Effects of -Linolenic Acid on Rice Seed Germination Under Drought Stress: Postprint

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Abstract

Abstract: -Linolenic acid is a polyunsaturated fatty acid of the -3 family and an essential component of cell membranes. Studies have demonstrated that under drought conditions, plants remodel cell membrane fluidity by releasing -linolenic acid. However, whether exogenous application of -linolenic acid can enhance plant drought resistance remains unclear. This study employed PEG6000 to simulate drought stress and investigated the effects of -linolenic acid on drought resistance during rice seed germination and seedling growth. The results indicated that under 14% and 16% PEG drought conditions, rice seed germination was delayed and seedling growth was inhibited. -Linolenic acid at concentrations of 25 mol·L 1 and 250 mol·L 1 alleviated the drought-induced inhibition of rice young root and seedling growth, with the alleviating effect intensifying as drought severity increased. Under 16% PEG conditions, 25 mol·L 1 and 250 mol·L 1 -linolenic acid increased seedling root length by 34.3% and 29.1%, shoot length by 67.8% and 52.0%, root weight by 43.9% and 35.2%, and shoot weight by 59.1% and 43.6%, respectively. -Amylase activity assays revealed that 25 mol·L 1 and 250 mol·L 1 -linolenic acid enhanced -amylase activity in rice seeds under drought conditions by 56.7%-70.7% and 36.8%-43.8%, respectively. Root vigor assays demonstrated that 25 mol·L 1 and 250 mol·L 1 -linolenic acid increased young root vigor under drought conditions by 11.4%-28.4% and 5.4%-22.2%, respectively. This paper also discusses the changes in -amylase activity and root vigor of rice seeds under various drought conditions. It is concluded that -linolenic acid primarily alleviates drought stress encountered during seed germination by enhancing -amylase activity, while its effect on rice root vigor is relatively limited.

Full Text

Preamble

Effect of -Linolenic Acid on Rice Seed Germination Under Drought Stress

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Abstract

-Linolenic acid (ALA) is a polyunsaturated fatty acid belonging to the -3 system and an essential component of plant cell membranes. Previous studies have shown that under drought conditions, plants release ALA to remodel membrane fluidity. However, whether exogenous ALA can enhance plant drought resistance remains unknown. This study investigated the effects of ALA on drought tolerance during rice seed germination and seedling growth using PEG6000 to simulate drought stress. The results showed that under 14% and 16% PEG-induced drought conditions, rice seed germination was delayed and seedling growth was inhibited. Application of 25 mol•L-1 and 250 mol•L-1 ALA alleviated the drought-induced inhibition of radicle and seedling growth, with the alleviating effect increasing as drought severity intensified. Under 16% PEG conditions, 25 mol•L-1 and 250 mol•L-1 ALA increased root length by 34.3% and 29.1%, shoot length by 67.8% and 52.0%, root fresh weight by 43.9% and 35.2%, and shoot fresh weight by 59.1% and 43.6%, respectively. -Amylase activity assays revealed that 25 mol•L-1 and 250 mol•L-1 ALA increased -amylase activity in germinating rice seeds under drought conditions by 56.7%-70.7% and 36.8%-43.8%, respectively. Root vigor measurements showed that 25 mol•L-1 and 250 mol•L-1 ALA increased radicle vigor under drought conditions by 11.4%-28.4% and 5.4%-22.2%, respectively. This paper also discusses the changes in -amylase activity and radicle vigor in rice seeds under different drought conditions. We conclude that ALA primarily alleviates drought stress during seed germination by enhancing -amylase activity, while its effect on rice radicle vigor is relatively limited.

Keywords: rice, -linolenic acid, drought, PEG, seed germination, -amylase

Introduction

Nearly all crop cultivation and horticultural plant propagation begin with seeds. Most crops grown by humans are reproduced generation after generation through seeds. Seed germination refers to the physiological process from water imbibition and swelling of mature seeds to the emergence of the radicle (Rajjou et al., 2012). Seed germination is the most critical stage in the plant life cycle, determining when a plant can enter natural or agricultural ecosystems (Weitbrecht et al., 2011). The most crucial factor for successful seed germination is a suitable environment. This process is not an independent biological process of dry seeds but rather a continuum connected with seed maturation, dehydration, and seedling establishment.

Biological membranes are the sites where cells exchange information and materials with the external environment. Damage to cells from various biotic and abiotic stresses primarily initiates at the cell membrane. Membrane lipids and proteins are essential components of the cell membrane, and their structure, composition, and interactions significantly influence the cell' s ability to resist stress (Wang et al., 2000). Biological membranes contain approximately 100 types of lipids, and changes in just 1%-2% of these components can affect cell survival (Wang et al., 2000). Dynamic changes in membrane structure represent an important aspect of plant stress resistance research. *Saccharomyces cerevisiae*, which contains only a single unsaturated fatty acid, serves as an ideal model for studying the functions of polyunsaturated fatty acids (PUFAs) (Yazawa et al., 2009). Studies using yeast have revealed that PUFAs possess protective functions under stress conditions, enhancing cellular tolerance to oxidative stress, low temperature, salinity, alcohol, and alkaline pH (Yazawa et al., 2009). Under stress conditions, alterations in membrane fluidity provide a stable environment for the function of important integral membrane proteins, such as those involved in photosynthesis. Evidence indicates that changes in oleic acid (18:1) content in *Arabidopsis* chloroplasts can trigger the expression of pathogen-responsive genes (Kachroo et al., 2001). Moreover, oleic acid (18:1) content influences plant defense gene expression (Kachroo et al., 2001) and affects *Aspergillus* propagation in seeds (Calvo et al., 1999).

-Linolenic acid (ALA) is a PUFA belonging to the -3 system and a crucial component of cell membranes. When responding to biotic and abiotic stresses, plants can release ALA to remodel membrane fluidity (Iba, 2002). Free linolenic acid acts as a signaling molecule under stress conditions and serves as a precursor for phyto-oxylipin biosynthesis (Blée, 2002). Transgenic tobacco overexpressing the -3 desaturase gene exhibited increased 18:3 unsaturated fatty acid content in membranes and enhanced resistance to salt and drought stresses (Zhang et al., 2005). This study investigated the effects of exogenous ALA application on rice seed germination, seedling growth, and related physiological characteristics under PEG-6000-simulated drought stress, providing insights for alleviating drought stress during rice germination.

1.2 Materials

The experimental material consisted of freshly harvested seeds of rice (*Oryza sativa* L. cv. Nipponbare). Seeds were collected from the Baodi experimental field in Tianjin and fully sun-dried before use.

1.3 Seed Germination Under Drought Conditions

Based on the effects of PEG6000 on water potential as proposed by Michel and Kaufmann (1973), different concentrations of PEG6000 were used to simulate drought stress. Rice seeds were sterilized with 0.1% potassium permanganate for 5 min, then rinsed 5-6 times with distilled water. Forty plump seeds were sown in petri dishes lined with filter paper, and 30 mL of PEG6000 solution at concentrations of 10%, 12%, 14%, and 16% (w/v) was added to each dish. The seeds were cultured at 28°C under a 12 h light/12 h dark photoperiod. Every two days, 5 mL of the corresponding PEG6000 solution was added to each dish. Germination was recorded when bud length exceeded 2 mm, and germination rates were counted regularly. At 10 days of culture, the 20 earliest germinated seedlings were selected from each dish to measure root length, shoot length, root fresh weight, and shoot fresh weight.

1.4 Effect of ALA on Seed Germination Under PEG Conditions

First, 1 mL of 70% ALA (commercial stock solution) was dissolved in anhydrous ethanol, then water was added dropwise with stirring to a final volume of 10 mL. The ALA emulsion was sonicated until the solution turned milky white, ensuring thorough mixing of ALA with water to prepare a 25 mmol•L-1 ALA stock solution. Forty plump seeds were cultured on filter paper containing 14% and 16% PEG6000 supplemented with 0, 1, 5, 25, or 250 mol•L-1 ALA. Culture conditions and statistical analysis of seed germination and seedling growth were the same as described above.

1.5 -Amylase Activity Assay

The -amylase assay kit from Suzhou Keming Biotechnology Co., Ltd. was used. The assay principle is based on -amylase hydrolyzing -1,4-glycosidic bonds in starch to produce reducing sugars such as glucose, maltose, and dextrin, which reduce 3,5-dinitrosalicylic acid to a brown-red compound. The color intensity, measured as absorbance, reflects the enzyme activity. Seeds germinated for 3 days under various conditions were selected, and the seed coat, radicle, and seedling were removed. The remaining tissue was thoroughly ground in a mortar with 1 mL of double-distilled water, then transferred to a centrifuge tube and centrifuged at 4500 rpm for 10 min. Five hundred microliters of the supernatant was transferred to a test tube and diluted with distilled water to 10 mL to obtain the amylase extract. The tube was incubated in a 70°C water bath for 15 min to inactivate -amylase. Both control and assay tubes received 3,5-dinitrosalicylic acid and were incubated at 40°C for 10 min. Absorbance was read at 540 nm, and ΔA was calculated as A_assay - A_control. One unit of enzyme activity was defined as the amount catalyzing the production of 1 mg of reducing sugar per gram of tissue per minute. -Amylase activity (U/g fresh weight) = $89.4 \times (\Delta A + 0.022) \div W$, where W is the fresh weight of seeds.

1.6 Root Vigor Assay

Following the method of Zhang et al. (2015), root vigor was assessed using the colorless, lipophilic, light-sensitive compound 2,3,5-triphenyltetrazolium chloride (TTC), which is reduced by respiratory hydrogen to red formazan (TTF).

Roots (0.05 g) from seeds germinated for 7 days in the ALA experiment were cut into approximately 1 cm segments and soaked in 0.6% TTC solution (dissolved in pH 7.4 phosphate buffer) at 30°C for 24 h. The staining solution was then discarded, and the roots were washed three times with distilled water. Formazan was extracted with 95% ethanol at 85°C for 20 min, and the extract absorbance was measured at 485 nm.

1.7 Statistical Analysis

Experiments were repeated three times, with three petri dishes per treatment condition, each containing 40 seeds. Physiological index data were obtained from three independent measurements. Statistical significance was analyzed using ANOVA followed by Student-Newman-Keuls test in SPSS 11.0, with a 95% confidence interval. Significant differences among groups under the same PEG or ALA treatment conditions are indicated by letters (a, b, c) or asterisks.

Results

2.1 Effects of Different PEG Concentrations on Rice Seed Germination and Seedling Growth

To investigate the effects of different PEG6000-induced drought conditions on rice seed germination, seeds were sown on filter paper soaked with 10%, 12%, 14%, 16%, 18%, and 20% (w/v) PEG6000 solutions. The results showed that as PEG concentration increased, rice seed germination was progressively delayed, but by day 5, nearly all seeds had germinated across all PEG concentrations (Table 1). Although germination rates reached 75% by day 7 under 18% and 20% PEG, the seeds turned black and seedling growth nearly ceased (data not shown). Therefore, 14% and 16% PEG6000 were selected as the drought treatment concentrations for subsequent experiments.

Table 1 Germination of rice seeds under different concentrations of PEG

PEG Concentration (%)	Day 3 Germination (%)	Day 5 Germination (%)	Day 7 Germination (%)
0	91.3±0.7 a	100.0±0.0 a	100.0±0.0 a
10	18.8±1.8 b	75.0±7.1 b	98.8±1.8 b
12	12.6±3.5 c	56.3±5.3 c	92.5±3.5 c
14	6.3±1.8 d	42.5±3.5 d	88.8±5.3 c
16	0.0±0.0 e	20.0±3.5 e	82.5±7.0 d

Note: Germination rate was recorded when bud length exceeded 2 mm.

To further explore the effects of different drought conditions on early rice seedling growth, we measured seedling growth status at 10 days post-germination under 14% and 16% PEG concentrations. Compared with the control, shoot length, root fresh weight, and shoot fresh weight under 14% PEG were 0.65-fold, 0.36-fold, and 0.42-fold of the control values, respectively; under 16% PEG, these values were 0.41-fold, 0.28-fold, and 0.29-fold of the control, respectively (Table 2). Thus, as drought severity increased, the inhibitory effect on seedling growth intensified. Interestingly, under 14% and 16% PEG, rice seedling root length was 1.63-fold and 1.26-fold of the control, respectively, although the number of crown roots decreased substantially (data not shown). Therefore, we hypothesize that under drought conditions, rice seedlings may resist drought stress by extending root length to maximize water absorption.

Table 2 Growth status of rice seedlings under different concentrations of PEG

PEG Concentration (%)	Root Length (cm)	Shoot Length (cm)	Root Fresh Weight (mg)	Shoot Fresh Weight (mg)
0 (Control)	3.88±0.88 b	4.23±0.56 a	191±12 a	313±23 a
14	6.31±1.55 a	2.77±0.55 b	133±6 b	4.88±1.15 b
16	1.72±0.33 c	69±2 c	91±8 b	53±4 b

Note: Data were acquired by measuring the 20 earliest germinated seedlings at 10 days in each culture plate.

2.2 Effect of ALA on Seed Germination and Seedling Growth Under Drought Conditions

To investigate the effects of ALA on seed germination and early seedling growth under drought conditions, rice seeds were sown in petri dishes containing 14% and 16% PEG6000 supplemented with 0, 1, 5, 25, or 250 mol•L-1 ALA. The results showed that ALA did not significantly improve germination potential or germination rate of rice seeds (Table 3).

Table 3 Effect of different concentrations of ALA on rice germination under PEG conditions

PEG Concentration (%)	ALA Concentration (mol•L-1)	Day 3 Germination (%)	Day 5 Germination (%)	Day 7 Germination (%)
14	0	13.8±0.7	97.5±0.0	100.0±0.0
14	1	12.5±1.4	96.3±2.1	97.5±1.4
14	5	21.3±0.7	100.0±0.0	100.0±0.0
14	25	23.8±2.1	100.0±0.0	100.0±0.0
14	250	13.7±0.7	97.5±1.4	98.8±0.7

PEG Concentration (%)	ALA Concentration (mol•L-1)	Day 3 Germination (%)	Day 5 Germination (%)	Day 7 Germination (%)
16	0	11.3±0.7	88.8±0.7	95.0±2.8
16	1	10.0±1.4	78.8±0.7	98.5±0.7
16	5	2.5±1.4	83.8±2.1	100.0±0.0
16	25	3.8±0.7	70.0±2.8	98.8±0.7
16	250	1.3±0.7	92.5±1.4	97.5±1.4

Note: Germination rate was recorded when bud length exceeded 2 mm.

Based on preliminary experiments on the effects of different ALA concentrations on seedling growth under drought conditions, 25 mol•L-1 and 250 mol•L-1 ALA showed the most significant effects. Therefore, we conducted a detailed analysis of the alleviating effects of 25 mol•L-1 and 250 mol•L-1 ALA on drought-induced inhibition of seedling growth (Figure 1 [Figure 1: see original paper]). The results showed that under 14% PEG conditions, both 25 mol•L-1 and 250 mol•L-1 ALA significantly affected rice seedling growth. Compared with the control, shoot length increased by 23.7% and 18.9%, root fresh weight by 47.0% and 51.0%, and shoot fresh weight by 35.3% and 29.7%, respectively, while root length only increased slightly. Under 16% PEG conditions, as drought severity increased, the promoting effects of 25 mol•L-1 and 250 mol•L-1 ALA on seedling growth were enhanced. Compared with the control, root length increased by 34.3% and 29.1%, shoot length by 67.8% and 52.0%, root fresh weight by 43.9% and 35.2%, and shoot fresh weight by 59.1% and 43.6%, respectively.

2.3 Effect of ALA on -Amylase Activity During Seed Germination Under Drought Stress

 -Amylase activity is a crucial indicator of seed vigor during rice seed germination. To investigate the effect of ALA on germination capacity of rice seeds under drought stress, we measured -amylase activity during early germination. The results showed that under 14% PEG conditions, treatment with 25 mol•L-1 and 250 mol•L-1 ALA increased -amylase activity by 70.7% and 43.8% compared with the control, respectively. Under 16% PEG conditions, 25 mol•L-1 and 250 mol•L-1 ALA increased -amylase activity by 56.7% and 36.8%, respectively. Furthermore, at the same ALA concentration, -amylase activity under 16% PEG was 25%-31% lower than that under 14% PEG conditions (Figure 2 [Figure 2: see original paper]).

2.4 Effect of ALA on Root Vigor During Seed Germination Under Drought Stress

The root system is the primary organ for water absorption in plants, and root vigor largely reflects drought tolerance. To investigate the effect of ALA on drought resistance of rice seedlings, we measured root vigor under 14% and 16% PEG6000 conditions with exogenous application of 25 mol•L-1 and 250 mol•L-1 ALA. The results showed that under 14% PEG conditions, 25 mol•L-1 and 250 mol•L-1 ALA increased root vigor by 28.4% and 22.2% compared with the control, respectively. Under 16% PEG conditions, radicle vigor increased by 11.4% and 5.4%, respectively. Moreover, at the same ALA concentration, root vigor under 16% PEG was 5%-42% higher than that under 14% PEG conditions (Figure 3 [Figure 3: see original paper]).

Discussion

The use of exogenous substances to enhance plant stress resistance has attracted increasing attention in plant resistance research. Previous studies have reported that exogenous application of methyl jasmonate (MeJA) (Dong et al., 2007), salicylic acid (SA) (Xia et al., 2014), nitric oxide (NO) donor sodium nitroprusside (SNP) (Mao and Wei, 2010), 5-aminolevulinic acid (5-ALA) (Zhang et al., 2010), and glycine betaine (GB) can improve plant stress resistance. These substances primarily alleviate osmotic stress by reducing membrane lipid peroxidation, promoting proline and soluble sugar accumulation, and enhancing the activities of protective enzymes such as SOD and POD (Mao and Wei, 2010; Xia et al., 2014). Linolenic acid is an important component of plant membrane lipids, but whether exogenous application can enhance plant stress resistance has not been reported.

This study used 14% and 16% PEG6000 to simulate drought and confirmed that exogenous ALA application can alleviate drought stress during rice seed germination. The optimal ALA concentrations were 25 mol•L-1 and 250 mol•L-1, but concentrations that were too high or too low did not produce obvious side effects on plant growth and development, suggesting that ALA is unlikely to function as a signaling molecule. Moreover, the alleviating effect of ALA on drought stress was mainly manifested in the relief of early seedling growth inhibition rather than improvement of seed germination rate (Table 3). The effects on early seedling growth were primarily observed in shoot length, shoot weight, and root weight, with no significant effect on root length. It appears that the extension of root length under drought conditions may be an innate response to enhance water absorption.

Rice is a starchy seed type, with starch being the main storage substance in the endosperm. During germination, amylases hydrolyze storage materials into simple organic compounds required for seed germination and seedling growth. Multiple amylases exist in rice seeds, with -amylase being the primary functional enzyme during early germination. Therefore, -amylase activity is critical for seed germination and seedling growth rates and serves as an important indicator of seed vigor. In this study, ALA application significantly increased -amylase activity under various drought conditions (14% and 16% PEG) by 36.8%-70.7% (Figure 2). Moreover, -amylase activity generally decreased as drought severity increased (Figure 2). Thus, -amylase activity in seeds is negatively correlated with the intensity of drought stress encountered. We therefore propose that -amylase activity can reflect the germination capacity of rice seeds under drought conditions, and that exogenous ALA alleviates drought stress by enhancing -amylase activity during seed germination.

The root system is an active absorption and metabolic organ in plants. Root vigor serves as an important indicator of water and nutrient absorption capacity and stress survival ability. The stronger the root metabolism, the greater the root vigor. Therefore, root vigor largely reflects drought tolerance. In this study, ALA application increased root metabolic vigor to some extent under various drought conditions (14% and 16% PEG) by 5.4%-28.4%. However, under high drought stress (16% PEG), the improvement in root vigor by ALA was very limited (5.4%-11.4%) (Figure 3). Moreover, root metabolic vigor generally increased with drought severity (Figure 3). We therefore hypothesize that enhanced root vigor under drought conditions may represent an adaptive response of plants to drought stress, and that the role of ALA in improving plant drought resistance through increased root vigor is very limited.

In summary, this study demonstrates from morphological and physiological perspectives that exogenous ALA alleviates drought stress primarily by enhancing -amylase activity in germinating rice seeds and root vigor in seedlings. Since -linolenic acid is a component of membrane lipids, future research should focus on its effects on membrane lipid composition and peroxidation levels to further elucidate the mechanisms underlying drought stress alleviation.

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