation belts, severely affecting metabolism. During long-term adaptation, flood-tolerant plants accelerate aerenchyma formation. Consistent with simulation studies, *T.*

*distichum* under prolonged complete submergence showed no significant changes in base diameter, indicating active response of height and base diameter to flooding.

Root organic acid metabolism plays a crucial role under flooding stress. Increased secretion of organic acids is an important defense mechanism against water stress. Malic acid can maintain transmembrane charge balance under stress, while organic acids like malic and oxalic acid can compensate charges during nitrate reduction, providing energy sources. Studies have found that *T. distichum* enhances flooding tolerance by secreting large amounts of malic and shikimic acids through lateral roots. Our results are similar: under MS stress, taproot malic acid content increased non-significantly, while under DS stress, taproot organic acid contents were lower than in SS, but total root tartaric and citric acids were significantly lower ( $P < 0.05$ ). This suggests that under moderate flooding, *T. distichum* roots maintain high organic acid metabolic activity, while under severe hypoxia from deep flooding, the tricarboxylic acid cycle is severely blocked, potentially causing significant citric acid reduction.

Correlation analysis revealed significant relationships between different organic acids, indicating close metabolic connections. Notably, total root shikimic acid showed significant correlations with starch and NSC, confirming the close relationship between organic acid and NSC metabolism. Under hypoxia, NSC stored as starch is utilized through soluble sugars for anaerobic metabolism, while organic acids like malic acid balance charges during nitrate reduction. Studies show that Taxodiaceae plants enhance long-term flooding tolerance by increasing root starch and soluble sugar contents. In our first batch, MS group showed significantly higher starch and NSC contents than SS group, while soluble sugar remained unchanged, indicating adaptation to moderate flooding. In the second batch, DS group showed significantly lower soluble sugar and NSC contents than SS group and initial values, possibly because severe stress consumed NSC to maintain physiological functions, though starch content remained similar to SS, suggesting *T. distichum* can maintain certain starch levels under prolonged deep flooding.

In conclusion, under in situ flooding stress in the Three Gorges hydro-fluctuation belt, root organic acid and NSC metabolism play important roles in maintaining physiological functions. *T. distichum* can adapt well to the hydro-fluctuation belt environment by maintaining certain NSC levels (especially starch) and organic acid metabolism levels similar to the control, with lateral root organic acid metabolism playing a superior role to taproots.

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