Chitosan Oligosaccharide Addition to Buddhist Pine (*Podocarpus macrophyllus* (Thunb) Sweet) under Drought: Responses in Ecophysiology and $\delta^{13}$C Abundance

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**Abstract:** Climate warming induces the necessity to increase the drought resistance of shade-obligate juvenile trees in sub-tropical forests. Chitosan oligosaccharide (COS) is a biopolymer derived from the marine resource that has attracted accumulative attention to induce and promote a plant’s resistance to abiotic stress. Buddhist pine (*Podocarpus macrophyllus* (Thunb)Sweet) seedlings were cultured as the model material whose natural distribution in sub-tropical areas of China has suffered severe summer drought events in the last 113 years. A split-block design was conducted with a simulated drought event (drought vs. irrigated control), the COS addition, and two samplings at the ends of drought and re-watered treatments. The COS addition increased the resistance to drought by inducing a starch allocation towards roots where $\delta^{13}$C abundance and antioxidant enzyme activities were upregulated. The COS addition can promote biomass allocation to roots and increase the number of new roots. The COS addition to drought-treated Buddhist pine seedlings resulted in robust diameter growth. Therefore, COS is an available polymer to promote the resistance of Buddhist pine to drought. More work is suggested to clarify the dose of COS addition that can induce a prominent response of biomass accumulation and carbohydrate metabolism.

**Keywords:** drought-resistance; soluble sugars; fine root morphology; seedling growth; biomass allocation; POD; SOD; CAT

1. **Introduction**

Climate change brings about increasing drought events that shape the global forest distribution and changes the within-forest structure in all biomes [1]. Sub-tropical forests exchange more carbon dioxide (CO$_2$) with the atmosphere than any other vegetation type; therefore, they are facing the most challenging droughts on the planet [2]. The pattern of precipitation shift exposes sub-tropical forests to foreseeing frequent drought events that may impair carbon (C) assimilation, even to the extent of vegetation dieback [3]. Facing frequent drought stresses, the functional composition of sub-tropical forests has been reformed from tall and strong dominant trees towards smaller and denser wooded dwarfs. During this process, the mortality of trees in sub-tropical forests has continuously occurred directly, or partially, due to water-deficit events. There is a large knowledge gap about the mechanism of drought-caused mortality in relation to sub-tropical forest trees [2,4]. People are helpless to cope with the drought impact on forest trees, even though it can be foreseen to come in high frequency.
The management and silvicultural practices are considered to adapt forests to drought under the guidance of a close-to-nature strategy. Density and diversity are two critical parameters that attract great attention considering modification towards a more drought-resistant forest ecosystem. Forests with a low tree population show solid adaptations to drought [5,6]. Sometimes heavy thinning management may be needed to remove as high as 60% of the basal area to promise the framework for the adaptation of forests to drought [6]. One of the most significant benefits of thinning is the creation of large gaps that promote undergrowth regeneration in forests exposed to a water-deficit condition [7]. Therefore, it is commonly suggested to bring more juvenile shade-tolerant trees to forest gaps to enhance the adaptation of the stand. The dependence on species screening will result in a low richness, which would, in turn, impact the sustainable development of the managed forest. An alternative approach is to improve the resistance to drought for newly introduced trees but, to our knowledge, no specific regime has been documented. Access to better adaptation appears to be enclosed if all strategies came only out of the approach derived from the terrestrial system.

Our society is established on the terrestrial biota, but a highly developed economic industry brings about an increasing demand for the recycling utilization of marine resources [8]. Chitin is a type of cationic amino polysaccharide made up of N-acetyl D-glucosamine repeat blocks joined by β-1,4 glycosidic bonds [9]. Chitin is derived from leftover seafood by extraction from the exoskeletons of crustaceans and shrimp crabs [8]. To facilitate utilization, chitosan is usually converted into the most commonly known derivative of a linear heteropolysaccharide polycationic called “chitosan” [10]. Chitosan is a biopolymer of glucosamine residues that can also be processed from seafood wastes [11]. Chitosan oligosaccharide (oligochitosan or COS) is the enzymatic-hydrolyzed product of chitosan that overcomes the drawbacks of insolubility at neutral pH and high viscosity of chitosan [12]. As at least five acetyl groups have been found to be carried, COS has a valuable attribute to activate the plant immune system and promotes the generation of an anti-oxidative metabolism [13–15]. COS addition was also found to function as a growth modifier that induced biomass allocation to roots and strengthened nutrient uptake and utilization [11,16]. Recently, COS was found to promote plant resistance to drought stress through modification of plant morphological, physiological, and metabolic responses [17,18]. This implies COS to be a potential modifier that may bring drought resistance of juvenile trees in sub-tropical regions. The specific responses in ecophysiology need to be detected to reveal the solid mechanism for using COS.

A non-structural carbohydrate (NSC; mainly soluble sugars and starch) metabolism is one of the earliest responses to drought stress as a link between responses of photosynthetic production and the subsequent utilization. NSC changes accompany the entire process of tree response to drought from growth deficit to essential mortality [19]. When facing a water-deficit event, both sugar and starch contents and partitioning in shoots are reduced due to the imposed impact on photosynthesis [20]. Starch is heavily consumed as hydrolyzing to sugars to fuel new root egress and elongation [21]. At the initial stage of root response to drought, the conversion from starch to sugars is stimulated for the supply of energy to fuel counter stress [22]. However, the heavy depletion of the starch reserve in roots exposed to drought can prevent the conversion to sugars, which primarily accounts for the mortality [23]. As a summary of the above-mentioned findings, NSC changes in response to the drought effect on both shoot and root parts can be taken as the scale to assess the effect of COS addition as a potential remission.

A drought event induces the depression on leaves’ stomatal conductance ($g_s$) to control transpiration; thereafter, intercellular CO$_2$ (Ci/Ca) is stimulated to accumulate to a higher concentration, which arouses the increase of a C isotope signature ($\delta^{13}C$) in newly formed photosynthates. It was also found that $\delta^{13}C$ declined in the fine roots of trees that were distributed in dry stands [24]. Recently evidence is still accumulating to employ $\delta^{13}C$ as the indicator to assess trees’ response to drought interruption [25,26]. Therefore, the detection of $\delta^{13}C$ can be a precise approach to quantify the effect of COS addition on the response of juvenile trees to drought. The activity of POD can assess the reactive oxygen species (ROS) scavenger, and active SOD rapidly converts the ROS into hydrogen peroxide and
water, which is catalyzed by CAT [14]. The activities of all these three enzymes are sensitive predictors of the antioxidant effect of COS addition on horticultural plants [27,28]. Therefore, they also have the full potential to be detected as an assessment for the anti-drought response of juvenile tree seedlings.

Buddhist pine (Podocarpus mascrophyllus (Thunb) Sweet) is a widely distributed species in sub-tropical regions of Southern China that received the least concern by the Red List of International Union for Conservation of Nature (IUCN) [29]. The sub-tropical regions in Southern China are the main area of Buddhist pine distribution [29]. All these regions have experienced severe drought in July and August since 1960 [30]. As a well-adaptative species to these regions, Buddhist pine has generated a resistance to extreme drought that has not been revealed well. It is a proper species that is further used to detect the response of COS addition under drought. In this study, a simulated drought was enforced on Buddhist pine seedlings with the hypotheses that (i) the decline in growth and photosynthetic products in drought-treated seedlings can be alleviated by COS addition but (ii) δ\(^{13}\)C would decline with COS addition in response to drought.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

Buddhist pine seeds were obtained from trees at Hangzhou (30°10′ N, 120°20′ E). Seeds were sterilized using potassium (K) permanganate (0.5%, w/w) and divided into two groups. One group was soaked in distilled water, and the other group was coated by soaking in COS solution (Qishanbao®, GlycoBio Co., Ltd., Dalian, Liaoning, China) at the concentration of 2 mg kg\(^{-1}\) (w/w) [11]. Both groups of seeds were soaked for 12 h, and subsequently sown 0.5 cm deep in sands with a moisture content of 80% at a constant temperature of 22 °C in the Laboratory of Combined Manipulation of Illumination and Fertility on Plant Growth (43°48.6′ N, 125°22.8′ E) (Zhilunpudao Agric. S&T Ltd., Changchun, China). Germinant seedlings were transplanted to plastic pots (7×7×7 cm, top diameter × bottom diameter × height) in April 2017. Initial height and root-collar diameter (RCD) were 4.0 cm and 1.4 mm, respectively. Pots were filled with substrates with a mixture of peat, spent-mushroom residue, and perlite (55:20:25, v/v/v) (Mashiro-Dust\textsuperscript{TM}, Zhiluntuowei A&F S&T Ltd., Changchun, Jilin, China). The initial substrate had an ammonium nitrogen (N) content of 79.99 mg kg\(^{-1}\), a nitrate N content of 1.67 g kg\(^{-1}\), an available phosphorus (P) content of 0.36 g kg\(^{-1}\), an organic matter content of 12.9% ± 0.15%, a pH of 4.82 ± 0.02, and an electrical conductivity (EC) of 1.62 ± 0.15 mS cm\(^{-1}\). The chemical analysis of these traits was adapted from previous studies [31]. The substrates with these characteristics have been successfully proven to culture Buddhist pine seedlings [32].

Buddhist pine seedlings were cultured by the sub-irrigation with fertilizers (N-P\(_2\)O\(_5\)-K\(_2\)O, 10-7-9) at the rate of 60 mg N plant\(^{-1}\), which was recommended to be the optimum nutritional status [32]. Potted seedlings were placed in plastic tanks (55.5 cm × 36.5 cm × 7 cm, length × width × height), where the water table was maintained to 3–5 cm. Seedlings were watered twice a week with a nutrient solution combined with the irrigation. The COS treatment was continuously applied to seedlings that were coated with seeds by spraying COS onto the foliage every time with irrigation. Light-emitting diode (LED) panels were employed to supply light for the growth of Buddhist pine seedlings with a spectrum of 85% red, 15% green, and 5% blue lights at the photosynthetic photon flux density of about 70 µmol m\(^{-2}\) s\(^{-1}\) 40 cm beneath the lighting surface. This spectrum has been proven to benefit tree seedling growth for Dalbergia odorifera T. Chen [16] and coniferous species [33].

2.2. Drought and Re-Water Treatments

In July and August, half of the Buddhist pine seedlings were subjected to the simulated drought treatment and the other half were watered ordinarily using the sub-irrigation regime with continuous watering to maintain the full moisture capacity. The drought treatment was achieved by adding a volume of 310 mL of water to the tank in a two-month time frame. This was simulated from averaged yearly precipitation from records in 191 meteorology stations across nine provinces and two provincial
municipalities in Southern China over the last 113 years (1900–2012) [30]. The southern parts of China contain the main distribution of Buddhist pine [29]. After the drought treatment, water-deficit seedlings were re-watered again using sub-irrigation and maintained for another month in September 2017. During the drought treatment phase, seedlings continuously received COS addition treatment.

2.3. Experimental Design and Seedling Sampling

The experiment was conducted as a split-block design, whereas the main blocks were the drought treatment with well-watered seedlings throughout the experiment and used as the control. The COS addition was taken within the sub-block that was replicated within three blocks. Seedlings were sampled twice at the end of drought treatment (August) and the re-watering treatment (September). At each sampling, 10 seedlings were sampled from one block of combined treatment. Therefore, a total of 240 seedlings were cultured and sampled in this study in an arrangement of 2 COS x 2 droughts x 2 samplings x 3 replicated blocks x 10 seedlings per block.

2.4. Sampled Seedling Measurement and Determination

Eight seedlings from the 10 sampled ones per combined treatment per block were used for measures on growth, biomass accumulation, carbohydrate concentration, nutrient concentration, and δ¹³C abundance. Sampled seedlings were divided into the shoot and root parts. Shoot growth was measured for seedling height and root-collar diameter (RCD). Fine roots were scanned to obtain images at a dots-per-inch (dpi) resolution of 118.11 pixels cm⁻¹ (HP Deskjet 1510 scanner, HP Inc., Palo Alto, CA, USA). Root images were subsequently analyzed using the WinRhizo software (Regent Instrument Inc., Calgary, Canada) to assess fine root length, surface area, diameter, and tip numbers. These fine roots, separately with tap roots and root parts, were oven-dried at 70 °C for 48 h and measured for dry weight biomass. Dried samples were ground to pass the 1 mm sieve and used for the determination of non-structure carbohydrate and δ¹³C abundance. Soluble sugars and starch were determined using a 0.5 g sample by the colorimetric method at 490 nm (UV-Visible 8453, Agilent Tech. Inc., Santa Clara, CA, USA) [21,34–37]. The δ¹³C abundance was measured using an isotopic ratio mass spectrometer (Finnigan MAT Delta V advantage, Thermo Finnigan, San Jose, CA, USA) according to the model [38]:

\[ \delta^{13}C \text{ (‰)} = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \times 1000 \]  

where \( R_{\text{sample}} \) and \( R_{\text{standard}} \) are the \( ^{13}C/^{12}C \) ratios in the sample and in the conventional Pee Dee Belemnite standard, respectively. The overall precision of the δ values was higher than 0.1‰ as determined from repeated samples.

The other two seedlings were used for enzymatic antioxidant assays on superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in leaves. Fresh leaf samples were centrifuged at 15,000 rpm at 4 °C to prepare the supernatant for future enzymatic determination. To determine SOD, the supernatant was extracted by ethylenediaminetetraacetic acid (EDTA) (75 mM), 1 mL of nitroblue tetrazolium (NBT) (50 mM), 950 µL of sodium phosphate buffer (50 mM), 1 mL of riboflavin (1.3 µM), and 0.5 mL of methionine (13 mM) and measured using a spectrophotometer at 560 nm. To determine CAT, the supernatant was extracted with 0.9 mL of \( H_2O_2 \) and phosphate buffer (50 mM) and measured for absorbance at 240 nm. The POD enzymic activity was assessed at 470 nm for the supernatant that was extracted by 400 µL of guaiacol (20 mM), 2 mL of sodium phosphate buffer (50 mM), and 500 µL of \( H_2O_2 \) (40 mM).

2.5. Statistical Analysis

All statistics were obtained using the SAS software ver. 9.4 in 64 bits (SAS Institute Inc., Charlotte, NC, USA). Data were analyzed using analysis of variance (ANOVA) with a mixed model to detect combined effects of drought x COS addition on repeated twice measures of variables. Firstly, data were tested for the response to one-way ANOVA detecting combined effects of drought x COS-addition x
When a significant effect was indicated ($\alpha = 0.05$), means were compared using the Tukey test at a significance level of 0.05.

3. Results

3.1. Shoot Growth

Factors of drought treatment, COS addition, and sampling time had an interactive effect on both height and RCD (Table 1). The irrigated control treatment with COS addition resulted in the highest seedling height and RCD at the second sampling, followed by the irrigated control treatment without COS addition in the second sampling (Figure 1). The drought treatment without COS addition at the second sampling resulted in a lower height and RCD. The drought-treated seedlings in the first sampling resulted in the lowest height and RCD, especially in the treatment without COS addition.

Table 1. $p$-values from mixed-model analysis of variance (ANOVA) of water availability ($W$), chitosan oligosaccharide addition ($O$), and sampling time ($T$) on growth, biomass accumulation, and carbohydrate content in Buddhist pine (Podocarpus macrophyllus) seedlings.

| Parameter       | $W$   | $O$   | $T$   | $W \times O$ | $O \times T$ | $W \times T$ | $W \times O \times T$ |
|-----------------|-------|-------|-------|--------------|--------------|--------------|-----------------------|
| Height          | $<$0.0001 | 0.0021 | $<$0.0001 | 0.9691     | 0.1576      | 0.1089      | 0.0075               |
| RCD $^2$        | $<$0.0001 | 0.0033 | $<$0.0001 | 0.5998     | 0.0454      | 0.8406      | 0.0162               |
| Shoot biomass   | $<$0.0001 | 0.0021 | $<$0.0001 | 0.6508     | 0.1170      | 0.0357      | 0.1745               |
| Root biomass    | $<$0.0001 | 0.0010 | $<$0.0001 | 0.6727     | 0.0017      | $<$0.0001   | 0.5005               |
| R/S $^3$        | $<$0.0001 | 0.0011 | $<$0.0001 | 0.6295     | 0.4677      | $<$0.0001   | 0.5413               |
| Shoot sugar     | 0.6865  | 0.3667 | 0.4590 | 0.6865     | 0.6099      | 0.6800      | 0.4377               |
| Root sugar      | 0.7205  | 0.9142 | 0.9417 | 0.8296     | 0.1842      | 0.0183      | 0.2975               |
| Shoot starch    | 0.0016  | 0.8723 | 0.0018 | 0.0135     | 0.4039      | 0.3682      | 0.4008               |
| Root starch     | 0.2817  | 0.0004 | 0.0957 | 0.3724     | 0.0168      | $<$0.0001   | 0.0040               |

Note: $^1$ Values in bold font indicate significant effect at 0.05 level; $^2$ RCD, root-collar diameter; $^3$ R/S, root to shoot ratio.

Figure 1. Height (A) and root-collar diameter (RCD) (B) in two harvests of Buddhist pine (Podocarpus macrophyllus) seedlings exposed to chitosan oligosaccharide (COS) addition (COS- vs. COS+) under contrasting water availabilities (drought vs. irrigated control). Different letters indicate a significant difference according to Tukey’s test at the 0.05 level. Error bars stand for standard errors.

3.2. Biomass Accumulation

Factors of drought and sampling time had an interactive effect on shoot and root biomasses (Table 1). Seedlings with the irrigated control treatment at the second sampling had the highest shoot biomass, which was higher than that in the drought treatment at the second sampling by 9% (Figure 2A). Shoot biomass in the irrigated control and drought treatments had a lower biomass by
21% and 30% in the first sampling than those in the second sampling, respectively. Root biomass was highest in the drought treatment at the second sampling, which was higher than that in the irrigated control at the same sampling by 36%. Root biomass in the second sampling was about 9-fold higher than that in the first sampling.

Factors of COS addition and sampling time also had an interactive effect on root biomass (Table 1). Seedlings in the second sampling generally had 12-fold higher root biomass than that in the first sampling (first sampling: 0.04 ± 0.02 g; second sampling: 0.52 ± 0.08 g) (Figure 2B). Although shoot biomass did not show a response to the interaction between COS addition and sampling time, shoot biomass was higher in the second sampling by 17–29% than that in the first sampling (Figure 2B).

The COS addition had a significant effect on the root to shoot biomass ratio (R/S) (Table 1). The COS addition caused a rise of R/S by 9% (Figure 3A). The drought treatment and sampling time generated an interactive effect on R/S (Table 1). The drought treatment at the second sampling resulted in the highest R/S, which was higher than that in the irrigated control treatment in the second sampling by 48% (Figure 3B). The drought treatment resulted in higher R/S in the first sampling when the general level was only about 40% of that in the second sampling (Figure 3B).
3.3. Carbohydrate Metabolism

Factors of drought treatment and sampling time had an interactive effect on soluble sugar concentration in roots (Table 1). The irrigated control treatment in the second sampling resulted in a lower root sugar concentration by 4–8% than that in the first sampling (Figure 4A).

![Figure 4](image-url)

Figure 4. Soluble sugar (A) and starch (B) contents in two harvests of Buddhist pine (*Podocarpus macrophyllus*) seedlings exposed to contrasting water availabilities (drought vs. irrigated control) and their interaction with chitosan oligosaccharide (COS) addition (COS vs. COS+). Different letters indicate a significant difference according to Tukey’s test at the 0.05 level. Lower-case letters represent shoot biomass; Greek letters represent root biomass. Error bars stand for standard errors.

Factors of drought treatment, COS addition, and sampling time had a combined effect on shoot starch concentration ($F_{7,16} = 5.13, p = 0.0033$). Shoot starch concentration was highest in the irrigated control treatment without COS addition in the first sampling, which was higher than that in the drought treatment without COS addition in the first sampling and that in most treatments in the second sampling, except the irrigated control treatment without COS addition (Figure 4B). The three factors also had an interactive effect on root starch concentration (Table 1). Root starch concentration was highest in the irrigated control treatment with COS addition in the first sampling and the drought treatment with COS addition in the second sampling (Figure 4B). The drought treatment without COS addition in the first sampling resulted in the lowest root starch concentration.

3.4. Fine Root Morphology

Factors of COS addition and sampling time had an interactive effect on fine root length ($F_{1,22} = 72.28, p < 0.0001$), diameter ($F_{1,22} = 6.22, p = 0.0207$), and root-tip number ($F_{3,20} = 12.84, p < 0.0001$). Seedlings receiving COS addition in the second sampling had higher fine root length, diameter, and tip number than those in the first sampling, whether or not COS was added (Figure 5A,C,E). Seedlings without COS addition in the second sampling also resulted in higher fine root length, diameter, and tip number than those in the first sampling, but only the root-tip number was different between the COS addition treatments in the second sampling (Figure 5E).
Factors of drought treatment and sampling time had an interactive effect on fine root surface area ($F_{3,20} = 19.47, p < 0.0001$) and tip numbers ($F_{3,20} = 9.57, p = 0.0004$). Seedlings in the drought treatment in the second sampling had higher fine root surface area and tip number in the second sampling than those in the first sampling, regardless of drought treatment.

3.5. The $\delta^{13}$C Abundance

Factors of drought treatment, COS addition, and sampling time had an interactive effect on $\delta^{13}$C abundance in both shoot ($F_{7,16} = 62.21, p < 0.0001$) and root parts ($F_{7,16} = 283.70, p < 0.0001$). Seedlings had the highest shoot $\delta^{13}$C abundance in the drought treatment with COS addition in the first sampling, followed by those without COS addition in the first sampling and those in the drought treatment in the second sampling (Figure 6A). Seedlings in the irrigated control treatment with COS addition had the lowest $\delta^{13}$C abundance in shoots. Root $\delta^{13}$C abundance was also highest in the drought treatment with COS addition in the first sampling and lowest in the irrigated control treatment with COS addition in the second sampling (Figure 6B).
Factors of drought treatment, COS addition, and sampling time had an interactive effect on the activities of POD ($F_{7,16} = 4.69, p = 0.0050$), SOD ($F_{7,16} = 2.85, p = 0.0393$), and CAT ($F_{7,16} = 422.43, p < 0.0001$). The POD activity was highest in seedlings with COS addition in the second sampling, followed by the drought treatment without COS addition in the first sampling (Table 2). The SOD activity was also highest in seedlings subjected to the drought treatment with COS addition in the second sampling, followed by those subjected to the drought treatments in the first sampling (Table 2). The CAT activity was highest in the drought treatment with COS addition in the second sampling, followed by that in the same treatment in the first sampling.

### Table 2. Effects of sampling time, water availability, and oligosaccharide addition on anti-oxidation enzyme activity in Buddhist pine (Podocarpus macrophyllus) seedlings.

| Water Availability | Oligosaccharide Addition | Sampling Time | POD $^1$ ($\Delta g_{10}^{-1}$ FW min$^{-1}$) | SOD $^2$ (U g$^{-1}$ FW) | CAT $^3$ (U g$^{-1}$ FW) |
|--------------------|--------------------------|---------------|---------------------------------------------|--------------------------|--------------------------|
| Drought            | No                       | First         | $11.54 \pm 3.79$ ab $^4$                      | $5.64 \pm 0.60$ ab       | $2.10 \pm 0.00$ g        |
|                    | Yes                      |               | $7.34 \pm 0.49$ bcd                         | $6.38 \pm 1.07$ ab       | $2.38 \pm 0.01$ b        |
| Irrigated control  | No                       |               | $7.01 \pm 1.67$ cd                          | $4.93 \pm 0.76$ b        | $2.31 \pm 0.00$ c        |
|                    | Yes                      |               | $6.47 \pm 2.14$ d                           | $3.64 \pm 1.07$ b        | $2.25 \pm 0.01$ e        |
| Drought            | No                       | Second        | $10.98 \pm 0.67$ abc                         | $5.58 \pm 0.92$ ab       | $2.28 \pm 0.02$ d        |
|                    | Yes                      |               | $14.35 \pm 2.74$ a                          | $8.26 \pm 1.85$ a        | $2.50 \pm 0.00$ a        |
| Irrigated control  | No                       |               | $10.60 \pm 1.30$ abcd                        | $4.08 \pm 1.53$ b        | $2.30 \pm 0.00$ c        |
|                    | Yes                      |               | $12.16 \pm 2.23$ a                          | $4.42 \pm 2.56$ b        | $2.17 \pm 0.01$ f        |

Note: $^1$ POD: peroxidase; $^2$ SOD: superoxide dismutase; $^3$ CAT: catalase; $^4$ different letters indicate a significant difference according to Tukey’s test at the 0.05 level.

### 4. Discussion

The decline in shoot height and RCD is a typical response of juvenile trees to drought-induced water deficit [39,40]. In this study, shoot height and RCD were all decreased by the drought treatment, which illustrated that the simulated drought event imposed a strong limitation on the shoot growth of Buddhist pine. Although we re-watered the drought-treated seedlings, the negative impact of drought on shoot growth was not alleviated, suggesting that the drought had a phenological impact on shoot growth [40,41]. The addition of COS increased the growth between seedlings subject to the same conditions.
water-deficit condition, which concurs with results regarding basil plants [42]. The increase in the height of Buddhist pine seedlings by the COS addition in our study was also found by Wang et al. [43]. In the second sampling, the decline of RCD in the drought treatment was alleviated by COS addition to a similar degree in the irrigated control treatment. According to Vander Mijnsbrugge et al. [41] and Wang et al. [11], the drought-induced phenological impact may last for more than a year. The observation of growth response in this study can be extended to a longer time in future work to further confirm the period of the COS effect on drought stress.

Shoot biomass was also depressed by the drought treatment which, however, cannot be alleviated by COS addition. Although Behboudi et al. [44] reported the remarkable improvement of biomass in wheat by adding chitosan nanoparticles, another study also failed to show any significant effect of COS addition on the biomass of drought-treated Thymus daenensis [45]. However, the COS addition promoted R/S and the number of fine root tips, which is a robust drought tolerance response to water deficit [46]. Neither soluble sugar nor starch concentrations in the shoots of our seedlings exposed to the drought were elevated by COS addition. In contrast, Li et al. [16] reported a decline in stem starch concentration in COS-treated Dalbergia odorifera seedlings. As the depletion of starch in stems was considered a consumption response to nutrient utilization, it is unlikely that COS addition to Buddhist pine seedlings fueled shoot consumption. The increase in root starch concentration in seedlings receiving COS addition resulted from the promotion of starch to roots to fuel new root egress. New root growth is tightly related to starch usage [21], and COS addition was found to benefit the number of new roots in Buddhist pine seedlings [11]. Thus, we can accept our first hypothesis.

The irrigated control treatment with COS addition in the second sampling resulted in the lowest $\delta^{13}$C abundance, suggesting the release of leaves’ stomatal conductance ($g_s$) to control transpiration and the decline of intercellular CO$_2$ (Ci/Ca) accumulation from newly formed photosynthates [47,48]. This combined treatment also resulted in the lowest root $\delta^{13}$C abundance, which concurs with the response of shrubs in dry stands [24]. It was interesting to find that, in drought-treated seedlings, COS addition can lower the $\delta^{13}$C abundance; however, in well-watered seedlings, COS addition tended to elevate the $\delta^{13}$C abundance, especially in the second sampling. These findings resulted from the mechanism of foliage spraying COS to control stomatal conductance as an anti-transpirant [49,50]. When Buddhist pine seedlings suffered drought stress, the addition of COS induced the stomatal closure to control transpiration; in contrast, when seedlings were well watered, the addition of COS promoted the openness of stomata to promote exchange. Therefore, we can accept our second hypothesis.

Our results revealed that the activities of these enzymes were all promoted by the COS addition in Buddhist pine seedlings. The addition of COS was also reported to promote these antioxidant enzymatic activities in chrysantheum (Chrysanthemum × morifolium (Ramat.) Hemsl.) [13], grape (Vitis vinifera L. “Touriga Franca” and “Tinto Cao”) [15], and potato (Solanum tuberosum L.) [14] when facing water stress. In the irrigated control treatment, the impact of COS addition was not as great as that in the water-stress condition. Investigation of the divergent effects of COS addition on drought-stressed seedlings is suggested as future work.

5. Conclusions

In this study, Buddhist pine seedlings were raised as the materials of juvenile trees with the distribution in regions subjected to historical drought events. The addition of COS can elicit the physiological acclimations in both shoot and root parts of seedlings exposed to drought without apparent changes in biomass allocation and fine root morphology. Therefore, COS can be recommended as an available polymer that is easily derived from unlimited sources of marine creatures for use in forest crops. The application of COS through aerial spraying is economically feasible as a potential anti-drought agent and warrants further application in more species. Currently from this study, no apparent side effect was observed, but details about the specific application regime (dose, time, form, and combined effects) should be considered in future work.
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