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Exogenous Trimethylamine *N*-Oxide (TMAO) Improves Apple Rootstock Drought Tolerance Through Physiological Modulation

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Abstract

Drought stress represents a major constraint on global apple production, with the widely used semi-dwarfing rootstock 'M.26' being particularly vulnerable to water deficit. Although the osmolyte trimethylamine N-oxide (TMAO) has been shown to improve abiotic stress tolerance in the model plant Arabidopsis, its potential role in enhancing drought resilience in woody fruit trees remains largely unexplored. Under prolonged moderate drought stress, exogenous TMAO application significantly promoted plant growth, mitigating the drought-induced suppression of plant height by 5.3–12.2% compared to untreated drought-stressed controls and alleviating the decline in above-ground biomass. This improvement was underpinned by a substantial alleviation of root growth inhibition, with TMAO restoring total root length and biomass from 37% in the control to only 6.1–9.5%. TMAO also fine-tuned the root-to-shoot ratio to favor resource allocation to roots. Consequently, TMAO-treated plants maintained superior leaf water status, exhibiting higher relative water content (drought-induced reduction limited to ~17.5% with TMAO versus 26.3% in the control). Physiologically, TMAO alleviated the drought-induced stomatal limitation of photosynthesis, sustaining higher net photosynthetic rate, stomatal conductance, and transpiration rate. Crucially, under severe drought stress, TMAO pretreatment markedly enhanced 'M.26' survival rates from approximately 39% in the untreated control to 60–68%, representing a relative increase of approximately 74%. Collectively, this study demonstrates that exogenous application TMAO significantly enhances drought tolerance in apple rootstock 'M.26', highlighting its potential as an effective and environmentally safe plant growth regulator for more sustainable cultivation of fruit trees under irregular/erratic irrigation conditions.

Keywords: apple rootstock; M.26; TMAO; drought tolerance; fruit development

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1. Introduction

Apple is one of the most popular temperate fruits widely grown in the world, valued for its high nutritional content, diverse flavor, and capability of generating considerable economic benefit. In China, the Loess Plateau stands as the largest apple-producing region, accounting for more than 25% of the global output [1–3]. Thanks to its suitable altitude and favorable sunlight conditions, apples from this region typically exhibit superior quality.

However, in the context of global climate change, water deficit events are occurring with increasing frequency, imposing a significant constraint to the development of the apple industry [4]. Therefore, enhancing drought stress tolerance in apple trees has become a crucial issue for sustainable production. As apple trees are commonly propagated through grafting, enhancing the drought tolerance of the rootstock is one of the crucial strategies to improve the drought tolerance of apple trees [5,6]. Improving the drought resistance of apple rootstocks is thus essential for ensuring stable and productive apple cultivation.

Plants respond to drought through a combination of physiological, biochemical, and metabolic adaptations [7–10]. One of the earliest responses to water deficit is stomatal closure, which reduces photosynthetic process and water loss through transpiration. The capacity to maintain leaf water content under drought conditions serves as a critical indicator of drought tolerance in crops, as it reflects the water saturation state of plant tissues [11,12]. When soil moisture declines and root water uptake is impaired, a decrease in leaf water content is among the first detectable physiological responses [8,13], with the magnitude of reduction positively correlated with stress intensity [14,15]. Additionally, stomatal closure under drought stress leads to a decline in both photosynthetic and transpiration rates [16,17]. Therefore, the ability to sustain high photosynthetic productivity while improving intrinsic water use efficiency under water-limited conditions is a key measure of a plant's overall drought resilience [10,13,18]. Moreover, plants frequently optimize their root system architecture by developing deeper and more extensive roots to enhance water uptake under drought; however, this reallocation of resources often comes at the expense of reduced shoot growth, particularly during the early stages of stress [19].

In response to drought stress, plants accumulate various endogenous osmolytes—such as sorbitol, proline, betaine, and sucrose—to increase cellular osmotic pressure and maintain water balance [9]. In parallel, stress-responsive phytohormones including abscisic acid (ABA), ethylene, jasmonic acid (JA), and salicylic acid (SA) are also induced to orchestrate the overall drought defense response [9,20]. Recent studies have shown that exogenous soil application of certain compounds before drought exposure—even in ungrafted apple rootstock plants—can effectively modulate plant drought responses. For instance, γ-aminobutyric acid (GABA) application in the soil enhances drought tolerance in apple rootstock 'M9-T337' by increasing net photosynthetic rate, relative leaf water content, root-to-shoot ratio, and water use efficiency of 'M9-T337' [21]. Similarly, the low-content sugar substitutes aspartame, sucralose, and erythritol improve tolerance to cold, drought, and salt stress in apple trees by promoting proline accumulation [22]. Other exogenous compounds such as dopamine, betaine, acetic acid, and SA have also been reported to mitigate drought-induced damage in apple rootstocks [23,24].

Trimethylamine N-oxide (TMAO), a well-known protein-stabilizing osmolyte, has been extensively studied in mammals for its role in cardiovascular and kidney diseases [25]. TMAO represents an oxidized state of trimethylamine (TMA), and TMA typically originates from dietary compounds ingested through the diet, such as those found in saltwater fish, eggs, liver, milk, and meat [26,27]. Subsequent to absorption, TMAO is transported to tissues for accumulation as an osmolyte; alternatively, it is more commonly excreted by the kidneys. In the context of human milk consumption, the plasma TMAO concentration rises to 2.08 μM, and this elevation does not exhibit any correlation with diseases [28,29]. In plants, the key gene involved in TMAO synthesis, $FMO_{GS\text{-}OX5}$, has been identified in Arabidopsis. Both overexpression of $FMO_{GS\text{-}OX5}$ and exogenous TMAO application enhance tolerance to abiotic stresses such as cold, salt, and osmotic stress in Arabidopsis [30]. However, whether this emerging osmolyte also functions in drought stress adaptation in woody plants, particularly in apple trees, remains unexplored.

'M.26' is a semi-dwarfing apple rootstock, which depended on scion variety, production system, and soil type and originally developed by the East Malling Research Station in the United Kingdom in 1929 [6,31]. As one of the adopted rootstocks, 'M.26' is valued for its good resistance to Phytophthora rot and early fruiting habit. However, its susceptibility to drought stress has generally restricted its use to regions with adequate water availability. In this study, we aimed to comprehensively reveal the dual role of TMAO in enhancing drought resistance of 'M.26', thereby providing both a theoretical basis and practical reference for the development of novel plant stress-resistance regulators and sustainable orchard management strategies.

2. Material and Methods

2.1. Plant Materials and Growth Conditions

The apple semi-dwarfing rootstocks 'M.26' were procured from Northwest Agriculture and Forestry University, located in Yangling, Shaanxi Province, China. Tissue-cultured 'M.26' plantlets were first rooted in glass culture vessels under sterile conditions and subsequently transplanted into soil in a controlled growth chamber set at 22 °C with a 16 h light/8 h dark photoperiod and 60% relative humidity. Following a one-month acclimatization period, the plants were transplanted to a greenhouse and positioned in plastic pots (15 cm × 20 cm, approximately 1.3 L) filled with a blend of garden soil and substrate (PINDSTRUP, Syddjurs, Denmark) (at a volume ratio of 1:1 in early June 2024. During the greenhouse cultivation period, ambient conditions were maintained at: daytime temperature of 25–30 °C, nighttime temperature of 18–22 °C.

2.2. TMAO and Drought Stress Treatments

Trimethylamine N-oxide dihydrate (TMAO, CAS No. 62637-93-8) was obtained from Sigma-Aldrich, Shanghai, China. The formal experiment commenced in mid-July 2024. Starting 15 July, one-month-old soil-adapted 'M.26' plants were irrigated weekly with 300 mL of aqueous solution containing 0 (control), 0.5, 1.0, or 2.5 mM TMAO via root drenching. This pretreatment phase lasted for one month, concluding on 15 August. Throughout the pretreatment, all plants were irrigated normally to maintain sufficient soil moisture.

Upon completion of the one-month pretreatment period, both short-term and long-term drought stress treatments were initiated on 16 August (specific experimental designs are detailed in Tables 1 and 2). For the long-term drought treatment group, soil volumetric water content (VWC) was monitored daily and supplemented accordingly to precisely maintain the target stress levels (moderate drought: 18–23% VWC; well-watered control: 43–48% VWC) over a two-month period, ending on 15 October.

Table 1. 'M26' apple plants used for short-term drought treatments.

Treatments	1	2	3	4
TMAO concentration (mM)	0	0.5	1.0	2.5
Numbers of 'M26' plants	30	30	30	30

Table 2. 'M26' apple plants used for long-term drought treatments.

Treatments	Well-Watered Control				Moderate Drought Stress			
Pre-treated TMAO concentration (mM)	0	0.5	1.0	2.5	0	0.5	1.0	2.5
Numbers of 'M26' plants	10	10	10	10	10	10	10	10

In the short-term drought treatment, 120 one-month-old 'M.26' apple plants were transferred in a greenhouse. Specifically, every 30 'M.26' rootstocks were pre-treated with 0, 0.5, 1.0, or 2.5 mM TMAO, and each group of 10 plants served as an independent biological replicate. Three biological replicates were established. All plants were completely deprived of irrigation to induce drought stress on 16 August (Table 1). After ten days without watering (26 August), the phenotypic status of the plants was documented photographically. The plants were then rewatered, and survival rates were assessed along with additional photographic records taken one week after rewatering. During the drought stress treatment, the pots were randomly repositioned on a daily basis to mitigate the potential influence of positional effects.

For the long-term drought treatment, soil volumetric water content (VWC) was monitored daily using a time-domain reflectometer (TDR; Model FS6430, Spectrum Technologies, Aurora, USA). The plants were partitioned into two groups (Well-watered control and moderate drought stress). With each plant treated as an independent biological replicate, ten plants from each group were maintained under well-watered control conditions, and the remaining ten were exposed to moderate drought stress (Table 2). Well-watered control plants were maintained at a VWC ranging from 43% to 48%. Under long-term drought conditions, irrigation was regulated daily to sustain VWC within the range of 18% to 23% (moderate drought stress). All treatment regimens were consistently applied over a continuous period of two months (16 August to 15 October). The pots were repositioned randomly on a weekly basis to alleviate the potential impact of positional effects.

2.3. Measurement of Photosynthetic Parameters

Photosynthetic parameters were measured using the LI-COR 6400 portable photosynthetic system (LI-COR 6400, Huntington Beach, CA, USA). The leaves positioned at the middle part of the shoots were selected for detection purposes. For each treatment, a total of 10 plants were utilized. The measurements included net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate. Determination of leaf photosynthesis was performed as described previously [21]. Measurement conditions were standardized at a photosynthetic photon flux density of 1000 μmol $m^{-2}s^{-1}$, an airflow rate of 500 μmol s^{-1}, a leaf temperature of 25 ± 2 °C, and a chamber relative humidity of 22 ± 2%.

2.4. Measurement of Chlorophyll Fluorescence

Chlorophyll fluorescence was measured using a pulse-modulated fluorometer (Heinz Walz, Effeltrich, Germany) to determine the maximum photochemical efficiency of PSII (Fv/Fm). Leaves located in the middle section of the shoots were chosen for detection. For each treatment, a total of 10 plants were employed at the same time of day.

2.5. Determination of Leaf Relative Water Content (RWC)

The relative water content (RWC) of leaves was determined on 15 October 2024, according to a previously described method [21]. Fresh leaf weight (FW) was first recorded using an analytical balance with 0.1 mg precision. One fully expanded leaf was collected from each of ten independent plants per treatment (n = 10). These leaves were immersed in distilled water for 12 h at 25 °C in the dark to reach full turgidity, after which the turgid weight (TW) was measured. Finally, the samples were oven-dried (70 °C) to a constant weight to obtain the dry weight (DW). RWC was calculated as:

$$\mathrm{RWC}(\%) = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100 \quad (1)$$

2.6. Root Morphology Analysis

The roots were rinsed with water to remove the soil surrounding the rhizosphere. Subsequently, photographs were taken to document the phenotypes. The length of the longest root was measured using a ruler, and the weight was determined with an electronic balance. Thereafter, the roots were transferred to an oven maintained at 70 °C under dark conditions for 96 h to obtain a constant weight (dry root weight). A total of ten plants were employed for the measurement of root length and weight.

2.7. Statistical Analysis

All quantitative results are expressed as means ± SD. The data were analyzed via two-way or one-way analysis of variance (ANOVA) in SPSS 21.0 (IBM, Armonk, USA) followed by Tukey's Honest Significant Difference Test (Tukey HSD test). For the same index, differences with distinct letters indicate statistically significant disparities ($p < 0.05$) in levels. Graphical representations were prepared using GraphPad Prism 10 (Dotmatics, San Diego, CA, USA).

3. Results

3.1. TMAO Enhances Biomass Accumulation of 'M.26' Under Prolonged Drought Stress

Based on previous studies, exogenous TMAO was administered to 'M.26' apple plants to explore its involvement in mitigating drought stress. Following the two-month treatment period under prolonged drought stress, all concentrations of TMAO promoted the growth of 'M.26' plants compared to the untreated control plants (Figure 1A). The most pronounced morphological effect was observed in plant height. While TMAO application did not lead to significant differences under well-watered conditions, exogenous TMAO substantially enhanced the height of 'M.26' under moderate drought stress (Figure 1B). Based on the statistical data, a two-month period of moderate drought significantly suppressed the height of 'M.26', reducing it from 81.5 cm to 62.4 cm. Conversely, the application of TMAO remarkably mitigated this inhibitory effect. Under drought stress conditions, the height of 'M.26' ranged from 68.6 cm to 69.4 cm. TMAO application partially mitigated the growth suppression caused by water deficit, resulting in a 5.3–12.2% increase in plant height compared to drought-stressed plants without TMAO treatment. In contrast, TMAO treatment had no significant effect on the stem diameter of 'M.26' plants (Figure 1C). Furthermore, prolonged moderate drought stress was found to inhibit 'M.26' growth, whereas TMAO treatments at different concentrations partially alleviated this inhibition. Specifically, TMAO treatments significantly improved the above-ground biomass (dry shoot weight) of 'M.26' under drought stress conditions (Figure 1D). In summary, long-term drought suppressed 'M.26' growth, but exogenous TMAO mitigated this, promoting growth and biomass. Optimal TMAO concentration is undetermined, yet 0.5 mM TMAO may enhance 'M26' drought tolerance.

3.2. TMAO Alleviates Drought-Induced Suppression of Root Development and Modulates Biomass Allocation in 'M.26' Rootstock

Given that the drought tolerance of apple trees is largely determined by the rootstock, we next investigated the specific effects of TMAO on the root system architecture of 'M.26' under drought conditions. Extended (two-month) moderate drought stress exerted a significant inhibitory effect on root growth. In comparison to the well-watered control group, the length of the longest root decreased from 20.20 cm to 15.45 cm. However, exogenous application of TMAO significantly alleviated this inhibition. Notably, treatment with 1.0 mM TMAO almost completely restored the root developmental trajectory, counteracting the negative effects of drought stress (Figure 2A,B). Also, drought stress markedly suppressed

root biomass production, resulting in a 37% reduction in dry root weight. All TMAO concentrations (0.5, 1.0, and 2.5 mM) effectively mitigated this suppression, reducing the inhibition ratio to a range of 6.1% to 9.5% (Figure 2C). Considering the concurrent inhibition of above-ground biomass (shoot growth) by drought, we analyzed the root-to-shoot ratio to understand biomass partitioning. As shown in Figure 2D, under drought stress, 'M.26' plants exhibited a shift in resource allocation toward root growth rather than shoot growth. This response was further enhanced by exogenous TMAO application, which resulted in a significantly higher root-to-shoot ratio in treated plants compared to the untreated control under moderate drought conditions. Collectively, these results demonstrate that exogenous TMAO application enhances the tolerance of 'M.26' rootstock to long-term drought stress. This is achieved by preserving root growth and biomass, fine-tuning the root-to-shoot ratio, and thereby potentially improving water and nutrient acquisition during soil water deficit.

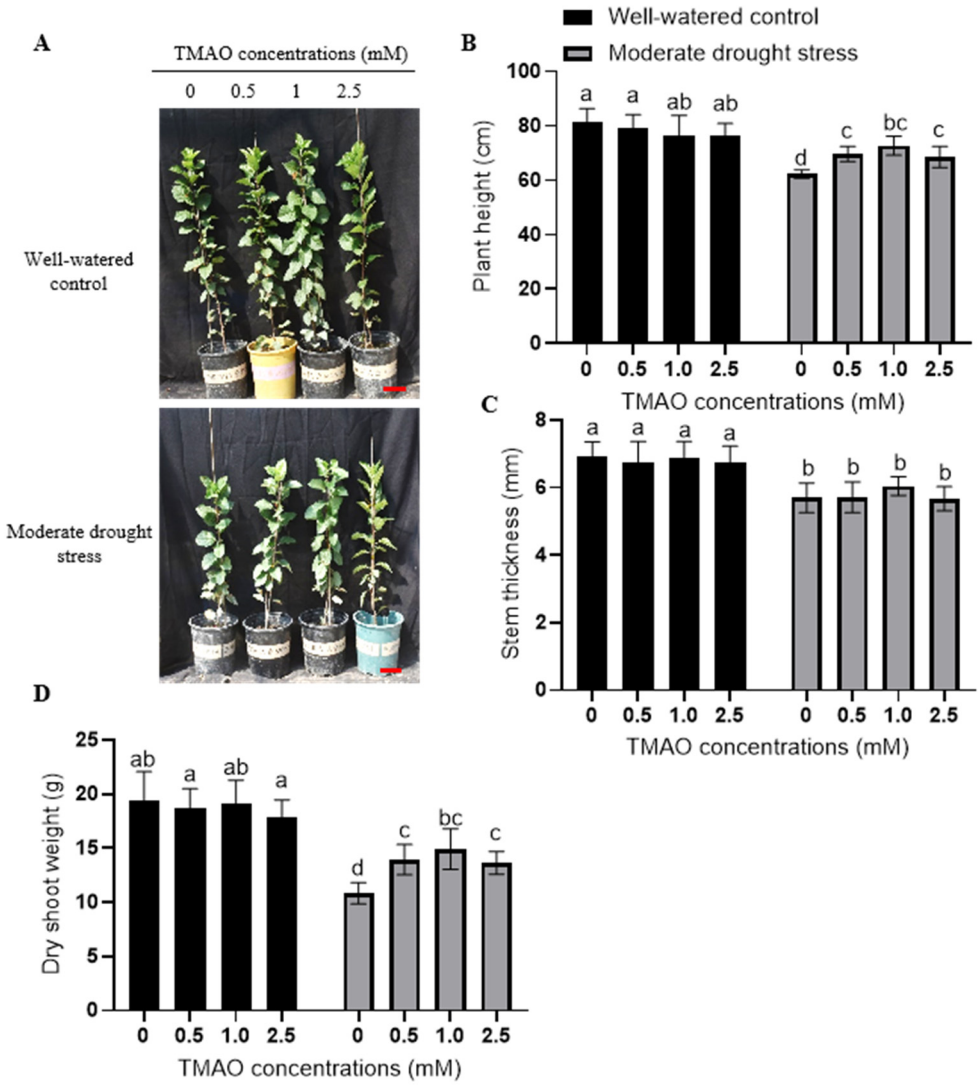

Figure 1. Growth potential of the apple rootstock 'M.26' following the application of different doses of exogenous TMAO under variable long-term moderate drought stress conditions. (**A**) Representative morphological phenotypes; (**B**) Plant height; (**C**) Stem diameter; (**D**) Dry shoot weight. In (**B**–**D**), error bars represent the standard deviation (SD) for $n = 10$. Different letters denote statistically significant differences ($p < 0.05$) in levels for the same index, as determined by two-way ANOVA followed by Tukey HSD Test. Scale bar: 5 cm (**A**).

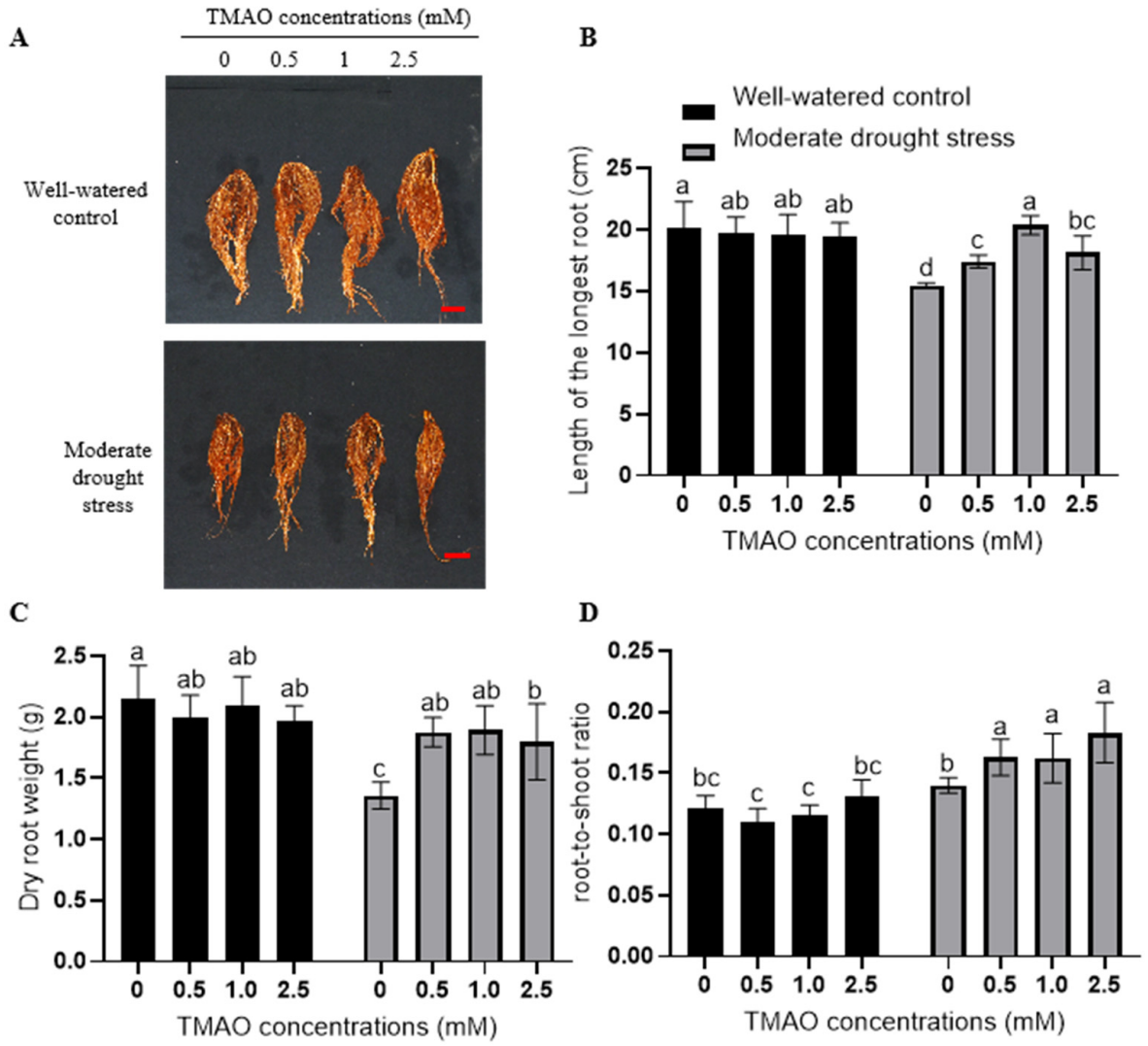

Figure 2. The root development status of apple rootstock 'M.26' following exogenous application of different doses of TMAO under variable drought conditions. (**A**) Representative images of root architecture; (**B**) Root length; (**C**) Dry root weight; (**D**) the root-to-shoot ratio. Error bars represent the standard deviation (SD) for $n = 10$. Different letters denote statistically significant differences ($p < 0.05$) in levels for the same index, as determined by two-way ANOVA followed by Tukey HSD Test. Scale bar: 5 cm (**A**).

3.3. TMAO Enhances Leaf Water Status of 'M.26' Under Long-Term Drought Stress

To further evaluate the role of exogenous TMAO in conferring long-term drought resistance in 'M.26' rootstock, we assessed the leaf physiological status under both well-watered and drought-stress conditions. Our findings demonstrate that prolonged moderate drought stress significantly reduced leaf fresh weight, dry weight, and relative water content (RWC) in 'M.26'. Although the application of TMAO did not yield any significant difference under the well-watered control condition, it exerted a significant alleviating effect of 'M.26' under the moderate drought stress condition. The drought treatment resulted in a 21.4% reduction in fresh leaf weight of 'M.26', whereas the TMAO treatment only led to a 2.2–8.9% decrease (Figure 3A). Notably, application of TMAO at various concentrations effectively mitigated the drought-induced decline in leaf RWC (Figure 3B). Specifically, drought stress caused a 26.3% reduction in leaf RWC, whereas TMAO treatments (0.5, 1.0, and 2.5 mM) alleviated this decrease, limiting the reduction to about 17.5% (Figure 3B). No significant differences in RWC were observed among the three TMAO concentrations

tested. Collectively, these results indicate that exogenous TMAO promotes the maintenance of adequate leaf hydration in 'M.26'.

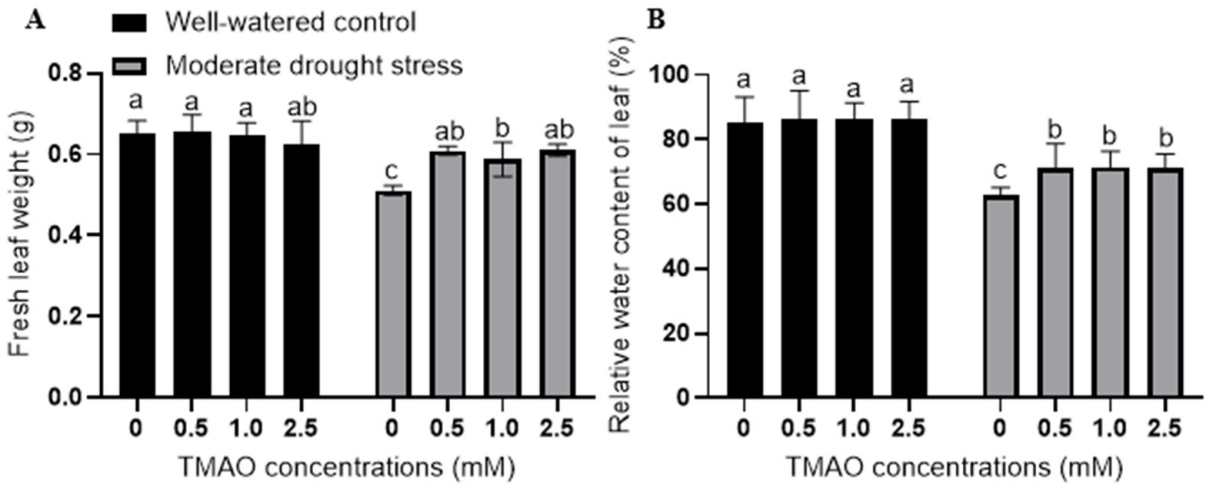

Figure 3. The leaf growth status of 'M.26' after exogenous application of different doses of TMAO under variable drought stress conditions. (**A**) Fresh leaf weight; (**B**) Relative water content of leaf. Error bars represent the standard deviation (SD) for $n = 10$. Different letters denote statistically significant differences ($p < 0.05$) in levels for the same index, as determined by two-way ANOVA followed by Tukey HSD Test.

3.4. Exogenous TMAO Improves the Photosynthetic Efficiency of 'M.26' Plants Under Long-Term Drought Stress Conditions

Given our previous observations that TMAO improved biomass and leaf water status, we investigated its specific effects on the photosynthetic apparatus of 'M.26' plants. We measured key photosynthetic parameters in TMAO-irrigated plants under both well-watered and prolonged drought-stress conditions. Under well-watered conditions, exogenous TMAO application did not significantly alter photosynthetic parameters, including the net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr). Following prolonged drought stress, untreated control plants exhibited a characteristic suppression of photosynthesis, with significant decreases in Pn, Gs, and Tr. Notably, the Ci remained largely unchanged. This pattern suggests that the initial limitation of photosynthesis was likely stomatal closure, a common response to conserve water under drought conditions that reduces carbon availability. Crucially, treatment with different concentrations of TMAO alleviated this drought-induced inhibition. TMAO-treated plants maintained significantly higher Pn, Gs, and Tr compared to the untreated controls under drought stress, with the 0.5 mM concentration demonstrating the most pronounced effect (Figure 4A–D). However, analysis of chlorophyll fluorescence parameters revealed that exogenous TMAO had no significant impact on the photochemical efficiency of photosystem II (PSII) in either control or drought-stressed leaves (Figure 4E). This indicates that the primary site of TMAO's action is not within the light-dependent reactions of the photosynthetic electron transport chain.

It is important to note that exogenous TMAO enhances photosynthetic performance in 'M.26' under drought stress primarily by mitigating stomatal limitation, as evidenced by the higher Gs and Tr, which facilitates CO_2 diffusion and sustains the net photosynthetic rate.

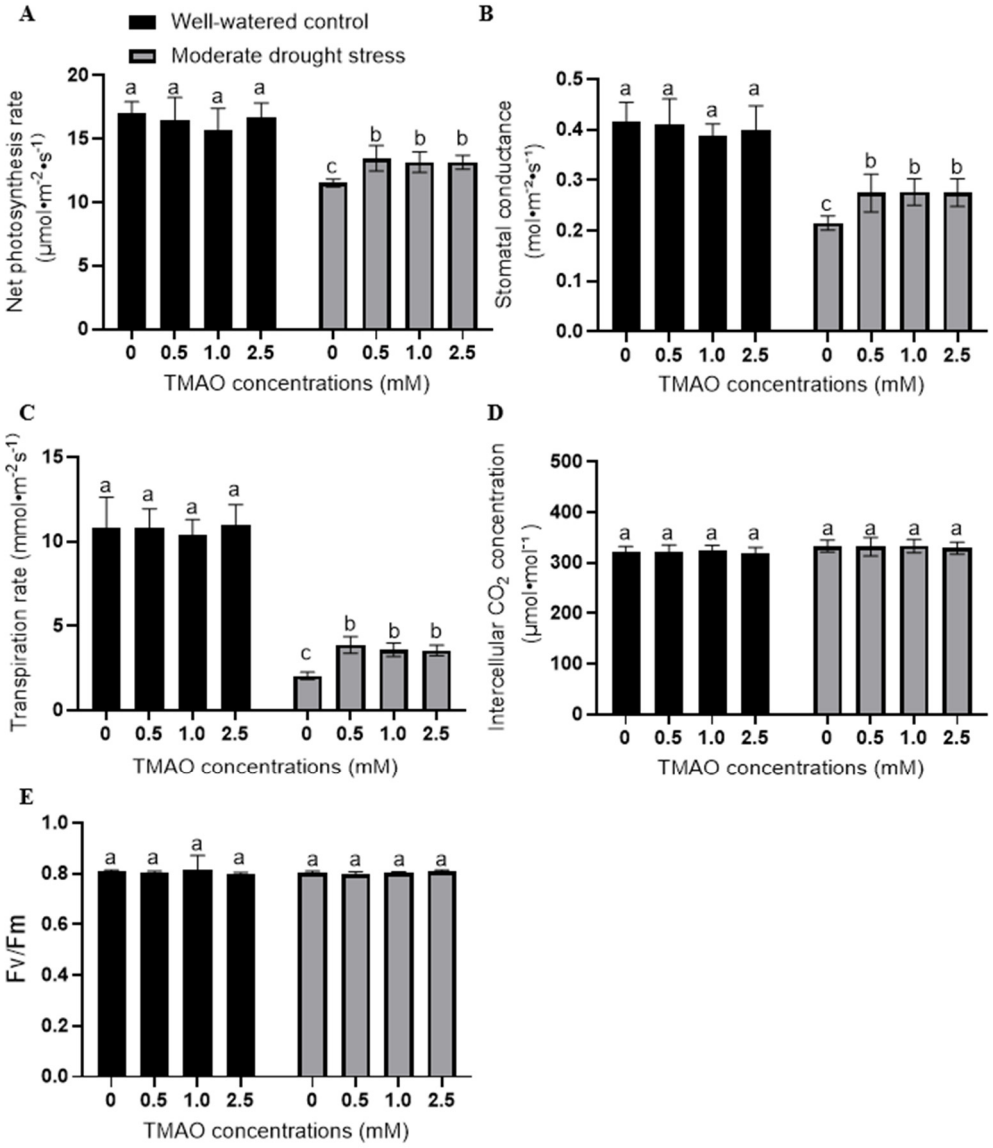

Figure 4. The photosynthetic characteristics of rootstock 'M.26' following exogenous application of different doses of TMAO under variable drought stress conditions. (**A**) Net photosynthesis rate; (**B**) Stomatal conductance; (**C**) Transpiration rate; (**D**) Intercellular CO_2 concentration; (**E**) maximum quantum yield of PSII (Fv/Fm). Error bars represent the standard deviation (SD) for $n = 10$. Different letters denote statistically significant differences ($p < 0.05$) in levels for the same index, as determined by two-way ANOVA followed by Tukey HSD Test.

3.5. Exogenous TMAO Improves the Survival Rate of 'M.26' Under Short-Term Drought Stress Conditions

While our previous experiments demonstrated that TMAO alleviates growth inhibition under prolonged moderate drought, we further investigated its role in severe drought scenarios. One-month-old 'M.26' plants were pretreated with weekly applications of TMAO (0, 0.5, 1.0, or 2.5 mM). Following this one-month preconditioning period, a short-term natural drought stress was initiated by withholding all irrigation. Prior to the drought induction, TMAO pretreatment alone had no significant effect on plant growth. Following a one-week period of water deprivation, nonetheless, plants pre-treated with TMAO demonstrated superior growth performance when compared to the untreated control group. The leaves tended to maintain a healthy state to endure drought stress (Figure 5A). Irrigation was resumed after 10 days of severe drought stress, and plant survival rates were subsequently assessed. The short-term drought resulted in approximately 39% survival

rate in untreated 'M.26' plants. In contrast, pretreatment with 0.5 mM TMAO significantly increased the survival rate to about 60%, and 1.0 and 2.5 mM TMAO provided the greatest protection, further improving survival to 68% (Figure 5B).

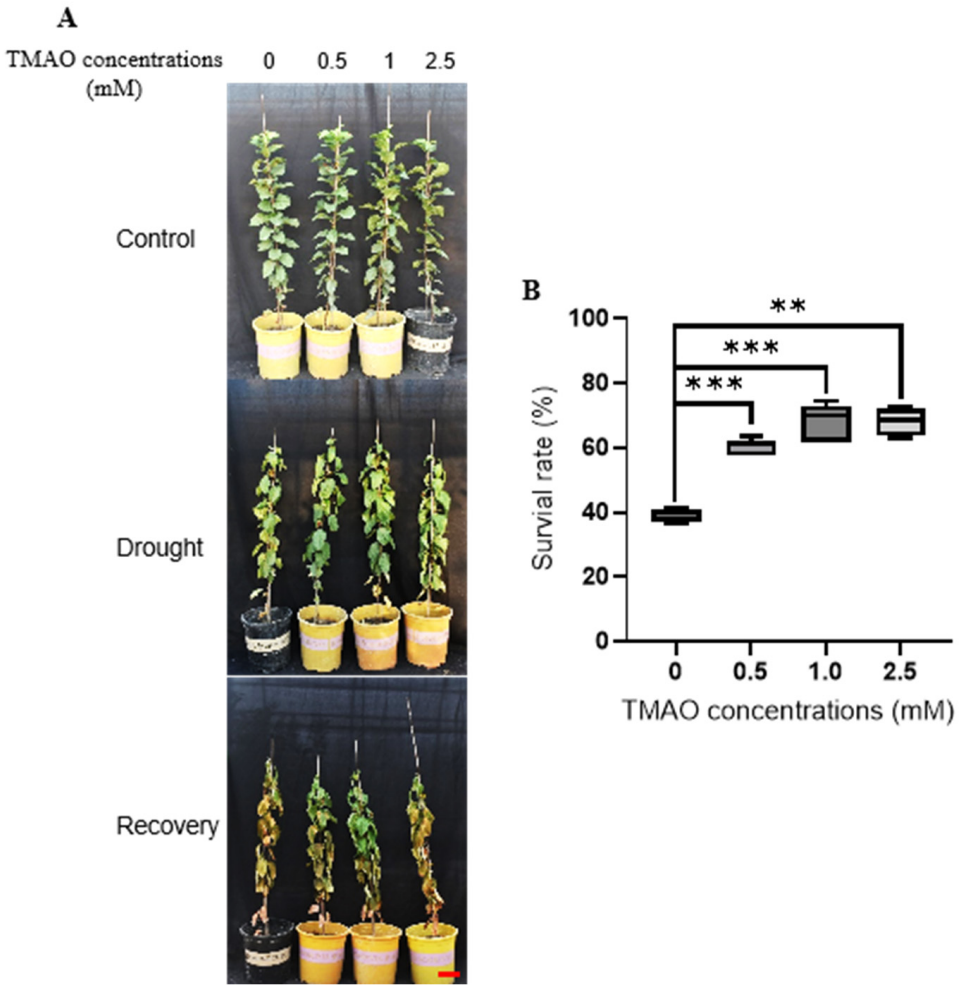

Figure 5. The morphology and survival rate of 'M.26' following exogenous application of TMAO under drought stress treatment. (**A**) Morphological phenotypes; (**B**) Survival rate. Asterisks indicate significant differences between TMAO-treated and untreated plants (** $p < 0.01$, *** $p < 0.001$; one-way ANOVA followed by Tukey HSD Test). Error bars represent standard deviation (SD). Scale bar: 5 cm (**A**).

3.6. Exogenous TMAO Improves the Photosynthesis Capacity of 'M.26' Under Short-Term Drought Stress Condition

Under well-watered conditions, exogenous TMAO application had no significant effect on the photosynthetic traits of 'M.26', indicating its non-perturbing nature in the absence of stress. However, short-term drought stress triggered a significant decline in key photosynthetic parameters, including the net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci). Notably, exogenous application of TMAO effectively alleviated this drought-induced photosynthetic inhibition at all tested concentrations, with the exception of the transpiration rate (Tr), which was not significantly improved (Figure 6D). These findings demonstrate that TMAO enhances the photosynthetic capacity of 'M.26' apple rootstock under short-term drought stress conditions.

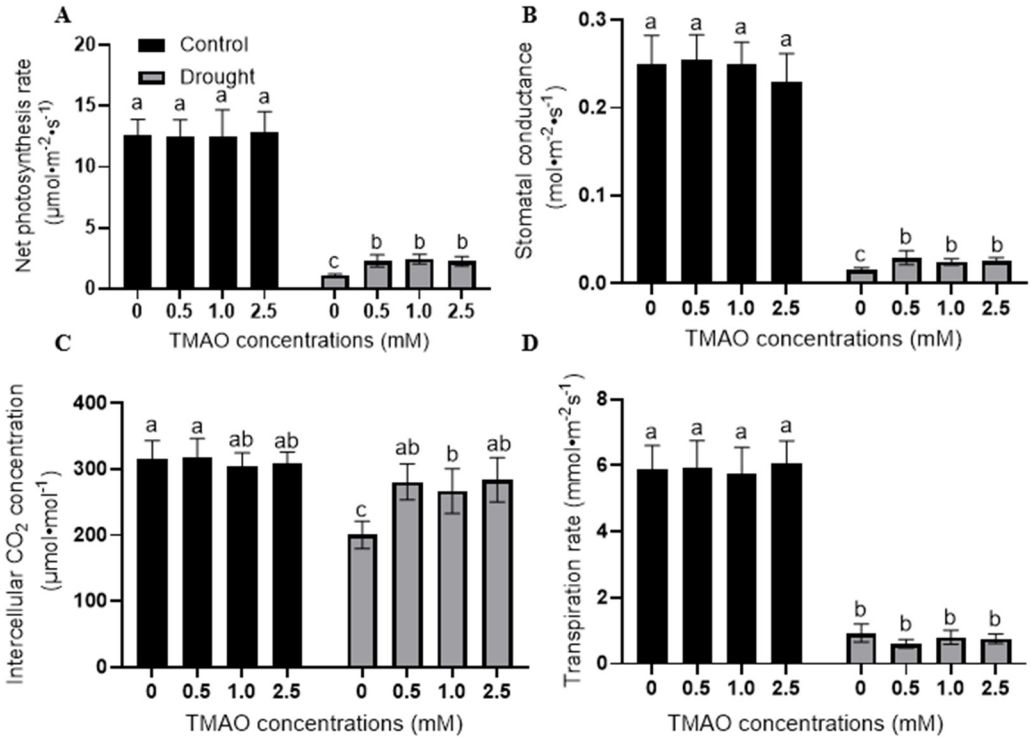

Figure 6. Effects of TMAO on photosynthetic parameters of 'M.26' under short-term drought conditions. (**A**) Net photosynthesis rate; (**B**) Stomatal conductance; (**C**) Intercellular CO_2 concentration; (**D**) Transpiration rate. Error bars represent the standard deviation (SD) for $n = 3$. Different letters denote statistically significant differences ($p < 0.05$) in levels for the same index, as determined by two-way ANOVA followed by Tukey HSD Test.

4. Discussion

Our study demonstrates that exogenous application of TMAO, an osmolyte traditionally studied in animal systems, effectively enhances drought tolerance in the apple rootstock 'M.26'. Previous research has found that TMAO is generated by N-oxygenation of trimethylamine by flavin-containing monooxygenases (FMOs) in animals [32]. Then, Catalá et al. identified the key gene involved in plants TMAO synthesis, *RCI5*/$FMO_{GS\text{-}OX5}$, which is induced by cold stress. Overexpression of $FMO_{GS\text{-}OX5}$ significantly increased the freezing, salt, and osmotic stress tolerance by mediating the TMAO accumulation [30]. Additionally, exogenous application of TMAO also alleviates Arabidopsis abiotic stress tolerance. Here, we found TMAO significantly improves the water stress tolerance in 'M26' apple rootstocks. This finding broadens the known scope of TMAO's functions.

The cornerstone of TMAO-induced drought resilience lies in its fundamental role as a compatible solute. We propose that TMAO facilitates osmotic adjustment—a crucial physiological process for maintaining cellular turgor under water deficit. By lowering the intracellular osmotic potential without disrupting cellular metabolism, TMAO creates a driving force for water influx, thereby stabilizing cell volume and membrane integrity. This mechanism directly explains the significantly enhanced root development observed in our study. Under the osmotic stress imposed by drought, the internal osmotic support provided by TMAO likely sustained root cell elongation and division, which manifested as the dramatic alleviation of drought's inhibitory effects on root length and root biomass [19]. A more extensive and functional root system is the primary architectural adaptation for enhanced soil exploration and water acquisition [33].

Beyond its canonical osmotic function, we posit that TMAO's profound impact on plant survival under severe drought stress is attributable to its renowned capacity as a chemical

chaperone. Severe dehydration leads to the loss of hydration shells around proteins, causing their denaturation, aggregation, and loss of function, which is often a primary cause of irreversible cellular damage and mortality [10]. TMAO is known to preferentially exclude itself from the surface of proteins, thereby creating a thermodynamic force that favors the properly folded, native state of proteins over the unfolded state [25,29,32]. This protein-stabilizing effect, extensively documented in animal and in vitro systems, likely constitutes a core mechanism by which TMAO pretreatment protected cellular structures—including key enzymes of the metabolic and respiratory pathways, as well as membrane proteins—during the severe desiccation of severe drought. This allowed for a successful metabolic reboot upon rehydration, culminating in the significantly improved survival rates we documented. This represents a pivotal, non-osmotic dimension of TMAO's action that is critical for extreme stress survival.

Our findings that exogenous TMAO enhances drought tolerance in'M.26' align with a growing body of literature demonstrating the efficacy of various exogenous compounds in mitigating water deficit stress in woody plants. For example, soil-applied γ-aminobutyric acid (GABA) was shown to improve drought resilience in apple rootstock 'M9-T337' by enhancing net photosynthetic rate, relative water content, and water use efficiency [21]. Similarly, low-calorie sweeteners such as erythritol and sucralose conferred cross-tolerance to drought, cold, and salinity in apple rootstocks, primarily through proline accumulation [22]. Other well-documented agents—including abscisic acid (ABA), salicylic acid (SA), betaine, and dopamine—have also been reported to alleviate oxidative damage and stabilize cellular homeostasis under drought conditions in apple rootstocks [24]. Notably, while many of these compounds primarily act through antioxidant defense or stomatal regulation, TMAO appears to exert a more pronounced effect on root system recovery and resource reallocation, as evidenced by the near-complete restoration of total root length and biomass under moderate drought—a feature less emphasized in prior studies. Moreover, the 68% survival rate achieved under severe drought following TMAO pretreatment represents one of the highest protective efficacies reported to date for a single exogenous application in apple rootstocks. Although direct comparisons are limited by differences in experimental conditions and genotypes, our results suggest that TMAO may offer a complementary or even superior strategy, particularly in scenarios where root integrity and post-stress recovery are critical.

The improved root system architecture and cellular hydration, underpinned by both osmotic and proteostatic mechanisms, created a robust foundation for sustaining aboveground physiological functions [19,33]. The maintenance of higher leaf relative water content (RWC) in TMAO-treated plants is a direct consequence of this enhanced foundational resilience. We further dissected the physiological implications of this superior water status and found that it was directly correlated with the mitigation of stomatal limitation to photosynthesis. Our data strongly suggest that TMAO sustains carbon assimilation primarily by facilitating stomatal opening and CO_2 diffusion under drought conditions. By maintaining a favorable plant water status, TMAO likely reduced the synthesis or signaling of the drought hormone abscisic acid (ABA) in the leaf, or modulated the sensitivity of guard cells to it, thereby permitting wider stomatal apertures. This ensured a steadier supply of CO_2 to the chloroplasts, a mechanism shared by other osmolytes like GABA [21]. This prioritization of hydraulic and gas exchange efficiency indicates that TMAO's primary role under moderate, chronic drought is to optimize physiological performance rather than merely providing passive cellular protection.

Furthermore, the observed shift in resource allocation towards the roots, as evidenced by the increased root-to-shoot ratio, signifies a systemic re-programming of plant development under TMAO influence. This is not merely a passive response to drought but

an active, TMAO-enhanced adaptive strategy. It is plausible that TMAO, either directly or indirectly through the improvement of plant water status, modulates the allocation of photoassimilates. A greater proportion of fixed carbon may be preferentially partitioned to the roots to support their extensive growth, a strategy that maximizes the return on investment (i.e., water uptake) under limiting conditions [19].

While our study focused on ungrafted 'M.26' rootstock plants under controlled conditions, the practical application of TMAO in orchard systems—where trees are typically grafted onto selected rootstocks—warrants consideration. Several feasible delivery methods could be explored for field use. For instance, soil drenching or drip irrigation during nursery production or early establishment stages could ensure efficient root uptake. Alternatively, foliar spray might offer a rapid and cost-effective approach, particularly during acute drought episodes, provided that TMAO can be effectively absorbed through the leaf cuticle (as demonstrated for other osmoprotectants like proline and glycine betaine). In grafted trees, application to the root zone would likely benefit both rootstock and scion via systemic translocation through the xylem. Future studies should evaluate TMAO efficacy across diverse scion–rootstock combinations and optimize timing, concentration, and delivery routes under real-world orchard conditions.

Critically, the agricultural application of TMAO is supported by its highly favorable safety and economic profile [26,27,29,30]. Unlike many synthetic pesticides or growth regulators, TMAO is a naturally occurring molecule present in common food sources like marine fish [28]. While elevated endogenous TMAO levels in humans have been linked to cardiovascular risk, exogenous application to plant roots—as used in this study—is unlikely to pose significant exposure risks to consumers. Moreover, TMAO is readily biodegradable in natural environments: recent studies have identified diverse bacteria—including *Paracoccus* sp. from marine habitats and gut-derived *Enterococcus faecalis*—that efficiently degrade TMA and TMAO through defined metabolic pathways [34,35]. This microbial degradability suggests minimal environmental persistence and a low ecological footprint for agriculturally applied TMAO. It should be noted that the present study was conducted on non-fruiting apple rootstock plants under controlled conditions, and TMAO was applied solely to the root zone. While our data demonstrate clear physiological benefits under drought stress, the potential accumulation or translocation of exogenously applied TMAO to edible fruit tissues in mature, grafted orchard trees remains unknown. Therefore, prior to any field-scale application in fruit production systems, thorough residue studies and food safety evaluations would be essential to ensure consumer safety and regulatory compliance. From a practical standpoint, the dual efficacy of TMAO in enhancing both survival under lethal stress and biomass accumulation under chronic stress translates into direct economic benefits for growers. By securing tree survival during extreme weather events and maintaining productivity during seasonal droughts, TMAO application can significantly reduce establishment costs and yield losses, offering a high return on investment in the face of increasing climate volatility.

5. Conclusions

In conclusion, our study shows that exogenous application of TMAO enhances drought tolerance in the apple rootstock 'M.26'. The most effective concentration under the experimental conditions was 0.5 mM, as it consistently and significantly improved key physiological parameters, including photosynthetic rate, stomatal conductance, and root growth, while providing substantial survival benefits (60% survival) under severe stress. TMAO functions dually: as an osmolyte to promote osmotic adjustment, thereby maintaining root development and leaf hydration, and as a chemical chaperone to stabilize proteins and enhance survival during severe dehydration. These integrated physiological improvements highlight TMAO's potential as an eco-friendly plant protectant. Future

research should focus on validating these effects under field conditions, testing a wider range of rootstock–scion combinations, and conducting in-depth molecular analyses to elucidate the signaling pathways through which TMAO modulates drought responses.

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