

## EFFECTS OF DROUGHT AND NITROGEN ADDITION ON GROWTH AND LEAF PHYSIOLOGY OF *PINUS MASSONIANA* SEEDLINGS

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### Abstract

Drought and nitrogen deposition are predicted to increase with global change, but the interactive effects of the two factors on tree physiology are still poorly understood. We investigated the individual and interactive effects of drought and nitrogen addition on growth and leaf physiology of *Pinus massoniana* seedlings. Seedlings were grown in growth chambers with two soil water (i.e. well watered and drought stress) and two nitrogen conditions (i.e. 0 and 40 kg N hm<sup>-2</sup> a<sup>-1</sup>). Biomass accumulation and allocation, leaf relative water content (RWC), free leaf water content (FWC), leaf chlorophyll (Chl<sub>a+b</sub>, Chl<sub>a</sub>, Chl<sub>b</sub>, Chl<sub>a/b</sub>), contents of carotenoid (Car), malondialdehyde (MDA) and proline (Pro), and superoxide dismutase (SOD) activity were examined throughout the entire experimental period. This study showed that the contents of MDA and Pro, SOD activity, root shoot ratio (R/S) and root mass ratio (RMR) were increased significantly in the drought treatment, while Chl<sub>a+b</sub>, Chl<sub>a</sub>, RWC and growth of seedlings were significantly decreased. In contrast, nitrogen addition had positive impacts on leaf chlorophyll content, leaf RWC and growth of seedlings. Leaf chlorophyll content had positive relationship with RWC irrespective of nitrogen supply, while MDA and Pro content had opposite trends. We also found that nitrogen addition could substantially ameliorate the negative impact of drought on *P. massoniana*, by increasing growth and limiting accumulation of MDA, Pro and SOD activity. This short term study suggests that current ambient nitrogen deposition in southern China may be helpful for establishment of *P. massoniana* seedlings in bare lands with limited water availability.

**Key words:** Drought; Nitrogen addition; Global change; Growth; Leaf physiology.

### Introduction

Water and nitrogen are important resources for plants, determining plant growth and physiological responses. However, drought stress is predicted to be enhanced in intensity and frequency with changing climate (Jefferson, 2015; Reichstein *et al.*, 2013), which would be further exacerbated by global warming (Duan *et al.*, 2014). Meanwhile, atmospheric nitrogen deposition has been globally elevated owing to increased fossil burning and agricultural nitrogen fertilization in recent years (Ren *et al.*, 2017). Global nitrogen deposition is expected to be double in 2050s (Schlesinger, 2009), which can have substantial effects on nitrogen and carbon cycling (Lu *et al.*, 2011). Furthermore, drought stress can interact with nitrogen processes and reduce nitrogen availability for plants (Misra & Tyler, 2000), thus negatively affecting carbon and nitrogen metabolism within plants and the following plant performance. To date, however, the interactive effects of drought stress and nitrogen addition on growth and functioning of trees remain poorly understood. Improved understandings of how drought stress interacts with nitrogen from the tree physiology perspective will provide insights into predicting tree responses to future climate changes.

Drought stress has effects on plant growth and physiological characteristics. On one hand, drought inhibits growth due to its negative impact on cell turgor (Mcdowell *et al.*, 2008; Woodruff *et al.*, 2015). According to the optimal allocation theory and functional balance hypothesis, when plants are under resource stress, the organ for exploring the resource would be favored given the highest priority (Bloom *et al.*, 1985). For example, drought has often been observed

to improve the root/shoot ratio (R/S), allocating more carbon to roots for improving the capacity of water absorption (Hao *et al.*, 2018). On the other hand, drought stress also affects plant physiological responses. For instance, earlier evidence shows that the contents of chlorophyll and carotenoid (Car) in leaves were reduced under severe drought stress (Bacelar *et al.*, 2006; Boughalleb & Hajlaoui, 2011).

Proline (Pro) has been found to increase under drought stress (Farouk & Qados, 2011), functioning as an osmolyte to maintain water balance (Blum *et al.*, 1996) and protecting cell structures and macromolecules (Farouk & Qados, 2011). Superoxide dismutase (SOD) has an important effect on cell redox and the activity of SOD is positively correlated with the capacity of plant anti-oxidation (Poleskaya *et al.*, 2004). In addition, SOD is thought to scavenge the increased reactive oxygen species (ROS) under drought stress (Pyngrope *et al.*, 2013). When ROS is excessive under drought conditions and can not be scavenged efficiently by antioxidant system, membrane lipids may suffer from substantial oxidative damage. The degree of oxidative damage can be reflected by the changes of malondialdehyde (MDA) (Chakhchar *et al.*, 2015).

Previous research showed that moderate nitrogen addition (11.2 g m<sup>-2</sup> a<sup>-1</sup>) could promote the growth of *Pinus tabulaeformis*, while doubled nitrogen addition (22.4 g m<sup>-2</sup> a<sup>-1</sup>) had contrasting impact on growth (Wang *et al.*, 2014). The content of chlorophyll in leaves is usually increased under nitrogen supply (Palmroth & Palmqvist, 2014). Moreover, the activity of SOD was observed to be enhanced by nitrogen addition (Zhang *et al.*, 2014). Nitrogen supply influences cell membrane

permeability yet, thereby inducing changes in MDA content (Guo *et al.*, 2010). The combined effects of drought and nitrogen addition on plant growth and physiology have also been previously documented. The inhibition of biomass and growth under drought stress can be alleviated by nitrogen supply. In addition, higher nitrogen supply could enhance the tolerance of plants to drought stress (Alandia *et al.*, 2016; Shi *et al.*, 2017). However, the degree of changes in drought endurance may largely depend on the rate of nitrogen supply and the intensity of drought. Drought can also reduce the absorption of nutrition from soils, thereby limiting the impacts of nitrogen addition on plants metabolism (Song *et al.*, 2010). Therefore, the interactive effects of drought and nitrogen addition on tree growth and physiological responses remain to be well understood.

*Pinus massoniana* is widely planted across southern China as a common pioneer forestation tree species for soil and water conservation, with high ecological and economic values (Justine *et al.*, 2015). The area of *P. massoniana* plantation has been rapidly extended over the last several decades (Kuang *et al.*, 2008). *P. massoniana* is usually planted in harsh environments with limited water availability. The soil in southern China is generally not nitrogen deficient, but the atmospheric nitrogen deposition is relatively high in this area (Zhang *et al.*, 2013). Consequently, better understandings of how *P. massoniana* cope with the interactive effects of drought and nitrogen deposition will provide more mechanistic insights into the capacity of *P. massoniana* to acclimate to changing climates and will be helpful for plantation management in the context of climate change. The objective of this paper is to examine the individual and interactive effects of drought and nitrogen addition on growth and physiological responses of *P. massoniana*. We predicted that: (i) Drought treatment alone would reduce total biomass (TB), RWC and the contents of  $Chl_{a+b}$  and Car compared with CK treatment, while it would increase the R/S, the contents of leaf MDA and Pro, and SOD activity; (ii) Compared with CK treatment, nitrogen addition treatment would increase the growth of seedlings and contents of leaf chlorophyll and Car; (iii) The negative impacts of drought on *P. massoniana* seedlings would be ameliorated by nitrogen addition.

## Materials and Methods

**Plant materials and growth condition:** One-year-old *P. massoniana* seedlings with approximate heights (about 50 cm in height; purchased from a nursery near Jiujiang, Jiangxi province, China) were transplanted into 4.0 L plastic pots filled with soil. The soil was obtained from the plantation nearby and soil characteristics were as follows: soil PH-4.18, the content of organic carbon-8.64 g/kg, total N-1.64 g/kg, total P-1.01 g/kg. Then twenty-four transplanted seedlings were placed into two growth chambers (one each with twelve seedlings). The chambers have a photosynthetic photon flux density of  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $25^\circ\text{C}/18^\circ\text{C}$  day/night temperature, 16 h/8 h day/night photoperiod and relative humidity of 55%. We watered seedlings daily and fertilized them once a week with a commercial liquid fertilizer (Mirale-Gro, Wuhan, China;  $\text{N} \geq 30 \text{ g/L}$ ,  $\text{P}_2\text{O}_5 \geq 14 \text{ g/L}$ ,  $\text{K}_2\text{O} \geq 16 \text{ g/L}$ ,  $\text{Fe} \geq 0.14 \text{ g/L}$ ,

$\text{Mn} \geq 0.06 \text{ g/L}$ ). Seedlings were rotated between chambers weekly to reduce the possible effects of chamber microenvironment.

**Experimental design:** After three weeks growth in chambers, four seedlings were harvested for the baseline (two for each chamber). Ten of the remaining seedlings in each chamber were divided into two treatments. In one chamber, five seedlings were assigned to ambient nitrogen plus well watered (CK) or ambient nitrogen plus drought (D) treatment, respectively. In another chamber, five seedlings were assigned to nitrogen addition plus well watered (N) or nitrogen addition plus drought (DN) treatment, respectively. Thereby, this experiment had a full factorial design of water  $\times$  nitrogen with two levels for each factor. However, two seedlings in D and one in DN died as the experiment progressed.

Seedlings in the CK and N treatments were watered to field capacity daily (soil volumetric water content was about 30%) throughout the entire experimental period, while seedlings in the D and DN treatments were allowed to desiccate completely. The soil volumetric water (SWC) content was measured by a portable Time Domain Reflectometry (TDR) probe (MXG-I, Shangdong, China) every three days.

Seedlings in the N and DN treatments were fertilized with  $\text{NH}_4\text{NO}_3$  prior to the experiment. Four gram  $\text{NH}_4\text{NO}_3$  was added to 500 ml deionized water and the solution was irrigated to each seedling to achieve the target local nitrogen deposition rate (i.e.  $40 \text{ kg N hm}^{-2} \text{ a}^{-1}$ ). The same amounts of water were added to seedlings in CK and D treatments. The target nitrogen deposition rate simulated the rate of ambient nitrogen deposition in local area (Nanchang City, Jiangxi province, China;  $28^\circ 41' 7'' \text{ N}$ ,  $115^\circ 51' 11'' \text{ E}$ ) and was comparable with many places in China (Liu *et al.*, 2011).

**Biomass measurements:** Four seedlings were destructively harvested as a baseline prior to the experiment, while all the other plants were harvested at the end of the experiment. Harvested seedlings were separated into roots, stem and leaves. Roots were washed free of soil with tap water. All plant organs were oven-dried at  $60^\circ\text{C}$  for 48 hours and biomass was then determined. Total biomass (TB) was calculated by the sum of root, stem and leaf biomass. Root/shoot ratio (R/S) was calculated as the ratio of root biomass to shoot biomass (i.e. leaf + stem). The leaf mass ratio (LMR) was calculated as leaf biomass/total biomass. Stem mass ratio (SMR) and root mass ratio (RMR) were calculated as stem biomass/total biomass and root biomass/total biomass.

**Leaf biochemical analyses:** On days 0, 7 and 28 of the experiment, fresh leaves (healthy, mature leaves) were sampled for the following biochemical analyses. Leaf (about 0.10 g) were extracted in 80% acetone, and the contents of chlorophyll ( $Chl_{a+b}$ ,  $Chl_a$ ,  $Chl_b$ ) and Car were then analyzed spectrophotometrically as described previously (Arnon, 1949). The chlorophyll a/b ( $Chl_{a/b}$ ) was calculated as  $Chl_a/Chl_b$ .

MDA was assayed by the thiobarbituric acid (TBA) reaction as described in a previous paper (Fu & Huang, 2001). Fresh leaves were homogenized in 5 ml of 0.1% trichloroacetic acid (TCA), and were then centrifuged at 10,000 g for 5 min. One ml of the supernatant was mixed

with 4 ml of 20% TCA which conclude 0.5% TBA. After heating at 95°C for half an hour, the mixture was quickly cooled and centrifuged for 10 min. The absorbance of the supernatant was measured at 600 and 532 nm, respectively. The calculation of MDA content can be found in a previous study (Cakmak & Horst, 2006). Pro content was analyzed by the method (Sulfosalicylic acid) of previous studies (Bates *et al.*, 1973). SOD activity was measured by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 625 nm following the method of an earlier study (Beauchamp & Fridovich, 1971).

**Relative water content (RWC) and free leaf water content (FWC) measurements:** RWC and FWC were measured throughout the experiment.

$$\text{RWC} = (W_f - W_d) / (W_t - W_d) \quad (1)$$

$$\text{FWC} = (W_f - W_d) / W_f \quad (2)$$

where  $W_f$  is the leaf fresh weight, while  $W_d$  is the leaf dry weight after 48 hours oven-dried at 60°C.  $W_t$  is the leaf turgid weight, which is obtained after the leaf were fully hydrated in distill water for 24 hours.

### Statistical analyses

Statistical analyses were conducted with SPSS 19.0 (USA). Two-way ANOVA was used to test the individual and interactive effects of water and nitrogen on biomass and allocation parameters at the end of the experiment. Other time-series parameters (i.e. RWC, FWC,  $\text{Chl}_{a+b}$ ,  $\text{Chl}_a$ ,  $\text{Chl}_b$ ,  $\text{Chl}_{a/b}$ , Car, MDA, Pro and SOD) were analyzed using two-way repeated-measures ANOVA to test temporal effects of water and nitrogen. One-way ANOVA was assessed to compare means among treatments. The normality and homogeneity were evaluated before all analyses. Significant difference was considered when  $P < 0.05$ .

### Results

**Soil water conditions:** The SWC in the CK and N treatments did not vary significantly throughout the entire experimental period (Fig. 1), while those in the D and DN treatments had substantial declines as drought progressed, to a greater extent in D than DN in the first week. Afterwards SWC was not significantly different between D and DN.

**Biomass accumulation and allocation:** Both drought treatments decreased TB of *P. massoniana* seedlings (Table 1,  $P = 0.021$ ), but D treatment lowered 24% TB than DN treatment, indicating that N treatment could reduce the negative impact of drought on seedlings growth. D treatment significantly increased R/S (+57%) and RMR (+46%) compared with CK treatment ( $P < 0.05$ ), while it did not affect SMR and LMR. N treatment increased TB by 58% compared with CK treatment ( $P < 0.05$ ) and caused a lower SMR (-23%) and higher LMR (+29%), but it did not have significant effects on R/S or RMR. DN treatment significant increased R/S (+23%) and RMR (+25%) compared with CK treatment ( $P < 0.05$ ).

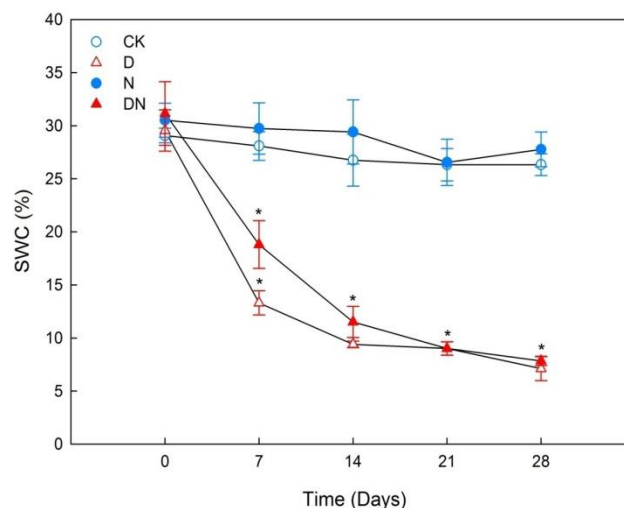


Fig. 1. Soil volumetric water content (SWC) at depth of 8 cm over the entire experimental period. Values are Means  $\pm$  SE ( $n = 3-5$ ). Treatment: CK-Ambient nitrogen plus well watered; D-Ambient nitrogen plus drought; N-Nitrogen addition plus well watered; DN- Nitrogen addition plus drought. The "\*" denotes the treatment had significant difference with CK

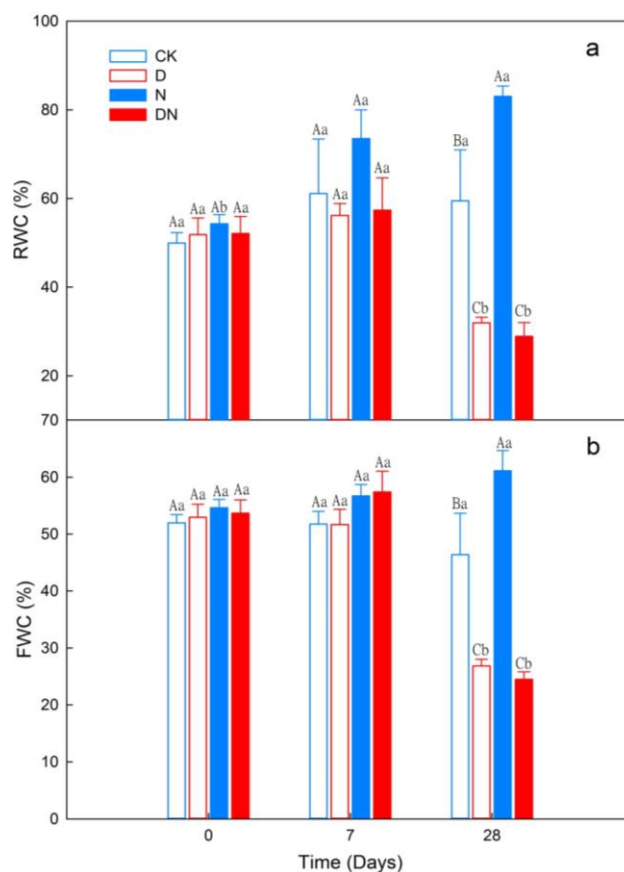


Fig. 2. Measured (a) leaf relative water content (RWC) and (b) free water content (FWC) of *Pinus massoniana* seedlings in four treatments over the entire experimental period. Values are Means  $\pm$  SE ( $n = 3-5$ ). Treatment: CK-Ambient nitrogen plus well watered; D-Ambient nitrogen plus drought; N-Nitrogen addition plus well watered; DN- Nitrogen addition plus drought. Different uppercase letters at the same time denote significant differences among four treatments, while different lowercase letters in same treatment indicate the significant differences among different time.

**Table 1. The total biomass (TB), root shoot ratio (R/S), root mass ratio (RMR), stem mass ratio (SMR), and leaf mass ratio (LMR) of *Pinus massoniana* seedlings at the end of the experiment. Values are Means  $\pm$  SE (n=3-5).**

Treatments	Total biomass	Root shoot ratio	Root mass ratio	Stem mass ratio	Leaf mass ratio
CK	14.943 $\pm$ 0.632 b	0.129 $\pm$ 0.011 c	0.114 $\pm$ 0.008 c	0.487 $\pm$ 0.039 a	0.399 $\pm$ 0.047 ab
D	6.672 $\pm$ 0.728 d	0.203 $\pm$ 0.009 a	0.167 $\pm$ 0.004 a	0.509 $\pm$ 0.045 a	0.324 $\pm$ 0.052 b
N	23.661 $\pm$ 1.794 a	0.121 $\pm$ 0.014 c	0.108 $\pm$ 0.010 c	0.377 $\pm$ 0.026 b	0.515 $\pm$ 0.035 a
DN	10.266 $\pm$ 1.015 c	0.167 $\pm$ 0.013 b	0.143 $\pm$ 0.012 b	0.516 $\pm$ 0.005 a	0.341 $\pm$ 0.013 b

**Treatment:** CK-Ambient nitrogen plus well watered; D-Ambient nitrogen plus drought; N-Nitrogen addition plus well watered; DN-Nitrogen addition plus drought. Different letters in the same column denote significant difference among four treatments ( $P < 0.05$ )

**Table 2. Summary of two-way repeated measures ANOVA on water, nitrogen treatment and time on physiological parameters of *Pinus massoniana* seedlings during the entire experiment.**

Parameter		Water	Nitrogen	Time	Water: Nitrogen	Water:Time	Nitrogen: Time	Water: Nitrogen: Time
Chl <sub>a+b</sub>	F	19.383	0.379	20.244	0.285	15.152	8.081	2.518
	P	<b>0.002</b>	0.867	<b>&lt;0.001</b>	1.310	<b>&lt;0.001</b>	<b>0.004</b>	0.112
Chl <sub>a</sub>	F	16.002	0.166	21.024	0.046	9.827	1.040	0.134
	P	<b>0.004</b>	0.695	<b>&lt;0.001</b>	0.836	<b>0.002</b>	0.376	0.876
Chl <sub>b</sub>	F	1.070	6.747	1.054	1.210	0.283	6.053	0.410
	P	0.331	<b>0.032</b>	0.371	0.303	0.757	<b>0.011</b>	0.670
Chl <sub>a/b</sub>	F	10.061	23.394	58.108	8.553	9.282	15.503	2.672
	P	<b>0.013</b>	<b>0.001</b>	<b>&lt;0.001</b>	<b>0.019</b>	<b>0.002</b>	<b>&lt;0.001</b>	0.100
Car	F	12.461	2.474	16.564	2.138	3.973	5.055	3.616
	P	<b>0.008</b>	0.154	<b>&lt;0.001</b>	0.182	<b>0.040</b>	<b>0.020</b>	0.051
MDA	F	100.775	1.187	220.825	3.183	152.358	1.806	6.639
	P	<b>&lt;0.001</b>	0.308	<b>&lt;0.001</b>	0.112	<b>&lt;0.001</b>	0.196	0.008
Pro	F	15.985	11.363	3.645	4.802	9.233	8.115	4.215
	P	<b>0.004</b>	<b>0.010</b>	0.050	0.060	<b>0.002</b>	<b>0.004</b>	<b>0.034</b>
SOD	F	26.842	2.336	6.290	6.940	5.579	2.930	2.167
	P	<b>0.001</b>	0.165	<b>0.010</b>	<b>0.030</b>	<b>0.015</b>	0.082	0.147
RWC	F	14.274	2.019	6.268	2.364	18.519	0.652	1.379
	P	<b>0.005</b>	0.193	<b>0.010</b>	0.163	<b>&lt;0.001</b>	0.534	0.280
FWC	F	42.509	9.647	24.055	4.572	23.903	0.520	2.095
	P	<b>&lt;0.001</b>	<b>0.015</b>	<b>&lt;0.001</b>	0.065	<b>&lt;0.001</b>	0.604	0.156

Significant values are indicated in table ( $P < 0.05$ )

**Leaf RWC and FWC:** Leaf RWC declined remarkably in both drought treatments towards the end of the experiment, compared with well watered treatments (Fig. 2a; Table 2), which was changed in accordance with SWC. We found that RWC had positive relationships with SWC irrespective of nitrogen availability, and the decline rate of RWC was increased as drought prolonged (Fig. 3a). Interestingly, at the end of the experiment, leaf RWC in N treatment was 39% higher than that in CK treatment (Fig. 2a). However, the positive effect of nitrogen addition was diminished by drought stress. Furthermore, leaf FWC had similar temporal patterns and treatment effects with leaf RWC (Fig. 2b and 3b; Table 2).

**Leaf chlorophyll and Car content:** The contents of leaf Chl<sub>a+b</sub>, Chl<sub>a</sub> and Chl<sub>b</sub> in CK did not have significant variations during the entire experiment, while leaf Chl<sub>a+b</sub> and Chl<sub>a</sub> in D and DN treatments declined as drought progressed (Fig. 4; Table 2). In contrast, drought stress did not have significant impact on Chl<sub>b</sub> (Fig. 4b). Nitrogen addition notably enhanced Chl<sub>a+b</sub> (averagely by 30%) only under well watered conditions, but

increased Chl<sub>b</sub> under both water treatments, reflecting the positive effect of nitrogen addition on the synthesis of chlorophyll. In the present study, drought and nitrogen treatment significantly decreased Chl<sub>a/b</sub>. The responses of leaf Car content to drought stress and nitrogen addition were similar with those of leaf chlorophyll content (Fig. 4d; Table 2).

**Leaf MDA, Proline and SOD activity:** Leaf MDA content was increased in D and DN treatments drought progressed (Fig. 5a; Table 2), indicating that cell membrane was damaged seriously under severe drought condition. Nevertheless, nitrogen addition did not have notable effect on leaf MDA content (Fig. 5a). Pro was increased (+101%) significantly in D treatment at the end of the experiment (Fig. 5b). However, Pro did not change much in DN treatment (Fig. 5b, Table 2). The activity of SOD was significantly increased under drought stress, particularly in the D treatment (Fig. 5c; Table 2), while it was not much affected by nitrogen addition. Additionally, there were significant interactive effects of drought and nitrogen addition on SOD activity (Table 2).

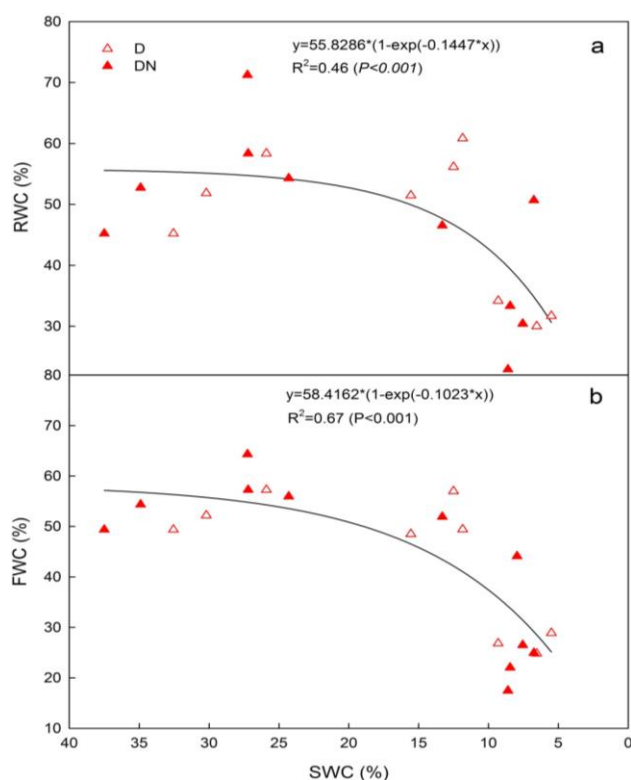


Fig. 3. (a) Leaf relative water content (RWC) and (b) free water content (FWC) of *Pinus massoniana* seedlings grown under D and DN conditions as function of soil volumetric water content (SWC). Data are fitted with linear functions ( $y=a*(1-\exp(-bx))$ ). Treatment: D-Ambient nitrogen plus drought; DN-Nitrogen addition plus drought.

**The relationships of leaf RWC and other physiological parameters:**  $Chl_a$  and  $Chl_{a+b}$  in leaves had positive relationships with RWC, while those did not show significant variation between nitrogen availabilities (Fig. 6). In contrast, leaf MDA and Pro content of *P. massoniana* seedlings had significant negative relationships with leaf RWC (Fig. 7a,b), especially when leaf RWC was below 45%, demonstrating that leaf water stress had greater impacts on MDA and Pro than SOD activity.

## Discussion

This paper focused on the individual and interactive impacts of drought and nitrogen addition on growth and leaf physiology of *P. massoniana* seedlings. Consistent with the first hypothesis, our results suggest that growth,  $Chl_{a+b}$ ,  $Chl_a$  and RWC were significantly reduced by drought stress, but contents of leaf MDA and Pro, SOD activity, and R/S were increased in the drought treatment. We also found that nitrogen addition elevated the leaf contents of chlorophyll and Car and growth of *P. massoniana*, which could partially support our second hypothesis. Compared with D treatment, the contents of leaf MDA, Pro and SOD were lower, but the growth of seedlings was increased in DN treatment, supporting the third hypothesis that nitrogen addition would ameliorate negative impacts of drought on *P. massoniana* seedlings.

**Plant growth and physiological responses to individual drought treatment:** The research has shown that individual drought stress had negative impact on total

biomass of *P. massoniana* seedlings, while R/S and RMR were increased under drought stress, indicating that not only the biomass accumulation but also the biomass allocation patterns are altered by drought stress (Aaltonen *et al.*, 2017). Assimilates are often partitioned from above ground to underground for greater root extension under drought stress (Rich & Watt, 2013). RWC is an important indicator that reflects plant leaf water status (Farouk & Qados, 2011). Leaf RWC and FWC declined sharply as drought progressed, which shows the severity of drought stress (Boughalleb & Hajlaoui, 2011; Jiménez *et al.*, 2013).

Leaf chlorophyll content is an important index for leaf health and can reflect the capacity of photosynthesis to some extent. In line with earlier evidence (Boughalleb & Hajlaoui, 2011), leaf  $Chl_a$  and  $Chl_{a+b}$  content in this study were significantly decreased by drought at the end of the experiment. Drought stress is reported to increase the activity of chlorophyll degrading enzyme, thus reducing chlorophyll content (Abbaszadeh *et al.*, 2007; Ajithkumar & Panneerselvam, 2014). Moreover, the reduction of chlorophyll content was related to drought duration and intensity (Anjum *et al.*, 2011). Interestingly,  $Chl_b$  of *P. massoniana* may be less sensitive to drought stress than  $Chl_{a+b}$  and  $Chl_a$ , and thus  $Chl_{a+b}$  was reduced under drought stress. In the present study,  $Chl_a$  and  $Chl_{a+b}$  had pronounced positive relationships with RWC under drought treatment, while this relationship was not detected for  $Chl_b$ , suggesting that the responses of  $Chl_a$  and  $Chl_{a+b}$  to leaf water status may be greater than  $Chl_b$ . Nonetheless, drought stress alone did not have significant effect on Car content compared with CK control, which is supported by other studies (Clement *et al.*, 2000; Shi *et al.*, 2017).

MDA is produced by lipid peroxidation, the content of which usually marks the degree of damage to cell (Arbona *et al.*, 2008; Smirnov, 1993). In this experiment, MDA content of *P. massoniana* seedlings did not change much at the earlier drought stage, but was increased at the severe drought stage, showing that cell membrane was impaired seriously by severe drought stress, which is supported by current knowledge (Liu *et al.*, 2011; Xu & Zhou, 2006). Our study also found that MDA content had negative relationship with RWC, indicating that changes in MDA were partly driven by leaf water status (Wang, 2014). Pro is one of the compatible solutes for plants, which contributes to osmotic adjustment and avoid the damage of cell membrane (Ahmed *et al.*, 2009; Hessini *et al.*, 2009). The extent of Pro accumulation was often related with the drought tolerance (Krasensky & Jonak, 2012). The present results indicated that D treatment increased Pro content compared with CK control at the end of the experiment, suggesting that *P. massoniana* can acclimate to drought stress by osmotic adjustment, which is consistent with previous studies (Liu *et al.*, 2011; Sekmen *et al.*, 2014). SOD plays a key role in plant antioxidant system (Liu *et al.*, 2009), which can promote the conversion of  $O_2^-$  into  $H_2O_2$  and thus reduces plant oxidization (Gill & Tuteja, 2010). Therefore, SOD was regarded as one of the most effective enzymatic antioxidants involved in stress tolerance (Sayfzadeh *et al.*, 2011). In our study, SOD activity was elevated as drought was prolonged in D treatment, which agrees with earlier studies (Sekmen *et al.*, 2014). Taken together, *P. massoniana* can acclimate to drought stress by morphological (i.e. increasing R/S, RMR) and biochemical (i.e. increasing proline and SOD activity) adjustments.

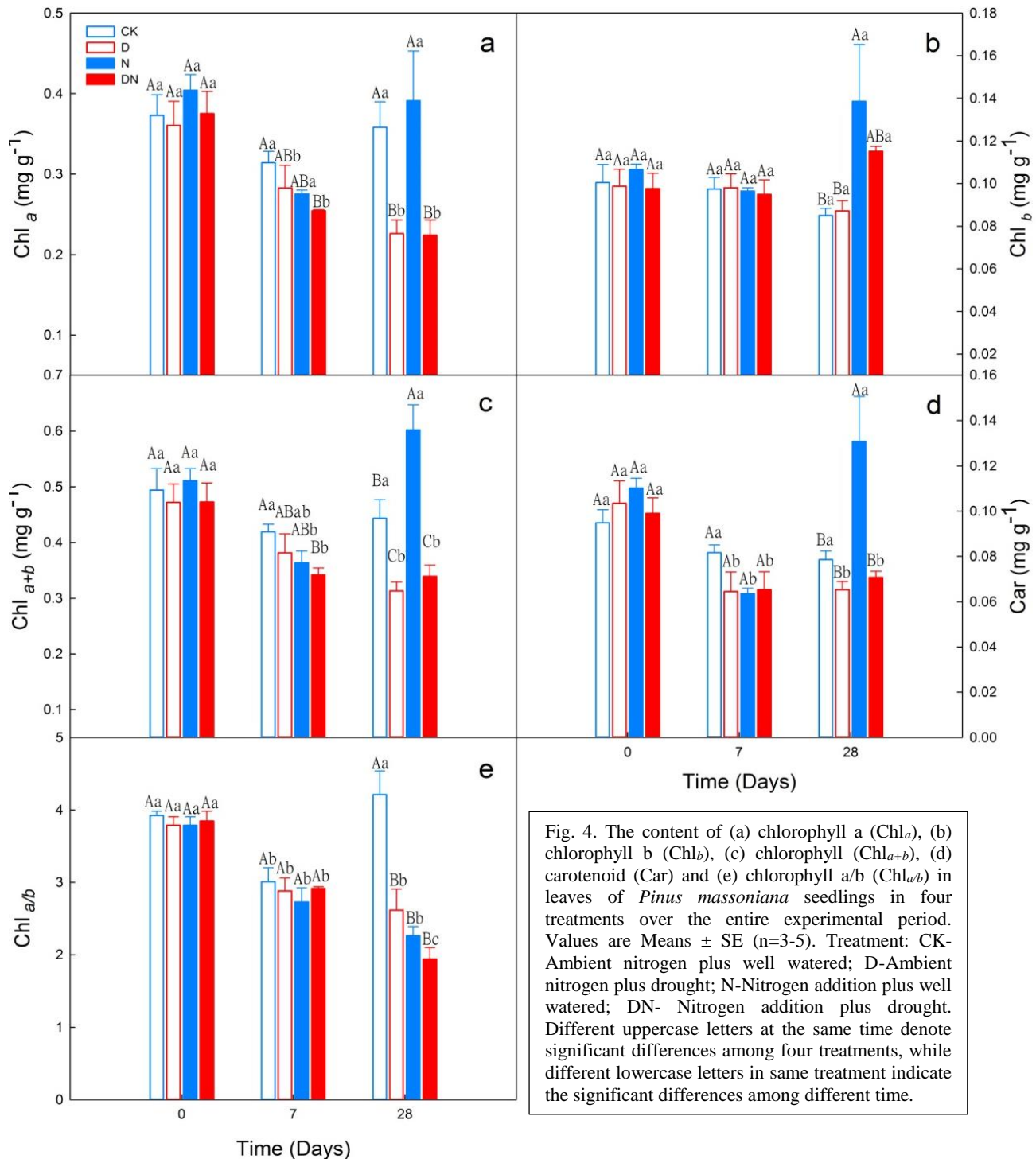


Fig. 4. The content of (a) chlorophyll a (Chl<sub>a</sub>), (b) chlorophyll b (Chl<sub>b</sub>), (c) chlorophyll (Chl<sub>a+b</sub>), (d) carotenoid (Car) and (e) chlorophyll a/b (Chl<sub>a/b</sub>) in leaves of *Pinus massoniana* seedlings in four treatments over the entire experimental period. Values are Means  $\pm$  SE (n=3-5). Treatment: CK-Ambient nitrogen plus well watered; D-Ambient nitrogen plus drought; N-Nitrogen addition plus well watered; DN- Nitrogen addition plus drought. Different uppercase letters at the same time denote significant differences among four treatments, while different lowercase letters in same treatment indicate the significant differences among different time.

#### Plant growth and physiological responses to individual nitrogen treatment:

In the present study, nitrogen addition had positive impact on biomass accumulation of *P. massoniana* seedlings, reflecting that the rate of nitrogen addition in this area is helpful for growth of *P. massoniana* seedlings. Biomass allocation was also altered by nitrogen addition. N treatment had positive effect on LMR compared with CK control, which is in agreement with other studies (Manninen *et al.*, 2009; Poorter *et al.*, 2012). But SMR was decreased in N treatment compared with CK control, and LMR response to N treatment was greater than SMR, indicating that leaf of *P. massoniana* seedlings maybe more sensitive to nitrogen addition than other organs. Moreover, compared

with CK treatment, leaf RWC and FWC were significantly increased by N treatment at the end of experiment, demonstrating that nitrogen addition had positive effect on plant water status.

Our study found that nitrogen supply significantly increased contents of leaf Chl<sub>a+b</sub>, Chl<sub>b</sub> and Car compared with CK, which agrees with earlier results (Palmroth *et al.*, 2014; Zhang *et al.*, 2014). For example, it showed that nitrogen addition increased leaf chlorophyll content of *Poplar* hybrid107 (Zhang *et al.*, 2014). Nitrogen addition did not have significant effect on MDA content in this study, suggesting that cell membrane was not impaired by the amount of nitrogen applied (Saneoka *et al.*, 2004; Zhang *et al.*, 2014).

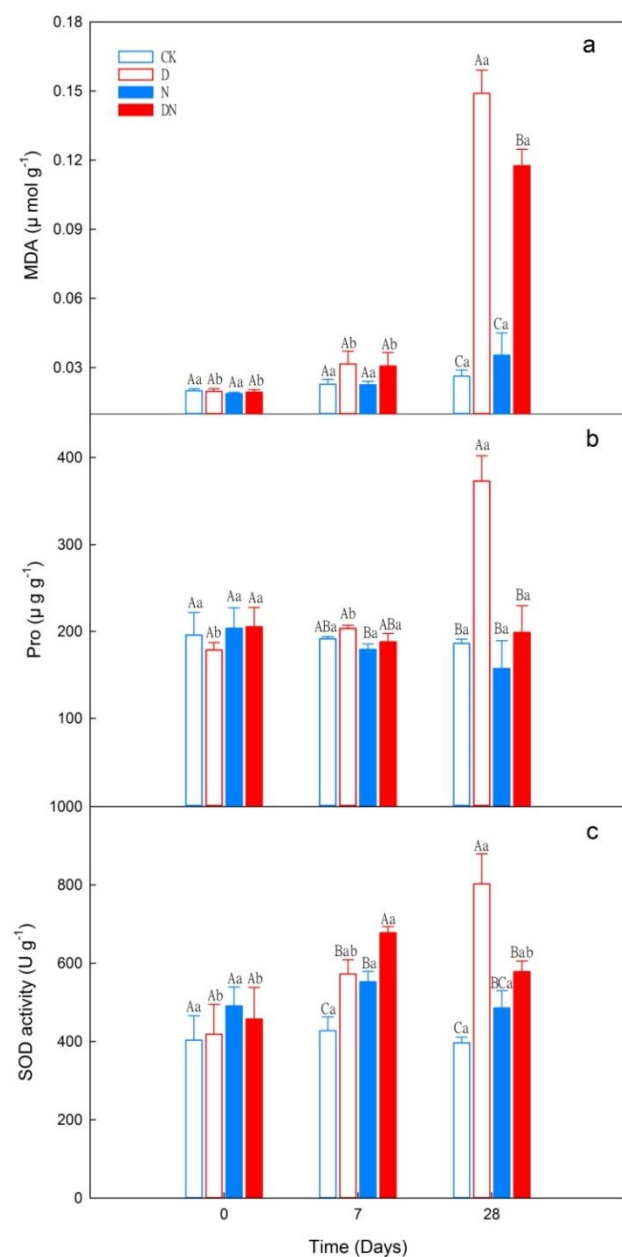


Fig. 5. The content of (a) malondialdehyde (MDA), (b) proline (Pro) and (c) superoxide dismutase (SOD) activity in leaves of *Pinus massoniana* seedlings under four treatments over the entire experimental period. Values are Means  $\pm$  SE ( $n=3-5$ ). Treatment: CK-Ambient nitrogen plus well watered; D-Ambient nitrogen plus drought; N-Nitrogen addition plus well watered; DN-Nitrogen addition plus drought. Different uppercase letters at the same time denote significant differences among four treatments, while different lowercase letters in same treatment indicate the significant differences among different time.

**Plant growth and physiological responses to combination of drought and nitrogen:** Compared to CK control, D treatment had larger negative impact on total biomass of *P. massoniana* seedlings than DN treatment, which is similar with some previous studies (Guo *et al.*, 2010; Wu *et al.*, 2008). It means that nitrogen addition could alleviate the inhibiting impacts of drought stress on growth (Ali & Golombek, 2016; Dzedek *et al.*, 2016). In the present study, however, RWC did not differ between D and DN treatments,

indicating that nitrogen addition had minimal effect on regulating plant water status. The content of  $\text{Chl}_{a+b}$  of *P. massoniana* was not increased under drought stress with nitrogen fertilization, which was inconsistent with earlier finding that leaf chlorophyll content of *Fraxinus mandshurica* were increased by two years nitrogen addition at lower precipitation (CK-30%) (Wang *et al.*, 2012). The discrepancy may be related with the duration of nitrogen addition. In addition,  $\text{Chl}_a$  and  $\text{Chl}_{a+b}$  had pronounced positive relationships with RWC similarly between D and DN treatments, suggesting that the sensitivity of chlorophyll content to RWC was not shifted with nitrogen supplies.

Compared with D treatment, the MDA content of *P. massoniana* in DN treatment was significantly decreased. Similar result was found in Saneoka *et al.* (2004) that the MDA content of *Agrostis palustris* was decreased with nitrogen fertilization under drought stress. Nitrogen addition has been found to reduce lipid peroxidation under drought stress condition (Saneoka *et al.*, 2004), thereby elevating the stability of cell membrane (Sudhakar *et al.*, 2001). We also found that the content of Pro was lower in DN compared with D treatment, which demonstrates that nitrogen addition reduced the effect of drought on Pro. It has been shown that nitrogen addition can alter carbon allocation to sinks, with more carbon for growth rather than for the improvement of stress tolerance (Zhou *et al.*, 2011). Similarly, Guo *et al.*, (2010) observed that Pro content of *Abies fabri* seedlings induced by drought stress were reduced by higher nitrogen supply. Moreover, in the present study, the responses of SOD activity to combination of drought and nitrogen are similar with MDA and Pro responses. Additionally, Wu *et al.*, (2018) found that nitrogen supply significantly reduced the SOD activity of *Moso bamboo* in water deficit condition. Our results indicated that nitrogen addition reduced the sensitivity of *P. massoniana* seedlings to drought stress by increasing the stability of cell membrane and decreasing SOD activity, thus ameliorating the negative impacts of drought to some extent.

## Conclusion

This study focused on whether nitrogen addition could ameliorate the negative impact of drought on *P. massoniana* seedlings. The contents of MDA and Pro, and SOD activity were significantly increased under drought treatment alone, while contents of  $\text{Chl}_{a+b}$ ,  $\text{Chl}_a$ ,  $\text{Chl}_{a/b}$  and RWC were significantly decreased. However, contents of MDA and Pro, and SOD activity were lower in the combination of drought and nitrogen addition than in single drought stress. We also found that the growth of *P. massoniana* seedlings was larger in the combination of drought and nitrogen compared with individual drought stress (Fig. 8). Therefore, our study suggests that nitrogen addition can ameliorate the negative impacts of drought on *P. massoniana* seedlings.

Results from our chambers study may have some variations with field conditions. For example, this study only investigated potted seedlings in a short duration, results from this study may not represent actual responses of mature trees and thus extrapolation needs careful consideration. However, our study demonstrated the possible mechanisms underlying responses of *P. massoniana* seedlings to combination of drought and nitrogen addition. This short term study suggests that current ambient nitrogen deposition rate in southern China may be helpful for establishment of *P. massoniana* seedlings in bare lands with limited water availability. Future longer term studies in the field are needed to more

accurately examine the combined effects of drought and nitrogen addition on pioneer tree species for degraded land restoration in the context of increasing drought stress and higher nitrogen deposition.

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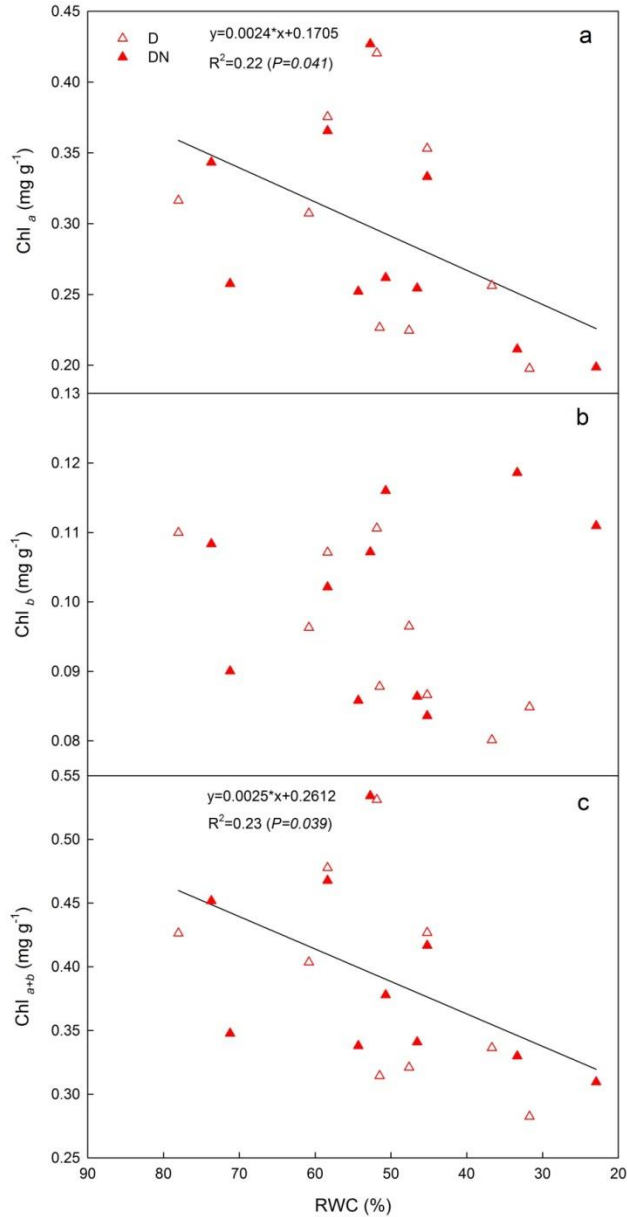


Fig. 6. (a) Leaf chlorophyll a ( $Chl_a$ ) content, (b) chlorophyll b ( $Chl_b$ ) content, and (c) chlorophyll ( $Chl_{a+b}$ ) content in leaves of *Pinus massoniana* seedlings grown under D and DN conditions as function of leaf relative water content (RWC). Data are fitted with linear functions ( $y=ax+b$ ). Treatment: D-Ambient nitrogen plus drought; DN-Nitrogen addition plus drought.

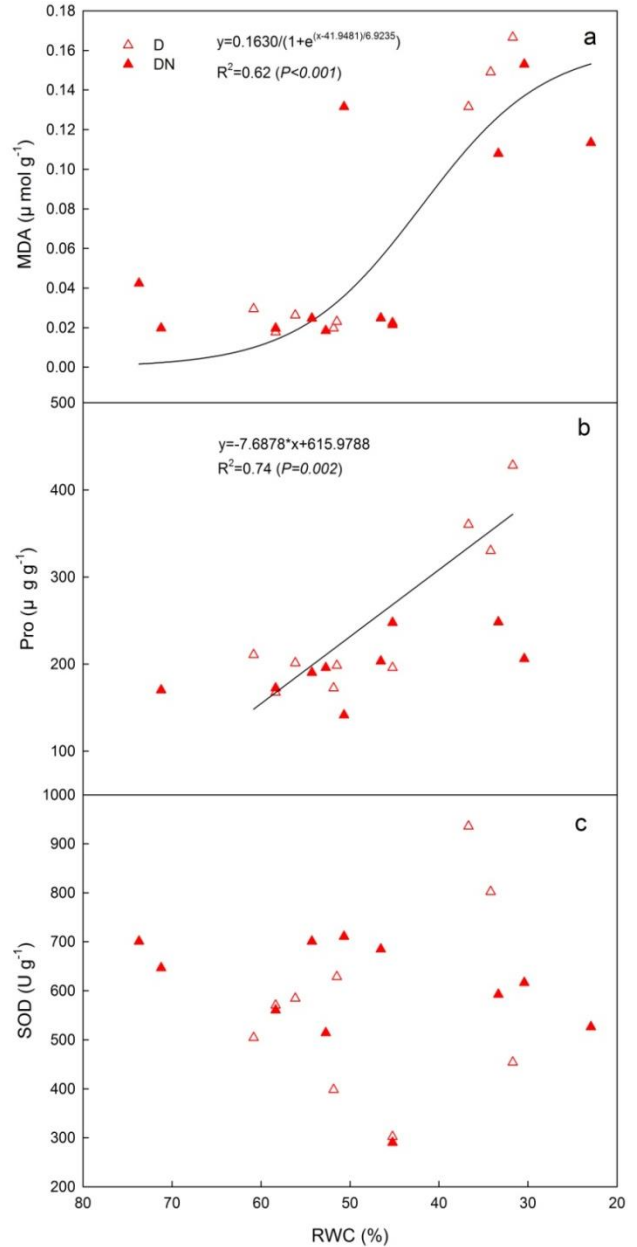


Fig. 7. (a) The content of Malondialdehyde (MDA), (b) proline (Pro), (c) Superoxide dismutase activity (SOD activity) in leaves of *Pinus massoniana* seedlings grown under D and DN conditions as function of leaf relative water content (RWC). Pro and SOD activity data are fitted with linear functions ( $y=ax+b$ ), but MDA data are fitted with sigmoid function ( $y=a/(1+\exp(-(x-x_0)/b))$ ). Only significant relationships are shown. Treatment: D-Ambient nitrogen plus drought; DN-Nitrogen addition plus drought.



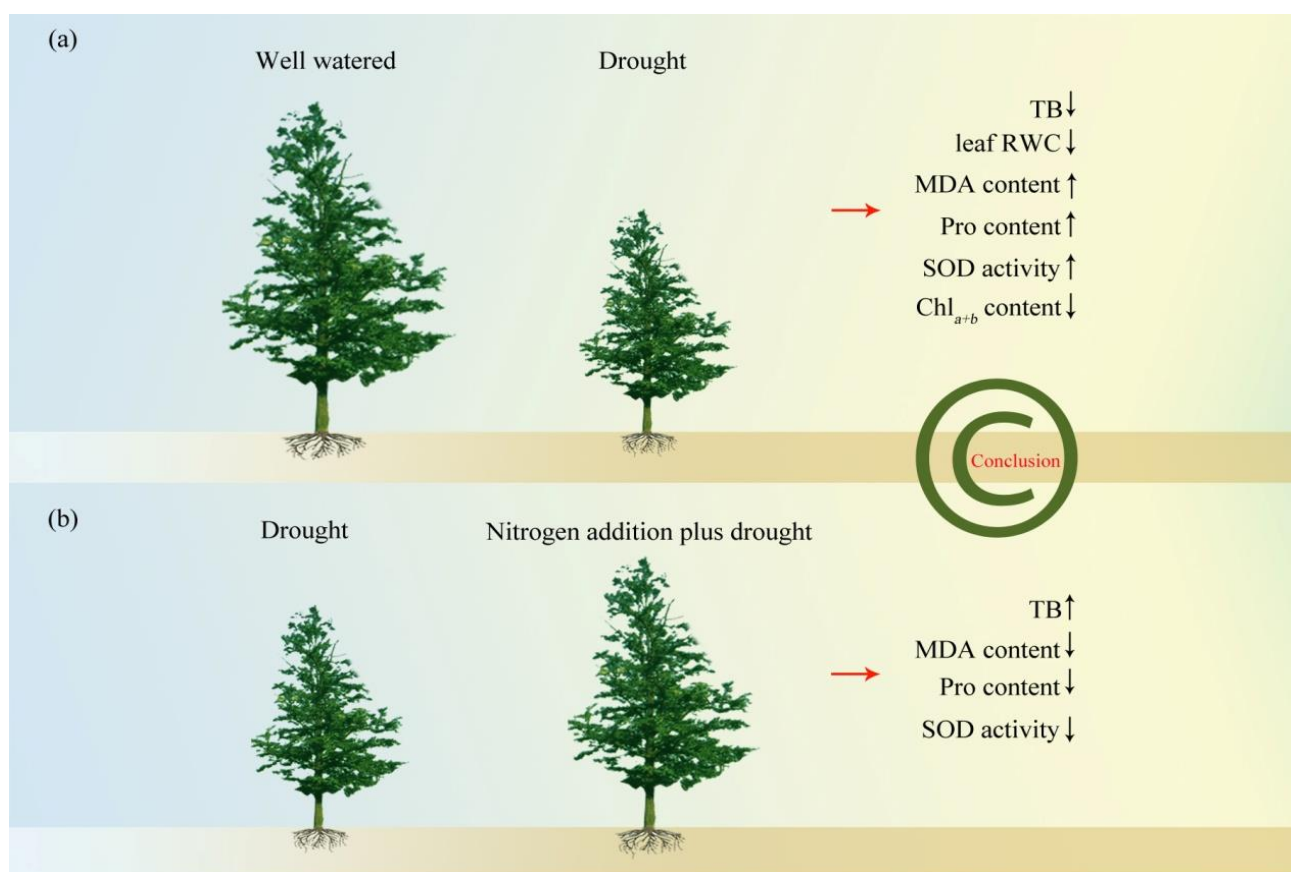


Fig. 8. (a) The effect of individual drought on the growth and leaf physiology of *Pinus massoniana* seedlings. (b) The effect of nitrogen addition on the growth and leaf physiology of *Pinus massoniana* seedlings under drought condition. Up arrow means values of the related traits increased; down arrow means values of the related traits decreased.

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