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Short title: *C. rotundus* in rice fields

Mature Tubers of *Cyperus rotundus* Confer Flooding Tolerance by Adopting A “Low-oxygen Quiescence Strategy”, which may Contribute to Its Emergence in Rice Fields

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Abstract

Purple nutsedge (*Cyperus rotundus* L.) is one of the world's resilient upland weeds, primarily spreading through its tubers. Its emergence in rice fields has been increasing, likely due to changing paddy farming practices. This study aimed to investigate how *C. rotundus*, an upland weed, can withstand soil flooding and become a problematic weed in rice (*Oryza sativa* L.) fields. The first comparative analysis focused on the survival and recovery characteristics of growing and mature tubers of *C. rotundus* exposed to soil flooding conditions. Notably, mature tubers exhibited significant survival and recovery abilities in these environments. Based on this observation, further investigation was carried out to explore the morphological structure, non-structural carbohydrates, and respiratory mechanisms of mature tubers in response to prolonged soil flooding. Over time, the mature tubers did not form aerenchyma but instead gradually accumulated lignified sclerenchyma fibers, with lignin content also increasing. After 90 days, the lignified sclerenchyma fibers and lignin contents were 4.0 and 1.1 times higher than those in no soil flooding (CK). Concurrently, soluble sugar content decreased while starch content increased, providing energy storage, and alcohol dehydrogenase (ADH) activity rose to support anaerobic respiration via alcohol fermentation. These results indicated that mature tubers survived in soil flooding conditions by adopting a "low-oxygen quiescence strategy", which involves morphological adaptations through the development of lignified sclerenchyma fibers, increased starch reserves for energy storage, and enhanced anaerobic respiration. This mechanism likely underpins the flooding tolerance of mature *C. rotundus* tubers, allowing them to endure unfavorable conditions and subsequently germinate and grow once flooding subsides. This study provides a preliminary explanation of the mechanism by which mature tubers of *C. rotundus* from the upland areas confer flooding tolerance, shedding light on the reasons behind this weed's increasing presence in rice fields.

Key words: *Cyperus rotundus* L.**;** soil flooding; flooding tolerance; rice field weeds

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Introduction

Purple nutsedge (*Cyperus rotundus* L.) is one of the most challenging weeds to control globally, due to its rapid and efficient regenerative capacity and the viability of its tubers, which serve as the primary reproductive organ (Peerzada et al. 2017). This species has been shown to adversely affect the growth of dryland crops such as sugarcane (*Saccharum officinarum* L.), maize (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.), and peanut (*Arachis hypogaea* L.) (Du et al. 2019; Durigan 2005; Salgado et al. 2002; Silva et al. 2015; Tuor and Froud-Williams 2002). Additionally, it can negatively impact rice (*Oryza sativa* L.) seedlings through the release of aqueous extracts and leachates from its leaves and tubers. In particular, the lowland ecotype of *C. rotundus*, when present at a density of 80 tubers per square meter in transplanted rice fields, has been shown to reduce rice shoot and root biomass by 54% and 60% (Donayre et al. 2021; Quayyum et al. 2000). *Cyperus rotundus* was initially reported to occur only sporadically or at low densities in rainfed lowland rice fields in the Philippines during the 1970s (Carbonell and Moody 1983). However, by the 1990s, it had became a dominant weed in paddy fields rotated with vegetables (Baltazar et al. 1999), leading to a reduction in transplanted rice grain yield by 14% to 38% (Donayre et al. 2021). Since then, this weed has become increasingly prevalent in the Philippines' rice fields (Donayre et al. 2015), and reports of *C. rotundus* in rice fields have surfaced in over 20 countries across tropical and subtropical regions (Kraehmer et al. 2016). In Latin America, for instance, *C. rotundus* is commonly found in both irrigated and dryland rice fields in countries like Mexico, Honduras, Nicaragua, Brazil, Costa Rica, and Peru (Gonzalez et al. 1983; Silveira and Aquino 1983). Similarly, in Asia, it has become a pernicious weed in rice fields, including dry direct-seeded paddy fields in the hilly areas of Jiangsu, China (Yu et al. 2022), intermittent plots of alternate wet and dry irrigation rice fields, Chiba, Japan (Chapagain et al. 2011), and rice fields across five districts of Punjab, Pakistan (Rabbani and Baiwa 2001). It has been observed that in areas where *C. rotundus* is prevalent in rice fields, its presence can often be traced back to previous cropping practices or the transition from dry to wet land preparation (Baltazar et al. 1999; Donayre et al. 2015).

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This suggests that *C. rotundus* can survive and reproduce in both the aerobic environment of upland areas and the anaerobic conditions of flooded rice fields. Understanding how this upland weed, which primarily reproduces through tubers, has adapted and propagated to become a noxious weed in rice fields is crucial.

In the cultivation of rice, water management is crucial throughout the various developmental stages, playing a significant role in determining crop yield and quality (Zhu et al. 2024). Most of the growth stages of rice, including the seedling stage, tillering stage, heading stage, and flowering stage, need to keep completely soil flooding. However, certain stages, such as the end of tillering, filling, and ripening stages, necessitate draining and sun drying (Li et al. 2018; Zhang 2021). While this water management strategy optimizes rice development, it also inadvertently creates conditions that favor the survival and spread of certain weeds. We hypothesize that *C. rotundus* is well-adapted to thrive in both the flooded and drained conditions typical of rice fields.

Plants have developed two primary strategies to endure flooding: low-oxygen escape and low-oxygen quiescence strategy (Akman 2012; Bailey-Serres and Voesenek 2008; Voesenek and Baily-Serres 2015). The plants employing the low-oxygen escape strategy typically develop more aerenchyma and adventitious roots, which enhance shoot elongation, allowing the plants to re-establish air contact by utilizing energy and consuming carbohydrates (Luo et al. 2011; Voesenek and Baily-Serres 2015; Yu et al. 2012), as seen in deepwater rice (Hattori et al. 2011). In contrast, plants following the low-oxygen quiescence strategy are characterized by traits that enable them to accumulate more carbohydrates by reducing energy consumption and inhibiting growth. This approach supports long-term survival under flooded conditions, ensuring sufficient energy reserves for growth recovery after the removal of flooding (Luo et al. 2011; Nakamura and Noguch 2020). Much of the research has focused on how *C. rotundus* plants survive and reproduce in the oxygen-deficient environment of flooded rice fields, particularly in relation to their morphological and physiological responses. These responses include changes in morphological and anatomical features, energy consumption and supply, and enzymes of the

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fermentation pathway. Pena-Fronteras et al. (2009) have reported that the flooding tolerance of lowland *C. rotundus* may be attributed to large carbohydrate content, amylase activity, and the ability to maintain high levels of soluble sugars in the tubers during germination and early growth. Fuentes et al. (2010) further suggested that higher carbohydrate content, larger stem diameters, and larger air spaces, along with the mobilization and utilization of carbohydrate reserves under hypoxia, were important adaptive traits for plants under flooded conditions. However, the specific adaptive strategies employed by *C. rotundus* tubers to thrive under the flooding conditions prevalent in rice fields remain to be elucidated.

Therefore, two different maturity levels of *C. rotundus* tubers from the upland areas, mature and growing tubers, were selected for soil flooding and recovery (the removal of soil flooding) treatment to simulate water management in paddy fields. This study hypothesizes that *C. rotundus* tubers subjected to soil flooding and subsequent removal of flooding will exhibit specific response mechanisms. These mechanisms are expected to be evidenced by changes in tuber vigor, morphological structure, carbohydrate levels, and anaerobic respiration.

Materials and Methods

Plant sourcing and collection

Tubers of *C. rotundus* were collected from a sugarcane field in Fusui, Guangxi, China (22°32'27"N, 107°50'18"E), where *C. rotundus* is the predominant weed species characterized by extensive coverage and high density. Subsequently, the collected tubers were propagated in the Agricultural Science Experimental field at Guangxi University, Nanning, China.

Experimental design

The experiment was conducted in the greenhouse of College of Agriculture, Guangxi University (22°51'01"N, 108°17'42"E) from August to December 2022. The soil used in the experiment was a mixture of peat, roseite, and sand proportioned 2:1:1, which was mixed, packed in bags, and sterilized in an autoclave. A total of 720 mature tubers (0.8-1.2 g, with hard, black-brown coat) and 720 growing tubers (0.1-0.3 g, with a softer texture, and brown

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coat that could be pared by hand) of *C. rotundus* were collected for the experiment.

The experimental design aimed to simulate paddy water management conditions, involving both soil flooding (maintaining a water layer) and drainage (recovery from soil flooding) treatments for *C. rotundus* tubers. Mature and growing tubers of *C. rotundus* were placed in plastic pots (17 cm lower diameter × 18 cm height × 20 cm upper diameter) with a bottom layer of 8 cm of soil and a top layer of 6 cm of soil. Water was then slowly added to cover the soil surface, maintaining a water level 3 cm above the soil.

Mature and growing tubers were subjected to four soil flooding treatments: (1) no soil flooding treatment as control (soil flooding 0 d, CK), (2) complete soil flooding under 3-cm-deep water for 30 days (SF-30), (3) complete soil flooding under 3-cm-deep water for 60 days (SF-60), (4) complete soil flooding under 3-cm-deep water for 90 days (SF-90). Each treatment had three replicates, with 20 tubers per replicate.

Subsequently, mature and growing tubers were subjected to four treatments to investigate their recovery following different durations of soil flooding: (1) no soil flooding treatment as control (CK), (2) the removal of SF-30 (RSF-30), (3) the removal of SF-60 (RSF-60), (4) the removal of SF-90 (RSF-90). To ensure consistency across the trials, all four treatments were sampled on the same day. Tubers in treatment (4) were subjected to 90 days of soil flooding before sampling. Similarly, tubers in treatment (3) underwent 60 days of soil flooding, and those in treatment (2) experienced 30 days of soil flooding before being sampled. No further flooding was applied after the sampling. The water was removed from all recovery treatments on the same day at the end of their respective soil flooding durations, and the recovery duration was monitored for 30 days. Three replicates were conducted for each treatment, with each replicate comprising 20 tubers.

Sampling and Date collection

After the soil flooding treatments, an assessment was conducted by examining the number of viable tubers, identified by their firm texture when pressed with fingers and the absence of any signs of rot or softening. Additionally, the fresh weight of the tuber samples was measured. Based on the data, the survival rate and the fresh weight retention rate were

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calculated. The fresh weight retention rate was determined by Equation 1:

$$y = \frac{A_1 - A_0}{A_0} \times 100\% \text{ [1]}$$

where *y* represents fresh weight retention rate; A_0 is fresh weight of *C. rotundus* tubers after soil flooding treatments, A_1 is the fresh weight of *C. rotundus* tubers before soil flooding treatments. One portion of the fresh samples was used for the observation of morphological characteristics and determination of tuber vigor, while another portion samples was frozen in liquid nitrogen and stored at -80°C for further research.

After the recovery treatments following soil flooding, the emergence rate of *C. rotundus* tubers was recorded within 30 days. The germination rate was assessed after 30 days of recovery treatments was investigated with both sprouted tubers and established seedling tubers counted as germinated. Additionally, samples were collected at two critical growth stages: the sprouting phase, when tubers had just begun to developed buds and the seedling rate ranged from 10% to 20%; the seedling stage, when *C. rotundus* tubers were observed to successfully produce seedlings 30 days post-recovery (i.e., 30 days after water removal). A portion of the fresh samples was randomly selected for the observation of morphological characteristics (three tubers) and determination of tuber vigor (three tubers), while another portion of samples were frozen in liquid nitrogen and stored at -80°C for further research.

Measurement of growth parameters

Growth parameters data were collected at 30 d after recovery treatments from soil flooding: (1) plant height (the length from stem base to top of plant with a ruler), (2) total fresh weight, (3) aboveground and underground fresh weight, (4) root to shoot ratio (underground fresh weight/aboveground fresh weight), (5) fresh weight of new tuber, (6) number of new tubers, and (7) single tuber growth rate. The single tuber growth rate was calculated by Equation 2.

$$y = \frac{a - b}{N} \times 100\% \quad \text{[2]}$$

where *y* represents the single tuber growth rate; *a* is fresh weight of *C. rotundus* plants after 30 days of recovery; *b* is fresh weight of original *C. rotundus* tubers and *N* is total number of tubers in each plastic pot. Each replicate had 20 tubers, and each treatment had three replicates. Principal component analysis (PCA) method and membership function analysis

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(MFA) (Liu 2022) were employed to evaluate the recovery capability of *C. rotundus* tubers after all recovery treatments.

Staining and determination of tuber vigor

The tubers vigor was determined by TTC (2,3,5-triphenyltetrazolium chloride) method (Lin et al. 2019). For each treatment, three tubers were randomly selected and sliced into thin sections (1 mm) using a double-edged razor blade. Each slice was then fully immersed in a solution containing 0.5% TTC and 75 mM phosphate buffer (pH 6.8). The slices were incubated in the dark at 37 °C for 3 h. After incubation, the reaction was stopped by adding 2 mL of 1 mol L^{-1} sulfate buffer. Images of the stained tuber slices were taken with a Canon digital camera (EOS Digital 70D, Canon Co., Tokyo, Japan). The red extract solution of tubers was extracted using ethyl acetate, followed by spectrophotometric determination at 485 nm.

Observation of morphological characteristics

The anatomical structure of tubers was observed by paraffin sections. Three tuber samples were randomly selected for each treatment and then washed and cut into small pieces no larger than 5 mm × 5 mm × 5 mm using a two-sided razor blade. Samples were first soaked in 15% hydrofluoric acid for 2 days, and then fixed in 70% formaldehyde-alcohol-acetic acid (FAA) for about 2 days before rinsing and sectioning. The tubers samples were fixed, dehydrated, and then embedded in paraffin following the method by Cheng et al. (Cheng et al. 2018). The cross-sections of *C. rotundus* tubers, 10 μm thick, were cut with Leica RM 2255 microtome (Leica, Bensheim, Germany) and observed under bright-field illumination using an upright fluorescence microscope (Zeiss, Jena, Germany). The staining intensity of sclerenchymal fibers in each cross-section was measured using Image J software (Ver. 1.43u, National Institutes of Health, USA) and expressed as integrated density. Three cross-sections were randomly selected for measurement in each treatment.

Measurement of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content

The concentration of malondialdehyde (MDA) was determined using the thiobarbituric acid (TBA) method, as described by Yan et al. (Yan et al. 2017) with modifications. The tubers

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samples were homogenized in trichloroacetic acid (10% TCA) solution, and the mixture was centrifuged at 4°C, 10000×g for 20 minutes. The resulting supernatant was reacted with 0.67% TBA solution at 100°C for 20 minutes. After cooling to room temperature, the absorbance was measured at 450 nm, 532 nm, and 600 nm. The H_2O_2 content was measured spectrophotometrically following the reaction with potassium iodide (KI) (Yiu et al. 2009). The tubers samples were homogenized in 0.1% TCA solution, and the mixture was centrifuge d at 4°C, 10000×g for 10 minutes. The supernatant was then mixed with phosphate buffer and KI solution, and the mixture was incubated at 28°C for one hour. The absorbance was read at 390 nm and the H_2O_2 content was quantified busing a standard curve.

Determination of non-structural carbohydrates

The contents of non-structural carbohydrates, including starch and soluble sugar, were determined by an improved sulfuric acid-anthrone colorimetric method (Wu et al. 2019). The dried sample was extracted three times with 80% ethanol, and then the supernatant was used for the determination of soluble sugars, while the residue was used for the determination of starch content. For soluble sugar determination, the supernatant was mixed with anthrone solution in 10 seconds, quickly cooled on ice, and then boiled in water for 10 minutes. The absorbance was measured at 620 nm after cooling. For starch determination, the residue was mixed with distilled water and boiled for 15 minutes, followed by the addition of 9.2 mol L^{-1} perchloric acid after cooling, and then boiled again for 10 minutes. The absorbance was measured at 620 nm after cooling. Starch and soluble sugar contents were quantified using a glucose standard curve.

The determination of structural carbohydrate lignin content was carried out in accordance with the acetyl bromide method (Liu et al. 2018). The tubers samples were extracted three times with 95% ethanol, and the deposit was washed with ethanol: n-hexane solution. The dried samples were treated with 25% bromoacetyl glacial acetic acid solution at 70°C for 30 minutes and the reaction was halted by the addition of NaOH. Acetic acid and hydroxylamine hydrochloride were then added to the mixture, and the absorbance of the centrifuged supernatant was measured at 280 nm.

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Measurement of ethanol and lactic acid contents, alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities

The ethanol and lactic acid contents, along with the activities of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) were determined by assay kits (BC5100, BC2230, BC1080, and BC0680, Solarbio Science & Technology Co., Ltd, China). The measurement procedure was performed in accordance with the manufacturer's instructions, which included the preparation of sample extracts, the addition of reagents, and the measurement of absorbance or fluorescence.

Statistical data analyzes

Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., CA, USA) for figure creation. Data were analyzed using one-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range test for comparisons among multiple treatments, or independent-sample *t*-tests for comparisons between two treatments; Statistical significance was set at $P < 0.05$. Each group of data was checked normal distribution and homogeneity of variance test before conducting Duncan's multiple range test and independent-sample *t*-tests. Normal distribution was confirmed by examining the histogram for a bell-shaped curve. For Duncan's multiple range test, the variance result (Levene's Test) based on the median showed that $p > 0.05$ satisfied the homogeneity of variance hypothesis; Groups with the same letter indicated no significant difference, while different letters denoted significant differences. For independent-sample *t*-tests, Levene's test for equality of variances showed $P > 0.05$, confirming homogeneity. Significance was determined at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$(***).

Results and discussion

Growth response of mature and growing tubers under soil flooding

Mature tubers of *C. rotundus* only exhibited a darker epidermal surface after soil flooding, with no significant change in hardness compared to the pre-soil-flooding period; whereas most growing tubers decayed or even perished, with their internal structure largely destroyed

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and the previously tender white epidermis turning into a greyish-brown mutilated outer skin (Figure 1A). Correspondingly, the fresh weight retention rate of mature tubers gradually decreased with prolonged soil flooding but remained at 93% after 90 days of flooding, with a survival rate consistently maintained at 100%. In contrast, the survival rate of growing tubers dropped to 5% after 90 days of flooding, with the fresh weight retention rate decreasing to 22% (Figure 1B).

During the subsequent recovery period, the germination rate of mature tubers after 30, 60, and 90 days of soil flooding was significantly higher than that of growing tubers by 62%, 60%, and 67%, respectively (Figure 1B). Mature tubers began to emerge about one week after all treatments, with the highest emergence rate maintained under RSF-30 treatment, whereas the emergence rate of growing tubers was severely affected, with only minimal germination under the RSF-30 treatment (Figure 1C). Further related growth data also revealed that mature tubers exhibited strong recovery growth, with the best recovery growth capacity under RSF-60 treatment (Table 1 and Table 2). As the duration of soil flooding treatment increases, the height of mature tubers groups during the recovery period is suppressed to a certain degree. Specifically, the plant height under the RSF-90 treatment during the recovery period is reduced by 30%. However, the total fresh weight, above-ground and under-ground fresh weight, new tuber fresh weight, and single tuber growth rate under RSF-30 and RSF-60 showed faster recovery ability compared to CK and RSF-90. Especially, the single tuber growth rate of RSF-60 was nearly double that of CK. However, the growth of growing tubers was significantly inhibited ($P < 0.05$, Table 1), likely due to their low survival rate under soil flooding treatments. In addition, the severe emergence of *C. rotundus* plants was observed in rice fields converted from dryland in Guangxi, China (Figure 1D). These results indicated that mature tubers could survive prolonged soil flooding, whereas growing tubers cannot. It is further suspected that the emergence of *C. rotundus* in rice fields may result from the sprouting and growth of mature tubers that have survived in the flooded environment.

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Responses of tuber vigor in mature tubers under soil flooding

The tuber vigor of mature tubers was significantly affected by soil flooding and recovery treatments (Figure 2). The vigor of mature tubers decreased sharply in the initial stage of soil flooding with only 13% of CK at SF-30, and subsequently levelled off at SF-60 and SF-90 (Figure 2A, 2C). During the recovery period, tuber vigor increased, with the RSF-60 and RSF-90 treatments surpassing the CK level at the seedling stage. However, the RSF-30 treatment, despite showing some recovery, did not fully return to the CK level at the same stage (Figure 2B, 2D). Especially, under RSF-60 conditions, the vigor at the sprouting and seedling stages was approximately 1.6 and 1.7 times higher than that of CK, respectively (Figure 2D). Collectively, despite an initial decline at the onset of soil flooding, the vigor of mature tubers stabilized in the later stage. This resilience allowed for a gradual recovery during the sprouting and seedling stages of the recovery period, indicating that mature tubers are capable of withstanding prolonged soil flooding.

Responses of morphological traits in mature tubers under soil flooding

Previous research suggests that the formation of aerenchyma and apoplastic barriers can be considered as the structural adaptation for flooding tolerance in plants (Evans 2003; Yang et al. 2013). In our study, no aerenchyma was observed in the internal structure of mature tubers of *C. rotundus*, even after 90 days of soil flooding (Figure 3A), which is similar with findings in both submerged unsubmerged terrestrial environments (Wei et al. 2022). Thus, the result indicated that the aerenchyma formation is not responsible for the survival of *C. rotundus* mature tubers under soil flooding conditions. Notably, significant changes were observed in the lignified sclerenchyma fibers on the epidermis of mature tubers during both the soil flooding and the recovery period (Figure 3B-3E). The intensity of sclerenchyma fibers gradually increased by approximately 60% at SF-60 and quadrupled at SF-90, respectively (Figure 3D). During the subsequent recovery period, the intensity of sclerenchyma fibers of in the RSF-30 and RSF-60 treatments can return to the levels comparable to CK at the seedling stage. However, the RSF-90 treatment, despite showing some recovery, did not fully reach the CK level (Figure 3E). Lignin, a key component in sclerenchyma fiber formation

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(Novo-Uzal et al. 2012), exhibited similar alterations across treatments. Specifically, it significantly increased to 1.1 times that of CK after 90 days of soil flooding. Notably, the RSF-60 treatment facilitated lignin levels to recover to those of the CK during the seedling phase (Figure 3F-3G). These results indicated that the regulation of lignified sclerenchyma fibers on the epidermis and the level of lignification in the tubers act as a barrier to protect the mature tubers of *C. rotundus* from flooding stress. This is consistent with previous studies indicating that lignified peripheral mechanical tissue in *C. rotundus* may enhance plant adaptation to flooding environments (Wei et al. 2022; Zheng et al. 2024).

We also determined the contents of associated two signaling molecules associated with lignin, MDA and H_2O_2 (Veal and Day 2011), in mature tubers of *C. rotundus* during the soil flooding and recovery period. As the duration of soil flooding increased, both MDA and H_2O_2 contents in the mature tubers steadily rose, reaching 1.3 and 4.7 times the levels of CK by 90 days of soil flooding, respectively (Figure 4A, 4C). During the recovery period (RSF-30, RSF-60, and RSF-90), both MDA and H_2O_2 levels decreased, with a particularly rapid decline in H_2O_2 content, suggesting that cellular peroxidation and oxidation gradually diminished (Figure 4B, 4D). Although the H_2O_2 content did not return to the level as the same period of CK at the seedling stage of RSF-30 treatment (Figure 4D), the MDA contents of mature tubers were restored to CK level (Figure 4B). These results suggested that soil flooding can regulate the contents of MDA and H_2O_2, affecting the degree of cellular peroxidation and oxidation. Additionally, MDA and H_2O_2 may act as molecular signals that collectively affected the level of lignification and the development of sclerenchyma fibers, contributing to the formation of protective barriers that shield mature tubers from flooding stress. This morphological adjustment represents a structural manifestation of flooding tolerance as well (Yang 2013). Furthermore, identifying the molecular signals, such as MDA and H_2O_2 that trigger these morphological changes could offer new avenues for intervention. By manipulating these signaling pathways, it may be possible to inhibit the formation of protective structures in weeds, thereby reducing their survival and competitiveness under flooded conditions.

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Responses of non-structural carbohydrate in mature tubers under soil flooding

Carbohydrate reserves, critical energy sources for plants under flooding conditions, are often positively correlated with higher levels of flooding tolerance (Yuan et al. 2023). Under soil flooding conditions, the content of soluble sugars in non-structural carbohydrates in mature tubers of *C. rotundus* dropped sharply in the initial stage with only 51% of CK at SF-30, and subsequently maintained steady at SF-60 and SF-90 (Figure 5A). Conversely, the starch content of mature tubers increased with prolonged soil flooding, surpassing CK levels by over 50% at SF-90 (Figure 5C). During following recovery period, soluble sugars content at the seedling stage of RSF-30, RSF-60, and RSF-90 all increased by over 120% compared to the that in the same period of CK (Figure 5B). Starch content in mature tubers increased under the RSF-30 and RSF-60 treatments but decreased under RSF-90. In particular, the starch content at the sprouting and seedling stages of RSF-60 returned to CK levels (Figure 5D). These results indicated that non-structural carbohydrates of mature tubers were altered and re-mobilized by the soil flooding environment. Similar to the strategy employed by *SUB1A* rice, which remains stunted to conserve energy during flash flooding at the seedling stage (Nagai et al. 2010), these mature tubers conserved energy during flooding and reactivate growth post-flooding, ensuring their survival and subsequent development.

Responses of respiratory in mature tubers under soil flooding

Fermentation serves as a crucial energy generation mechanism in soil flooding condition (Tadege et al. 1999), primarily through the action of two key enzymes—alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH), which reflect plants' adaptability to such low-oxygen conditions. Under soil flooding conditions, the ethanol content and ADH activity in mature tubers of *C. rotundus* both increased with the duration of flooding. By SF-90, they were approximately 5 times and 2 times higher than that in CK, respectively (Figure 6A, 6C). Notably, the ethanol content of mature tubers returned to or fell below the level of the same period of CK at the sprouting and seedling stages of RSF-30, respectively (Figure 6B). The ADH activity of mature tubers also declined at the seedling stage after RSF-30, RSF-60, and RSF-90 compared to SF-30, SF-60, and SF-90 (Figure 6D). While

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LDH activity varied across different soil flooding time treatments (Figure 6G), lactic acid content did not show significant changes (P=0.078, Figure 6E). During recovery, lactate content and LDH activity in mature tubers showed an increase compared to the soil flooding treatment, except RSF-30 treatment. However, these levels remained either equal to or notably lower than those observed in the sprouting and the seedling stages of RSF-30 and RSF-60, relative to CK (Figure 6F, 6H). Moreover, LDH activity was consistently lower than ADH activity across treatments, suggesting that alcohol fermentation is the primary anaerobic respiratory pathway. These results indicated that mature tubers of *C. rotundus* enhance their resilience to soil flooding by upregulating the alcohol fermentation pathway, allowing them to resume growth once the flooding subsides and reducing the accumulation of toxic substances. Similar findings were reported for lowland ecotypes of *C. rotundus* by Fuentes and Peña-Fronteras et al (Peña-Fronteras et al. 2009; Fuentes et al. 2010), showing a relatively modest energy yield from ethanol fermentation (Sun et al. 2020). This metabolic adaptation thus emerges as a critical flooding tolerance mechanism.

Our findings, combined with previous research, suggest that mature tubers of *C. rotundus* can endure soil flooding conditions. This tolerance may be attributed to the mature tuber's adoption of "a low-oxygen quiescence strategy" (Figure 7), characterized by three primary adaptations. Morphological adaptations: mature tubers developed lignified sclerenchyma fibers, which subsequently accumulated lignin content and act as a protective barrier; Carbohydrate metabolism: mature tubers conserved energy by reducing the utilization of soluble sugars and increasing starch reserves for energy storage. Respiratory adjustments: mature tubers heighten ADH activity to strengthen the anaerobic respiration pathway of alcoholic fermentation, thus alleviating the energy crisis imposed by flooding to some degree.

Cyperus rotundus primarily affects regions with temperate and tropical monsoon climates, which are also the main rice-cultivating areas. Changes in cultivation practices, such as converting upland fields to paddy fields or implementing paddy-upland rotations, can leave a significant number of *C. rotundus* tubers behind. These tubers have the potential to serve as seed banks for the proliferation of *C. rotundus* during rice production. Given the

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water management measures of actual rice cultivation fields (Zhang et al. 2021), it is common for water layer to be absent at the end of the tillering and yellow ripening stage, which provides favorable conditions for germination and growth environment of mature tubers. Further, during this period, mature tubers can germinate rapidly and grow in the paddy field through enhancing consumption of soluble sugars, weakening alcoholic fermentation, and reducing levels of lignification (Figure 7). Once *C. rotundus* plants grow above the water surface, they can ensure high photosynthetic productivity and utilize the ability of clonal integration to co-resist flooding adversity, thereby expanding their reproduction (Zhang et al. 2010).

In conclusion, this study revealed that the mechanism of mature tubers from the upland are tolerant to soil flooding by adopting a low-oxygen quiescence strategy. This finding not only sheds light on how *C. rotundus* from the upland emerges in rice fields and becomes a noxious rice weed but also opens new avenues for future research. Specifically, further exploring of the genetic basis of flood tolerance in *C. rotundus* tubers could offer valuable insights for the development of flood-resistant crop varieties. Understanding how these mechanisms can be harnessed or mitigated could have significant implications for weed management in rice fields and the improvement of agricultural practices.

Abbreviations

SF, soil flooding; RSF, removal of soil flooding; MFA, membership function analysis; PCA, Principal component analysis; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase;

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Competing Interests.

The authors declare no competing interests.

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Table 1. Plant growth parameters of *Cyperus rotundus* at 30 days after recovery treatments from soil flooding.

Type of tuber	Treatment[a]	PH[b]	TFW[c]	AFW[d]	UFW[e]	R/S[f]	NTFW[g]	NTN[h]	STUGR[i]
		cm	g	g	g		g	number	%
Mature tubers	CK	21.4 6 ± 0.75 a	24.11 ± 0.85 b	4.10 ± 0.37 a	20.01 ± 0.33 c	5.09 ± 0.4 b	1.11 ± 0.19 ab	20.00 ± 2.05 a	1.53 ± 0.17 b
	RSF-30	20.82 ± 0.45 a	29.01 ± 0.42 a	4.90 ± 0.08 a	24.11 ±0.18 b	4.92 ± 0.06 b	1.56 ± 0.31 a	23.67 ± 0.27 a	2.73 ± 0.11 a
	RSF-60	16.35 ± 1.23 b	30.49 ± 1.04 a	4.85 ± 0.58 a	25.64 ±0.62 a	5.70 ± 0.53 b	1.32 ± 0.19 a	19.67 ± 1.52 a	2.87 ± 0.32 a
	RSF-90	15.16 ± 0.25 b	21.11 ± 0.45 b	1.16 ± 0.14 b	19.95 ± 0.21 c	17.19 ± 1.96 a	0.27 ± 0.04 b	9.50 ± 1.44 b	0.07 ± 0.01 c
Growing tubers	CK	18.84 ±1.67 a	6.88 ± 0.85 a	1.65 ± 0.29 a	5.23 ± 0.63 a	3.17 ± 0.38 b	0.18 ± 0.00 a	11.00 ± 0.71 a	2.50 ± 0.63 a
	RSF-30	8.62 ±1.96 b	1.41 ± 0.25 b	0.07 ± 0.03 b	1.34 ± 0.23 b	19.14 ± 2.30 a	0.00 ± 0.00 b	0.00 ± 0.00 b	-2.96 ± 0.32 b
	RSF-60	0.00 ± 0.00 c	0.58 ± 0.17 c	0.00 ± 0.00 b	0.58 ± 0.17 b	—	0.00 ± 0.00 b	0.00 ± 0.00 b	-4.06 ± 0.27 c

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RSF-90	0.00 ± 0.00 c	0.34 ± 0.04 c	0.00 ± 0.00 b	0.34 ± 0.04 c	—	0.00 ± 0.00 b	0.00 ± 0.00 b	-4.44 ± 0.05 c

[a]Treatment: CK, no soil flooding treatment; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding. [b]plant height; [c]total fresh weight; [d]aboveground fresh weightand; [e]underground fresh weight; [f]root-shoot ratio; [g]new tuber fresh weight; [h]new tuber number; [i]single tuber growth rate. Data are shown as the mean ± SD (N ≥ 3). The different letter in the same column of mature or growing tubers represents s a significant difference (P<0.05, one-way ANOVA).

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Table 2. Evaluation results of mature tubers of *Cyperus rotundus* at 30 days after recovery treatments from soil flooding.

Treatment[a]	PCA[b]		MFA[c]		Comprehensive ranking
	Rating value	Ranking	Rating value	Ranking	
CK	-0.047	3	0.552	3	3
RSF-30	0.059	2	0.894	2	2
RSF-60	0.104	1	0.940	1	1
RSF-90	-0.115	4	0.116	4	4

[a]Treatment: CK, no soil flooding treatment; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding.

[b]PCA: principal component analysis.

[c]MFA: membership function analysis.

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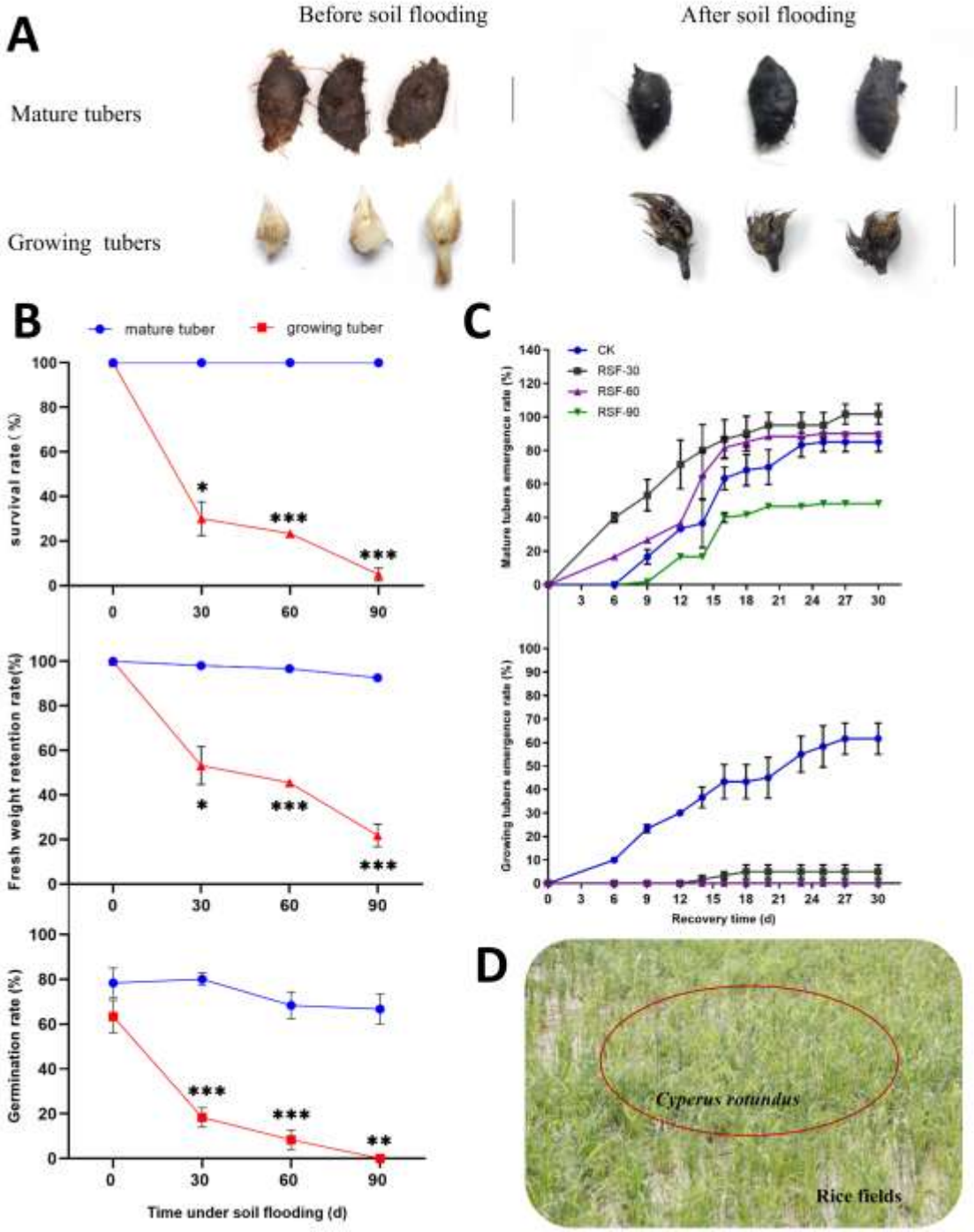

Figure 1. Phenotypic differences of mature tubers and growing tubers of *Cyperus rotundus* under soil flooding. (A), Phenotypes change of mature tubers and growing tubers of *Cyperus rotundus* before and after soil flooding. scale bars=1 cm. (B), The survival rate, fresh weight retention rate, and emergence rate of mature tubers and growing tubers under soil flooding treatments. (C), The emergence rate of mature tubers and growing tubers within 30 days after recovery treatments. (D), *Cyperus rotundus* appears in rice fields converted from dryland. Abbreviations: CK, no soil flooding treatment; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding. Data are shown as the means ± SE (N = 3). Independent-sample *t*-tests were used to determine differences at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$(***).

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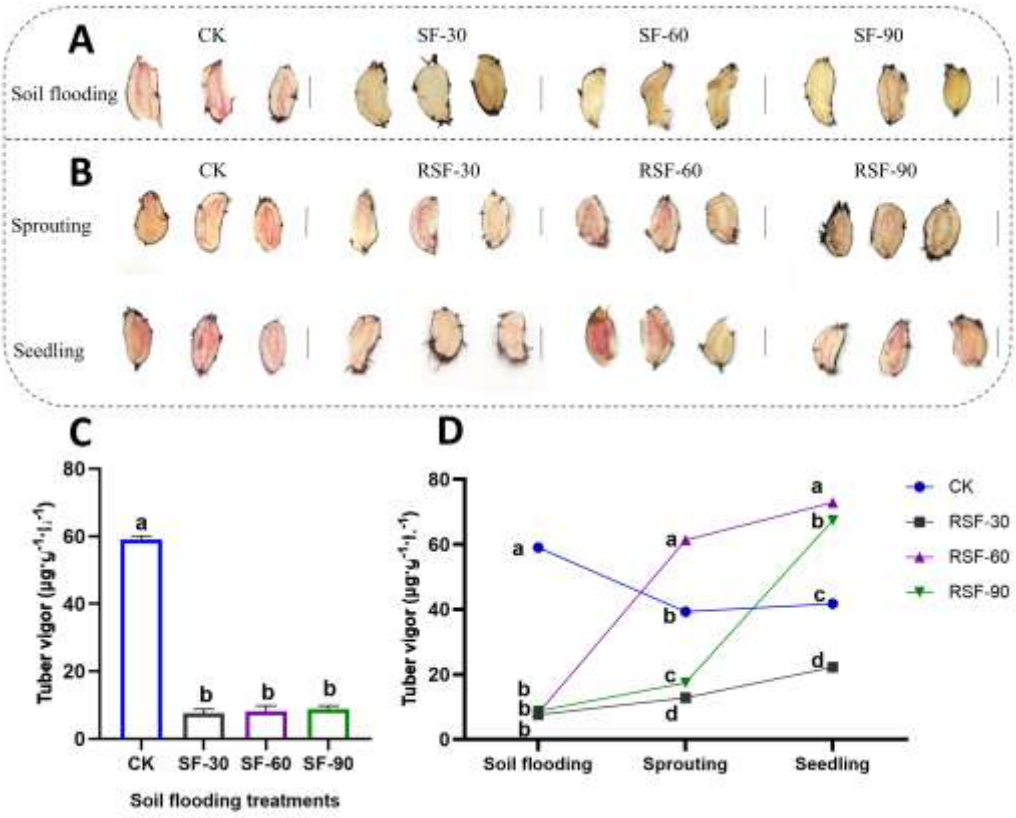

Figure 2. Responses of vigor of mature tubers of *Cyperus rotundus* under soil flooding. (A), Staining chart of mature tubers under soil flooding treatments. (B), Staining chart of mature tubers at the sprouting and seedling stage of recovery treatments. (C), Changes of tuber vigor of mature tubers under soil flooding treatments; different letters in graphic indicate significant differences ($P < 0.05$, one-way ANOVA). (D), Changes of tuber vigor of mature tubers after recovery treatments; different letters in each column indicate significant differences ($P < 0.05$, one-way ANOVA). Values are means ± SE (N = 3). Abbreviations: CK, no soil flooding treatment; SF-30, soil flooding for 30 days; SF-60, soil flooding for 60 days; SF-90, soil flooding for 90 days; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding.

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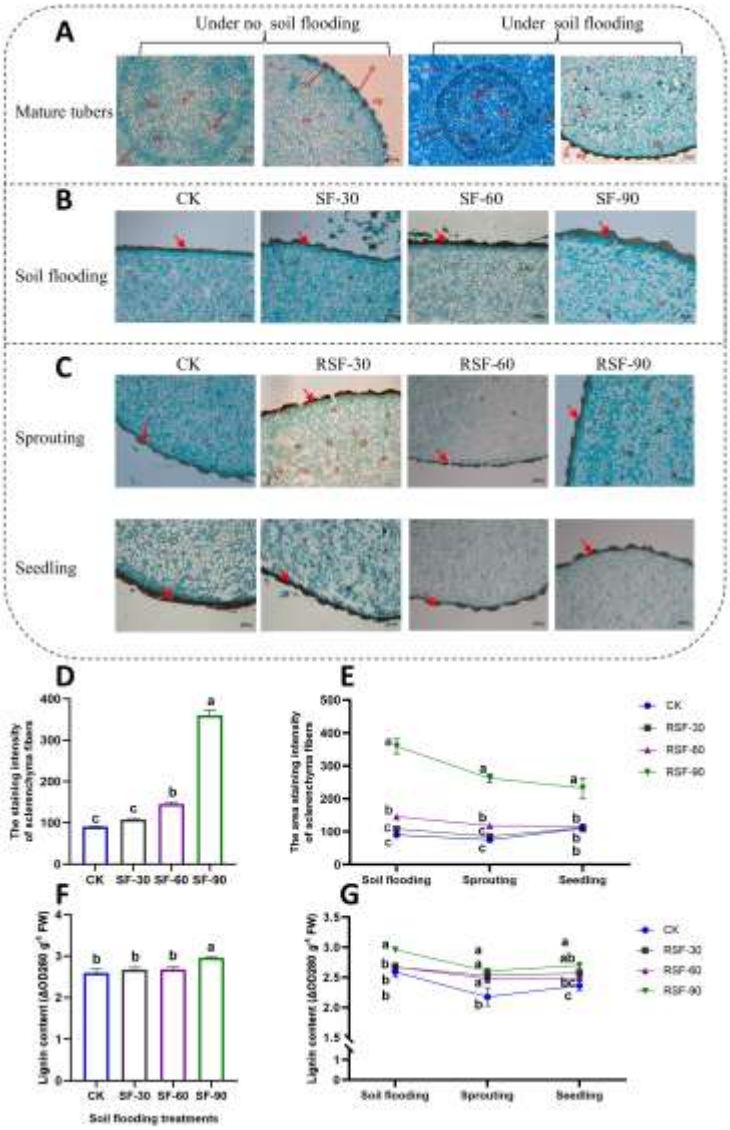

Figure 3. Responses of morphological traits of mature tubers of *Cyperus rotundus* under soil flooding. (A), Anatomical structure of the inner structure of tubers under no soil flooding and under soil flooding. Abbreviations: ep, epidermis; sl, sclerenchyma fibers; cu, cuticle; co, cortex; en, endodermis; pi, pith; vb, vascular bundles; mx, metaxylem; ph, phloem. (B), Diagram of changes of sclerenchyma fibers in mature tubers under soil flooding treatments. (C), Diagram of changes of sclerenchyma fibers in mature tubers at the sprouting and seedling stage after the recovery treatments. (D), Changes of staining density of sclerenchymal fibers in mature tubers under soil flooding treatments. (E), Changes of staining density of sclerenchymal fibers of mature tubers after recovery treatments. (F), Changes of lignin content in mature tubers under soil flooding treatments. (G), Changes of lignin content in mature tubers after recovery treatments. The red arrows represent sclerenchyma fibers, Staining: saffron and solid green. Values are means ± SE (N = 3). D and F: different letters in graphics indicate significant differences ($P < 0.05$, one-way ANOVA). E and F: different letters in each column indicate significant differences ($P < 0.05$, one-way ANOVA). Abbreviations: CK, no soil flooding treatment; SF-30, soil flooding for 30 days; SF-60, soil flooding for 60 days; SF-90, soil flooding for 90 days; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding.

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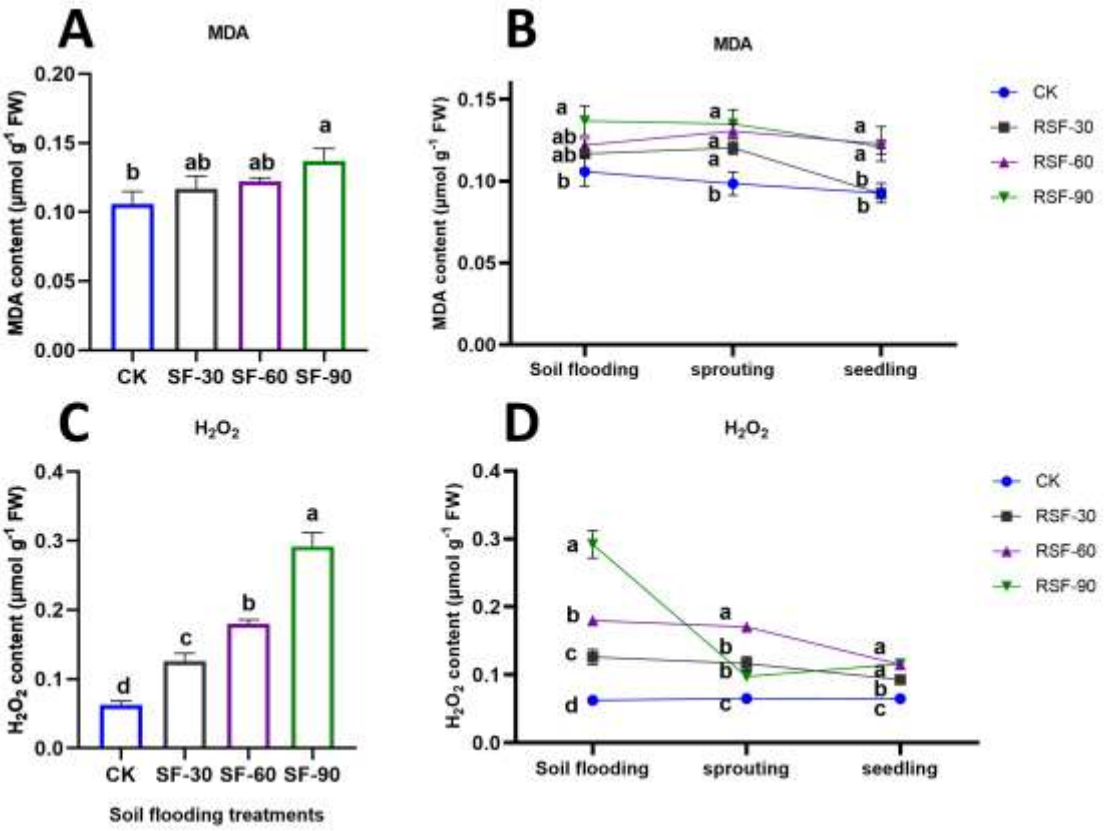

Figure 4. Responses of MDA and H_2O_2 of mature tubers of *Cyperus rotundus* under soil flooding. Changes of MDA content of mature tubers under soil flooding treatments (A) and after recovery treatments (B). Changes of H_2O_2 content of mature tubers under soil flooding treatments (C) and after recovery treatments (D). Values are means ± SE (N = 3). A and C: different letters in graphics indicate significant differences ($P < 0.05$, one-way ANOVA). B and D: different letters in each column indicate significant differences ($P < 0.05$, one-way ANOVA). Abbreviations: CK, no soil flooding treatment; SF-30, soil flooding for 30 days; SF-60, soil flooding for 60 days; SF-90, soil flooding for 90 days; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding.

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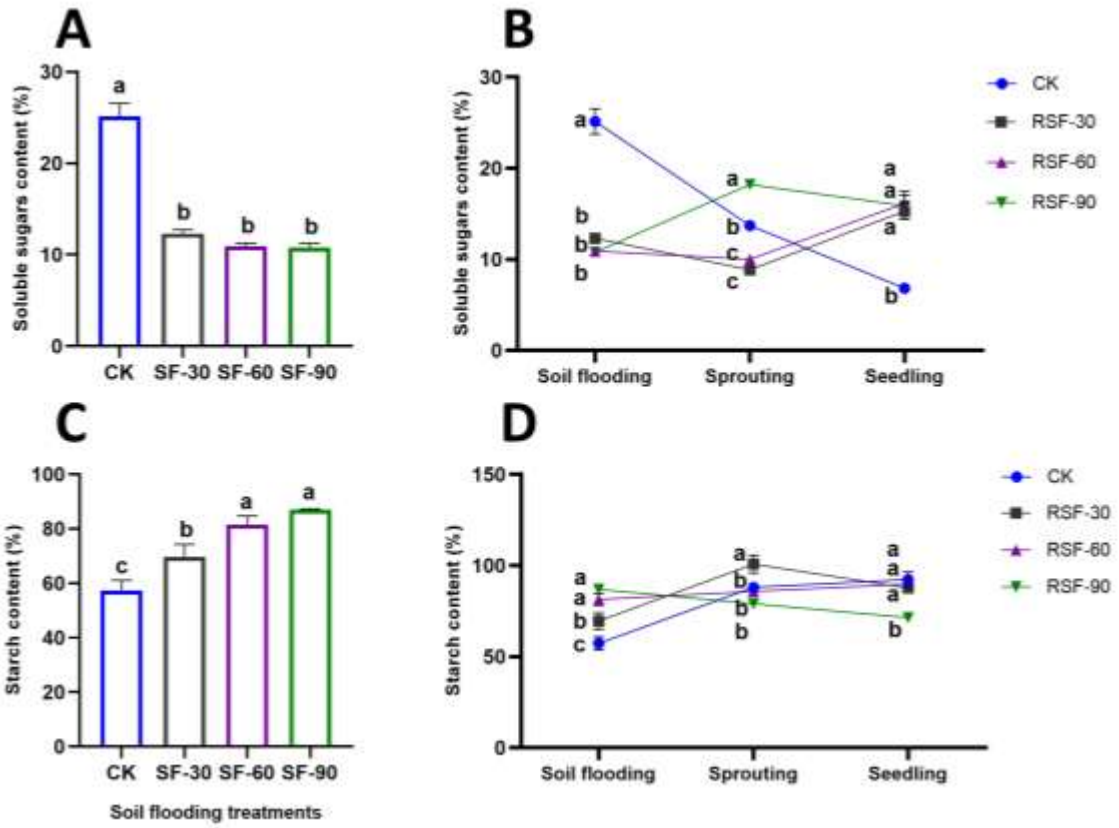

Figure 5. Responses of non-structural carbohydrate of mature tubers of *Cyperus rotundus* under soil flooding. Changes of soluble sugars content of mature tubers under soil flooding treatments (A) and after recovery treatments (B). Changes of starch content of mature tubers under soil flooding treatments (C) and after recovery treatments (D). Values are means ± SE (N = 3). A and C: different letters in graphics indicate significant differences ($P < 0.05$, one-way ANOVA). B and D: different letters in each column indicate significant differences ($P < 0.05$, one-way ANOVA). Abbreviations: CK, no soil flooding treatment; SF-30, soil flooding for 30 days; SF-60, soil flooding for 60 days; SF-90, soil flooding for 90 days; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding.

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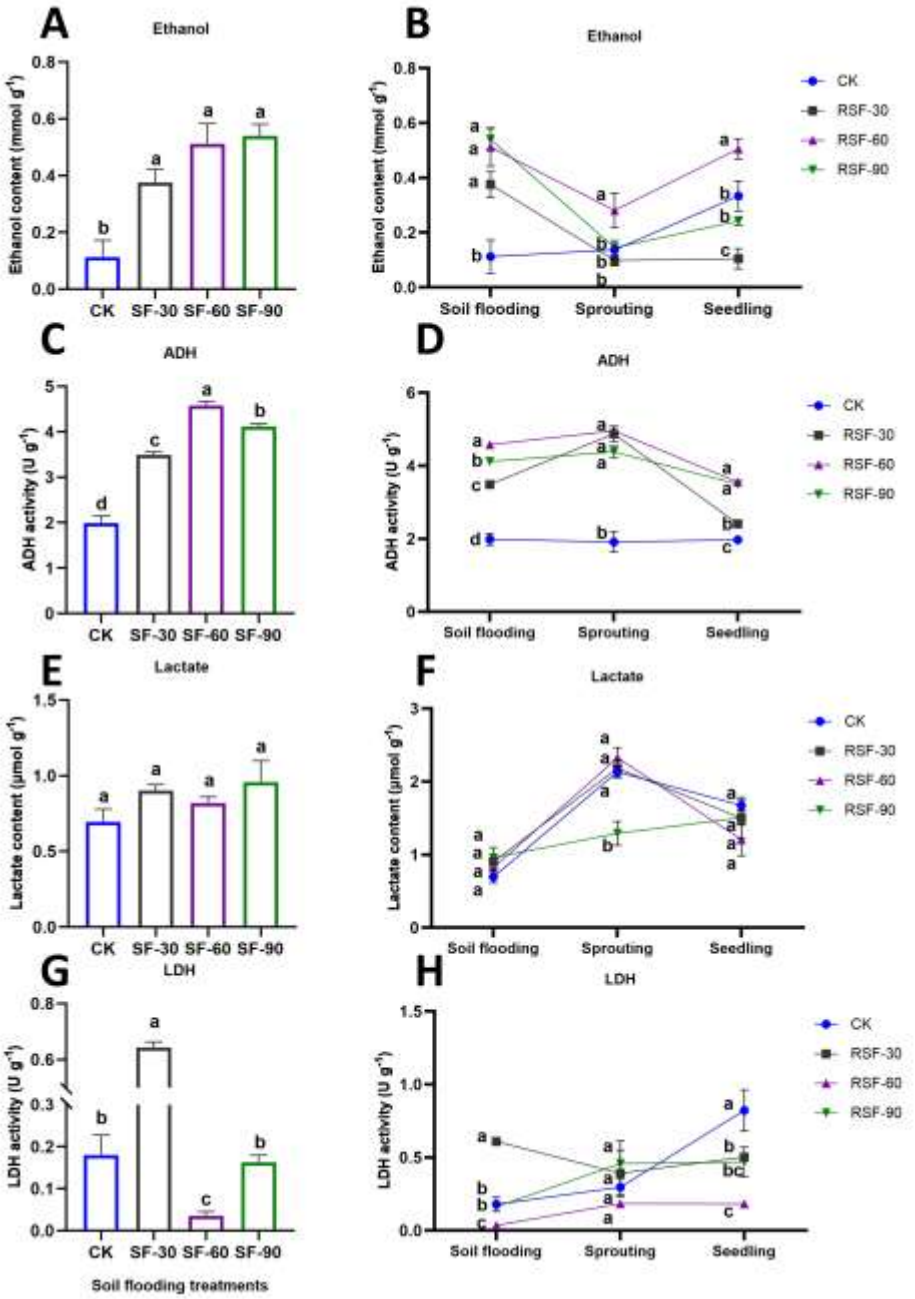

Figure 6. Responses of respiratory of mature tubers of *Cyperus rotundus* under soil flooding. Changes of ethanol content of mature tubers under soil flooding treatments (A) and after recovery treatments (B). Changes of ADH activity of mature tubers under soil flooding treatments (C) and after recovery treatments (D). Changes of lactic acid content of mature tubers under soil flooding treatments (E) and after recovery treatments (F). Changes of LDH activity of mature tubers under soil flooding treatments (G) and after recovery treatments (H). Values are means ± SE (N = 3). A, C, E and G: different letters in graphics indicate significant differences ($P < 0.05$, one-way ANOVA). B, D, F, and H: different letters in each column indicate significant differences ($P < 0.05$, one-way ANOVA). Abbreviations: CK, no soil flooding treatment; SF-30, soil flooding for 30 days; SF-60, soil flooding for 60 days; SF-90, soil flooding for 90 days; RSF-30, the removal of soil flooding at 30 days post-flooding; RSF-60, the removal of soil flooding at 60 days post-flooding; RSF-90, the removal of soil flooding at 90 days post-flooding.

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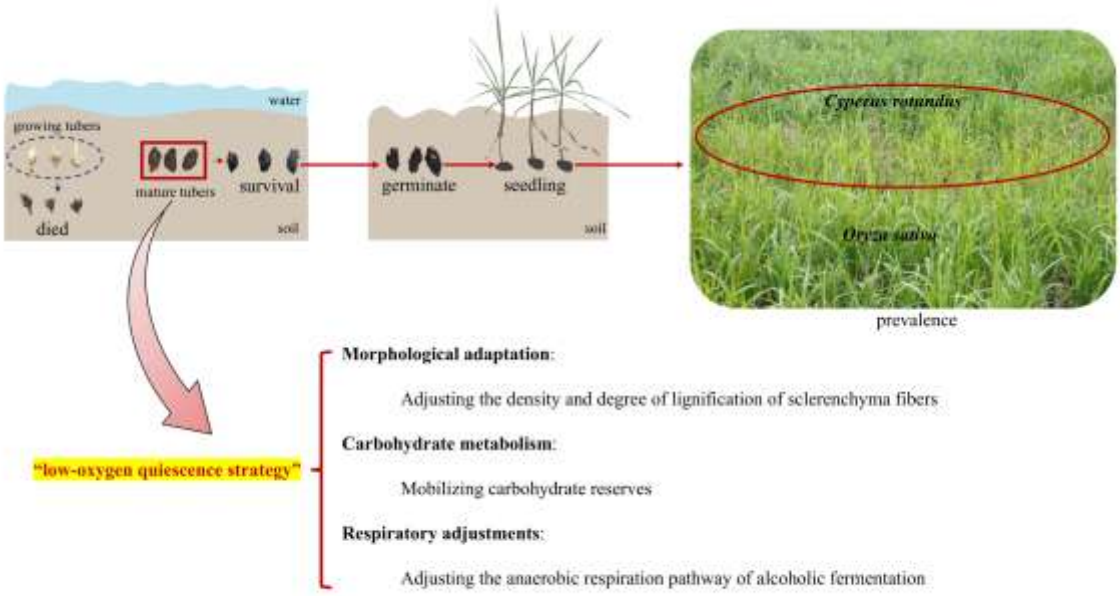

Figure 7. Mature tubers of *Cyperus rotundus* from the upland are tolerant to soil flooding by adopting a "low-oxygen quiescence strategy", thus explaining the reason for the emergence of *Cyperus rotundus* from the upland in rice fields.

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