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THE PHYSIOLOGICAL RESPONSES OF *NOUELIA INSIGNIS* TOWARDS DROUGHT AND LOW TEMPERATURE

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Abstract

Climate change was increased the frequency and magnitude of drought events in southwest China, particularly in winter and spring. However, the effects of drought and low temperature on physiological characteristics of the endangered *Nouelia insignis* whose natural regeneration is poor are not clear. Seedlings subjected to each drought treatment (achievement by withholding irrigation for 0, 5 and 9 d, respectively) were randomly divided into three groups and treated at 25, 5 and -5 °C for 1.5 h, respectively. Leaf relative water content, effective quantum yield of PSII-Y(II), photochemical quenching coefficient (qp) and electron transport rate (ETR) decreased significantly and superoxide dismutase (SOD) activity increased significantly with the duration of withholding water. Pigment content, malondialdehyde (MDA) content, peroxidase (POD) activity and proline content increased significantly and maximum quantum yield of photosystem II (Fv/Fm) decreased significantly for seedlings subjected to severe drought. Y(II), qP and ETR decreased with the duration of withholding water for seedlings treated at 5 and -5°C. Fv/Fm, Y(II), qp and ETR decreased significantly, by 26.02%, 35.90%, 27.37% and 37.33%, respectively and MDA content increased significantly by 26.19% for seedlings subjected to -5°C. Fv/Fm of drought-stressed seedlings recovered to the original level after 2 d of recovery but that of freezing-treated seedlings did not recover which decreased with the duration of withholding water. In conclusion, photosynthetic activities and membrane of *N. insignis* were negatively affected by both severe drought and freezing temperature and the seedlings were more tolerant to drought than freezing temperature which might be due to the increased activities of antioxidant enzymes and accumulation of some osmolytes. Additionally, tolerance of *N. insignis* to low temperature was not enhanced by pretreatment of seedlings with drought and even diminished after being subjected to severe drought. Therefore, extreme drought and low temperature, particularly their combination, might be the important factors which limited natural regeneration of *N. insignis*.

Key words: Antioxidant enzymes, Chlorophyll fluorescence parameters, Combined stress, Osmotic adjustment.

Introduction

Abiotic stresses are key environmental factors limiting the growth, distribution and survival of plants. There exists cross talk between different stresses (Fernando, 2018). For example, water deficit stress, extreme temperature and salinity could trigger cellular dehydration and lead to osmotic stress of plants (Krasensky & Jonak, 2012; Santander *et al*., 2017). Under stresses, excessive reactive oxygen species (ROS) could accumulate which lead to oxidative stress in plants. Cell membranes, photosynthetic pigment, nucleic acids and other cellular functions will then be disrupted (Asada, 2006; Gill & Tuteja, 2010). Thylakoid membranes of chloroplasts are primary sensors of environmental changes and the photochemical process of photosynthesis is therefore liable to be affected by stresses (Kalaji *et al*., 2016; Singh & Thakur, 2018). However, plants have evolved multiple mechanisms combat various stresses. Studies have showed that plants generally activate changes at the morphological, physiological, biochemical and molecular levels to establish a new cellular homeostasis compatible with stress conditions (Barchet *et al*., 2013). For example, plants could avoid photodamage through adjusting the pigment content and dissipating excessively absorbed energy, could alleviate oxidative stress by enhancing the antioxidant defense system, and could prevent the osmotic stress by accumulating the compatible solute (Zivcak *et al*., 2016; Guo *et al*., 2018).

Most researches have focused on plant responses to individual stress. However, plants are often simultaneously exposed to different stresses in the field. Some studies have shown that combined stress alter the gene expression and metabolism of plants in a novel pattern that is different from that caused by individual stress (Bowler & Fluhr, 2000; Rizhsky *et al*., 2004; Mittler, 2006; Mahalingam, 2015). The combined effect of multiple stresses might be antagonistic or synergistic (Yadav *et al*., 2017). Therefore, the adaptation of plants to combined stress can not be extrapolated from the response of plants to individual stress. Extreme temperatures including cold and heat are generally accompanied by low soil moisture in the field. The effects of combined heat and drought on plants have been explored by many researchers (Johnson *et al*., 2014; Sattar *et al*., 2020). However, how the combination of drought and low temperature affects plants at the physiological, biochemical and molecular levels is not well-understood and related researches are rare. Zhou *et al*., (2017) reported that there existed overlaps and cross-talk between cold and drought stress response pathways in *Dianthus spiculifolius*. According to the finding of Li *et al*., (2015), mild drought treatment could improve the tolerance of wheat to subsequent low temperature.

Nouelia is a monotypic genus of the Asteraceae, and the *N. insignis* is a rare woody plant in the family. *N. insignis* is distributed in Jinsha and Nanpan drainage areas in southwest China, chiefly in the dry-hot valley of Jinsha river (Peng *et al*., 2003). Therefore, *N. insignis* possesses great value to study the phylogenetic development of Asteraceae and the flora of dry-hot valley. However, *N. insignis* has become endangered due to habitat destruction and overexploitation for firewood. According to the investigations by Luan *et al*., (2006), fewer than 80-100 individuals of *N. insignis* were found in most of its populations. Extreme weather such as drought and cold could limit plant growth and even lead to plant death. In recent years, the frequency and magnitude of drought events have been increased in southwest China, particularly in winter and spring. Drought, low temperature and their combination have affected plant growth and even led to the death of some plant species in this area. We hypothesize that the poor regeneration of *N. insignis* is related to the climate change. The stages of seed germination and seedling establishment are most vulnerable to various stresses (Leck *et al*., 2008; Kolb & Barsch, 2010). Our preliminary work showed that seed germination of *N. insignis* was sensitive to both drought and low temperature. Seed germination of *N. insignis* decreased significantly in 0.10 g ml^{-1} Polyethylene glycol and few seeds could germinate below 15°C. However, the adaptation of *N. insignis* to drought and low temperature at the early seedling stage is not clear.

The present study is to determine the physiological responses of *N. insignis* to drought, cold and combination of drought and cold in terms of leaf relative water content, pigment composition, chlorophyll fluorescence, malondialdehyde (MDA) and osmotic substance content, antioxidant enzyme activities. This study will be conducive to understand the ecological adaptation of *N. insignis* to extreme weather events and its endangered mechanisms. Therefore, it will help to increase reproduction of *N. insignis* for conservation and ecosystem restoration of the area.

Materials and Methods

Plant materials and treatments: Mature cypselas of *N. insignis* were collected from Panzhihua, Sichuan province in April 2016. The cypselas were sown in a seedling bed filled with fine sand. After 30 d, each seedling was transferred to a plastic pot filled with laterite soil and humus (1:1 v/v). The seedlings were grown in a greenhouse in which the average temperature was about 25°C and the daytime photosynthetic photon flux density was about 250-300 μmol m^{-2} s^{-1}. One-year-old seedlings were used to conduct the study. The experiment was arranged in a completely randomized design with two factors. The first factor was drought duration: drought was achieved by withholding irrigation for 0, 5 and 9 d, respectively. The second factor was low temperature: seedlings subjected to each drought treatment were randomly divided into three groups and transferred to climate chambers set at 25, 5and -5°C for 1.5 h, respectively. The conditions of treatments were chosen based on the preliminary study. Our preliminary study showed that leaves of well-watered seedlings of *N. insignis* were injured seriously after being treated at -5°C for 2.5 h and all the leaves of seedlings treated at 25°C became seriously wilted after 11 d of withholding water. Considering the combination of drought and low temperature, the duration of withholding water was set up to 9 d and the time of low temperature was set for 1.5 h. There were 12 seedlings for each treatment and the physiological characteristics of seedlings subjected to each treatment were determined. Additionally, the maximum quantum yield of photosystem II (PSII) (Fv/Fm) of seedlings that were treated at each treatment were evaluated after 2 d of rewatering at 25°C.

Relative water content of leaves: 0.5 g of leaf sample was collected from four plants per treatment. Relative water content (RWC) of leaves were determined as follows:

$$\text{RWC}\ (\%) = [(\text{FW}-\text{DW})/(\text{FW})] \times 100$$

where FW and DW are fresh weight and dry weight, respectively. DM was obtained after drying the leaf sample in an oven at 75°C for 48 h.

Pigment content: Chlorophyll and carotenoid concentrations were determined according to Wang (2006). The absorbance of the extract was measured at 665 nm, 649 nm, and 470 nm wave lengths (UV-2450, Shimadzu, Japan). Chlorophyll a (Ca), chlorophyll b (Cb), carotenoid (Car) content were evaluated using the following formulae:

Ca (mg g^{-1})=[13.95 (OD 665)-6.88 (OD 649)] ×V/(1000×W)
Cb (mg g^{-1})=[24.96 (OD 649)-7.32 (OD 665)] ×V/(1000×W)
Total cholorphyll (mg/g)=Ca+Cb
Car (mg g^{-1})= [1000 (OD 470)-2.05 Ca-114.8 Cb] ×V/(245×1000×W)

where, V=final volume made (ml), W= fresh leaf sample weight (g).

Leaf chlorophyll fluorescence: Four seedlings were selected from each treatment, and one leaf was sampled from each seedling. Fluorescence parameters were tested at indoor temperature using a chlorophyll fluorometer (PAM-2500, Walz, Germany). Seedlings were dark-adapted for 30 min before measurements were conducted. In this paper, the maximum quantum yield of photosystem II (PSII) (Fv/Fm), effective quantum yield of PS II (Y(II)), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (NPQ), non-regulated (Y(NO)) and regulated (Y(NPQ)) non-photochemical energy loss in PS II as well as electron transport rate (ETR) were measured.

MDA content: MDA content was determined according to the methods described by Wang (2006). Absorbance was read at 450, 532, and 600 nm (UV-2450).

Proline content: Proline content was determined according to the methods described by Wang (2006). Absorbance was read at 520 nm (UV-2450).

Soluble sugar: Soluble sugar content was determined according to the methods described by Farhad *et al*., (2011). Absorbance was read at 625 nm.

Antioxidant enzyme activity: Peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) activities were measured as described by Wang (2006). For determination of POD, SOD and CAT activity, the absorbance of the supernatant was read at 470nm, 560 nm and 240 nm, respectively.

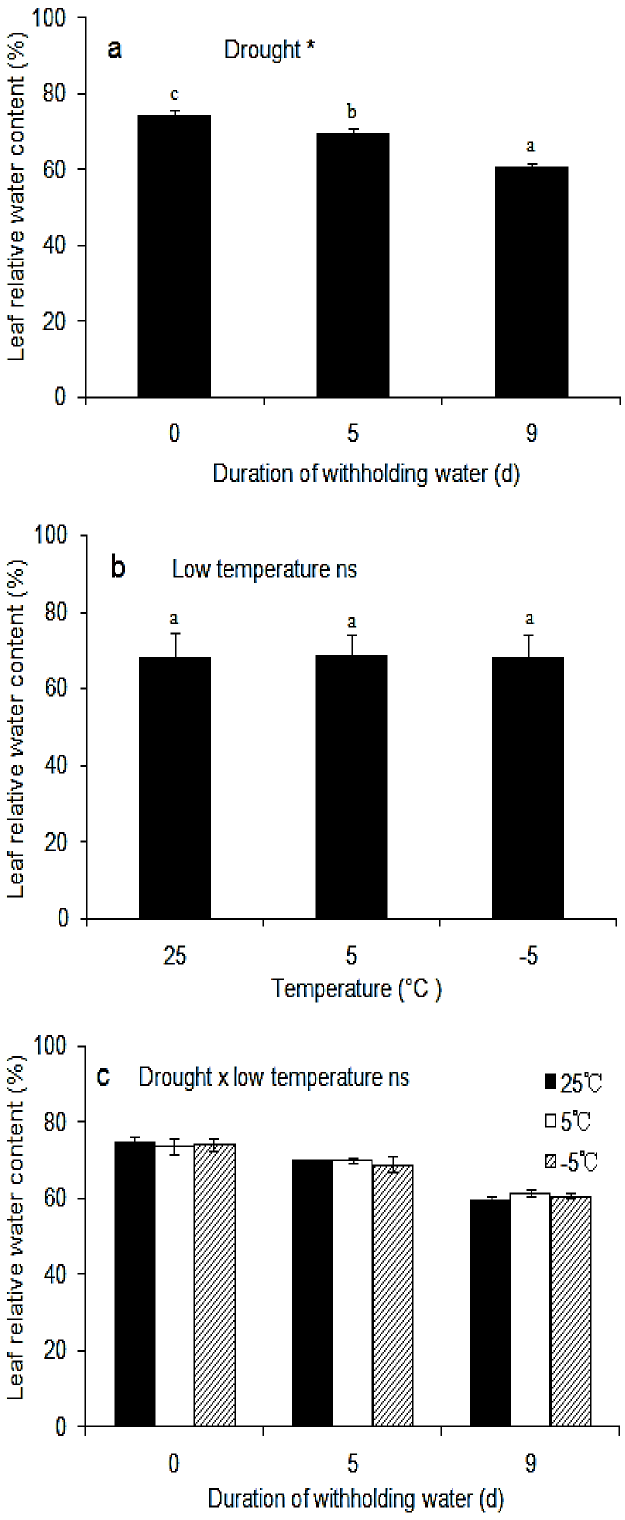

Fig. 1. Leaf relative water content of *Nouelia insignis* seedlings under drought, low temperature and combination of drought and low temperature. Two-way ANOVA analysis was performed in the general linear model. **a** Drought effect, drought was achieved by withholding water for 0, 5 and 9 d, respectively. **b** Low temperature effect, low temperature was conducted by treating seedlings at 25, 5 and -5°C for 1.5 h, respectively. **c** The interactive effect between drought and low temperature. The data represent means for each factor and means ± SD for each treatment. Comparison of the means by LSD ($p \leq 0.05$) were shown for the significant interaction between drought and low temperature. Different letters above the bars indicate significant differences among data within the same factor or interaction.

Statistical analyses: There were four replicates for each physiological parameter. All data were analyzed by two-way analysis of variance (ANOVA) in the general linear model (GLM) using SPSS 15.0 software (IBM Corp., Armonk, NY). When treatment interaction terms were significant ($p \leq 0.05$), means were separated by Fisher's least significant difference test (LSD).

Results

Leaf relative water content: Leaf relative water content was decreased significantly after 5 and 9 d of withholding water, by 6.20% and 18.43%, respectively (Fig. 1). Low temperature had no significant effects on leaf relative water content and no significant interactive effects were found between drought and low temperature.

Pigment composition: Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content did not change significantly for seedlings subjected to 5 d of withholding water but increased significant for seedlings subjected to 9 d of withholding water (Table 1). Chlorophyll a/b was decreased significantly after 5 d of withholding water but then increased to the original level after 9 d of withholding water (Table 1). Low temperature had no significant effects on pigment content and composition and no significant interactive effects were found on chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content between drought and low temperature. However, drought and low temperature had significantly interactive effect on chlorophyll a/b. Compared to the control, chlorophyll a/b was decreased significantly in seedlings subjected to combination of 5 d of withholding water and low temperature (5 and -5°C).

Chlorophyll fluorescence parameters: Both drought and low temperature had significant effects on leaf chlorophyll fluorescence parameters with significant interaction between the two factors (Table 2).Compared to that of the control, Fv/Fm and Y(NO) did not change significantly in seedlings subjected to 5 d of withholding water but Fv/Fm was decreased significantly and Y(NO) was increased significantly in seedlings subjected to 9 d of withholding water. Y(II), qP and ETR were decreased significantly with the duration of withholding water. All the tested fluorescence parameters did not change significantly in seedlings subjected to 5°C but Fv/Fm, Y(II), Y(NPQ), qP, NPQ and ETR were decreased significantly and Y(NO) was increased significantly in seedlings subjected to -5°C. Y(II), qP and ETR were decreased and Y(NO) was increased with the duration of withholding water for seedlings treated at 5 and -5°C. Drought had no significant effects but low temperature had significant effects on Fv/Fm of seedlings recovered for 2 d and no significant interactive effects were found between the two factors (Fig. 2). After 2 d of recovery, Fv/Fm of seedlings treated at -5°C did not recover to the control level which decreased with the duration of withholding water.

MDA content and antioxidant enzyme activities: Independent of temperatures, MDA content was increased significantly in seedlings subjected to 9 d of withholding water. MDA content did not change in seedlings treated at 5°C but increased significantly in seedlings treated at -5°C. No significant interactive effects were found between drought and low temperature (Table 3). CAT activity did not vary significantly with the duration of withholding water (Table 3). Low temperature had significant effects on CAT activity and significant interaction was found between drought and low temperature. Compared to the control, CAT activity was decreased significantly in seedlings subjected to 5°C but increased to the original level in seedlings subjected to -5°C. POD activity did not change significantly after 5 d of withholding water but increased significantly after 9 d of withholding water. The effect of low temperature on POD activity was not significant but the interactive effects between drought and low temperature were significant. SOD activity was increased significantly with the duration of withholding water. Low temperature had no significant direct or interactive effects on SOD activity.

Soluble sugar and proline: Neither drought nor low temperature significantly affected soluble sugar content with no significant interaction between the two factors (Fig. 3). Both drought and low temperature had significant effects on proline content with a significant interaction between the two factors (Fig. 4). Proline content did not change significantly after 5 d of withholding water but increased significantly after 9 d of withholding water, by 202.69% compared to the control. Independent of water conditions, proline content was decreased significantly with the decrease of temperature. Compared to the control, the proline content of seedlings treated by 5°C and -5°C was decreased by 25.86% and 55.80%, respectively.

Table 1. Leaf pigment content of *Nouelia insignis* seedlings under drought, cold and combination of drought and cold.

	Chlorophyll a (mg g^{-1})	Chlorophyll b (mg g^{-1})	Chlorophyll a+b (mg g^{-1})	Chlorophyll a/b	Carotenoid (mg g^{-1})
Main factors					
Drought					
0d	1.592 ± 0.309a	0.691 ± 0.137a	2.283 ± 0.438a	2.313 ± 0.185b	0.291 ± 0.054a
5d	1.423 ± 0.320a	0.676 ± 0.123a	2.099 ± 0.434a	2.100 ± 0.196a	0.250 ± 0.059a
9d	1.992 ± 0.219b	0.862 ± 0.081b	2.854 ± 0.287b	2.312 ± 0.158b	0.356 ± 0.045b
Cold					
25	1.761 ± 0.237a	0.758 ± 0.084a	2.519 ± 0.314a	2.320 ± 0.148a	0.319 ± 0.048a
5	1.620 ± 0.486a	0.728 ± 0.189a	2.348 ± 0.662a	2.223 ± 0.295a	0.290 ± 0.084a
-5	1.626 ± 0.354a	0.743 ± 0.144a	2.368 ± 0.495a	2.182 ± 0.101a	0.288 ± 0.068a
Interaction					
0d					
25	1.783 ± 0.270	0.747 ± 0.061	2.530 ± 0.329	2.379 ± 0.183c	0.322 ± 0.055
5	1.580 ± 0.425	0.672 ± 0.237	2.252 ± 0.658	2.393 ± 0.201c	0.293 ± 0.065
-5	1.413 ± 0.079	0.653 ± 0.049	2.067 ± 0.123	2.166 ± 0.093bc	0.258 ± 0.023
5d					
25	1.607 ± 0.261	0.701 ± 0.083	2.308 ± 0.342	2.284 ± 0.135bc	0.289 ± 0.054
5	1.188 ± 0.166	0.631 ± 0.113	1.819 ± 0.276	1.897 ± 0.127a	0.211 ± 0.039
-5	1.474 ± 0.395	0.695 ± 0.178	2.169 ± 0.572	2.119 ± 0.080b	0.248 ± 0.065
9d					
25	1.892 ± 0.101	0.826 ± 0.066	2.718 ± 0.150	2.296 ± 0.149bc	0.345 ± 0.018
5	2.093 ± 0.330	0.881 ± 0.121	2.974 ± 0.433	2.379 ± 0.224c	0.366 ± 0.067
-5	1.989 ± 0.170	0.880 ± 0.052	2.869 ± 0.219	2.260 ± 0.095bc	0.358 ± 0.047
Significance					
Drought	*	*	*	*	*
Cold	ns	ns	ns	ns	ns
Drought × Cold	ns	ns	ns	*	ns

Drought was achieved by withholding water for 0, 5 and 9 d, respectively. Low temperature was conducted by treatment of seedlings for 1.5 h at 25, 5 and -5°C, respectively. Two-way ANOVA analysis was performed in the general linear model. The data represent means ± SD for each factor and each treatment. * indicates $p \leq 0.05$ and ns indicates not significant. Comparison of the means by LSD ($p \leq 0.05$) were shown for the main factors of drought and low temperature and the interaction between drought and low temperature. Different letters (a-c) in the same column indicate significant differences among data within the same factor or interaction

Table 2. Chlorophyll fluorescence parameters of *Nouelia insignis* seedlingsunder drought, cold and combination of drought and cold.

	Fv/Fm	Y(II)	Y(NPQ)	Y(NO)	qp	NPQ	ETR
Main factors							
Drought							
0d	0.740±0.045b	0.462±0.103 c	0.267±0.107a	0.272±0.027a	0.752±0.103c	0.999±0.423a	43.025±9.733c
5d	0.760±0.025b	0.342±0.090b	0.366±0.108b	0.292±0.041a	0.598±0.102b	1.298±0.461b	30.800±7.788b
9d	0.562±0.274a	0.197±0.148a	0.264±0.182a	0.542±0.318b	0.407±0.227a	0.792±0.565a	18.125±13.636a
Cold							
25	0.757±0.021b	0.376±0.125b	0.332±0.120b	0.292±0.033a	0.632±0.143b	1.150±0.458b	34.958±12.087b
5	0.745±0.026b	0.384±0.107b	0.332±0.086b	0.283±0.042a	0.668±0.132b	1.183±0.309b	35.083±9.752b
-5	0.560±0.275a	0.241±0.194a	0.232±0.186a	0.530±0.328b	0.459±0.270a	0.756±0.351a	21.908±9.700a
Interaction							
0d							
25	0.753±0.020b	0.524±0.065c	0.188±0.063b	0.288±0.020b	0.792±0.070c	0.657±0.221b	49.400±6.257c
5	0.753±0.010b	0.469±0.095c	0.283±0.107bc	0.248±0.024a	0.777±0.076c	1.164±0.466c	43.225±8.855c
-5	0.714±0.074b	0.392±0.118bc	0.328±0.114c	0.280±0.025ab	0.688±0.141bc	1.177±0.395c	36.450±10.858bc
5d							
25	0.775±0.019b	0.318±0.044b	0.421±0.058c	0.262±0.014ab	0.577±0.048b	1.619±0.309c	29.025±3.989b
5	0.761±0.032b	0.393±0.079bc	0.332±0.088c	0.275±0.010ab	0.684±0.073bc	1.215±0.360c	35.325±6.220bc
-5	0.744±0.017b	0.317±0.127b	0.345±0.160c	0.339±0.037c	0.534±0.121b	1.061±0.576bc	28.050±11.234b
9d							
25	0.743±0.009b	0.287±0.083b	0.387±0.058c	0.327±0.025c	0.526±0.118b	1.176±0.087c	26.450±7.685b
5	0.721±0.016b	0.292±0.076b	0.381±0.040c	0.327±0.037c	0.542±0.121b	1.170±0.036c	26.700±7.032b
-5	0.223±0.211a	0.013±0.015a	0.024±0.028a	0.971±0.024d	0.155±0.180a	0.030±0.034a	1.225±1.415a
Significance							
Drought	*	*	*	*	*	*	*
Cold	*	*	*	*	*	*	*
Drought×cold	*	*	*	*	*	*	*

Fv/Fm: maximum quantum yield of photosystem II (PSII), Y(II): effective quantum yield of PSII, Y(NPQ): regulated non-photochemical energy loss in PS II, Y(NO): non-regulated non-photochemical energy loss in PS II, qp: photochemical quenching coefficient, NPQ: non-photochemical quenching, ETR: electron transport rate

Drought was achieved by withholding water for 0, 5 and 9 d, respectively. Low temperature was conducted by treatment of seedlings for 1.5 h at 25, 5 and -5°C, respectively. Two-way ANOVA analysis was performed in the general linear model. The data represent means ± SD for each factor and each treatment. * indicates $p \leq 0.05$ and ns indicates not significant. Comparison of the means by LSD ($p \leq 0.05$) were shown for the main factors of drought and low temperature and the interaction between drought and low temperature. Different letters (a-d) in the same column indicate significant differences among data within the same factor or interaction

Discussion

Stress stimuli could activate some signalling pathways which induce plant injury or plant adaptation. The pathways that triggered by different stresses might be co-activated under combined stress and some pathways specific for combined stress might also be activated (Rizhsky *et al.*, 2004). Therefore, plants might require unique molecular and metabolic adjustments to acclimate the combination of different stresses. Drought and low temperature are two factors which could limit plant development and distribution. With the increase of climate variability, plants are more frequently subjected to combined drought and low temperature in some areas. However, plant responses to their combined stress are not very clear.

Leaf relative water content could reflect physiological water status of plants (Sekmen *et al.*, 2014), it has often been used to indicate leaf sensitivity to drought and other types of stresses. Leaf relative water content was decreased by 6.20% and 18.43% after 5 and 9 d of withholding water, respectively. It suggested that 5 and 9 d of withholding water led to the water loss of leaves and seedlings of *N. insignis* were subjected to drought stress to a different extent. Chilling and freezing could also lead to drought stress due to reduced water absorption and cellular dehydration induced by extracellular ice formation (Hiilovaara-Teijo & Palva, 1999; Ferrando *et al.*, 2004). Although low temperature had no effect on leaf relative water content of *N. insignis*, the possibility could not be ruled out that seedlings of *N. insignis* treated at -5°C might simultaneously suffer from combined freezing and osmotic stress. In the dry-hot valley, the dry season may last for several months and drought also occurs even during the wet season due to high evaporation. Therefore, water deficit is a key factor affecting seedling growth and even the seedling survival of *N. insignis*.

Table 3. Malondialdehyde (MDA) and antioxidant enzyme activities of *Nouelia insignis* seedlings under drought, cold and combination of drought and cold.

	MDA (µmol g^{-1})	CAT (U min^{-1} g^{-1})	POD (U min^{-1} g^{-1})	SOD (U g^{-1})
Main factors				
Drought				
0d	0.030 ± 0.010a	18.667 ± 6.750a	4245.536 ± 1474.293a	313.214 ± 213.701a
5d	0.035 ± 0.012ab	19.941 ± 6.690a	4381.335 ± 1291.525a	886.072 ± 198.341b
9d	0.041 ± 0.012b	25.805 ± 17.560a	6737.421 ± 1528.531b	1098.279 ± 143.615c
Cold				
25	0.031 ± 0.010a	25.249 ± 16.199b	4947.047 ± 1342.167a	739.296 ± 459.554a
5	0.033 ± 0.012a	15.270 ± 6.676a	5323.118 ± 2485.906a	822.102 ± 300.368a
-5	0.042 ± 0.010b	23.894 ± 7.705b	5094.126 ± 1549.767a	736.166 ± 398.581a
Interaction				
0d				
25	0.021 ± 0.003	12.061 ± 4.156ab	3531.871 ± 254.018ab	141.861 ± 24.685
5	0.030 ± 0.011	21.311 ± 7.089b	4570.898 ± 1841.733ab	528.533 ± 239.263
-5	0.038 ± 0.005	22.629 ± 3.332b	4633.837 ± 1868.652ab	269.246 ± 77.782
5d				
25	0.032 ± 0.007	21.784 ± 6.041b	5223.737 ± 1279.190b	953.586 ± 130.392
5	0.035 ± 0.020	15.500 ± 3.353ab	3259.218 ± 1308.962a	823.674 ± 178.277
-5	0.038 ± 0.005	22.540 ± 8.748b	4661.050 ± 192.494ab	880.956 ± 290.096
9d				
25	0.039 ± 0.010	41.902 ± 17.052c	6085.533 ± 635.412b	1122.442 ± 154.551
5	0.034 ± 0.003	9.000 ± 0.892a	8139.238 ± 738.633c	1114.100 ± 114.823
-5	0.050 ± 0.015	26.513 ± 10.786b	5987.491 ± 1918.569b	1058.296 ± 187.915
Significance				
Drought	*	ns	*	*
Cold	*	*	ns	ns
Drought×cold	ns	*	*	ns

CAT: catalase, POD: peroxidase, SOD: superoxide dismutase

Drought was achieved by withholding water for 0, 5 and 9 d, respectively. Low temperature was conducted by cold shock of seedlings for 1.5 h at 25, 5 and -5 °C, respectively. Two-way ANOVA analysis was performed in the general linear model. The data represent means ± SD for each factor and each treatment. * indicates $p \leq 0.05$ and ns indicates not significant. Comparison of the means by LSD ($p \leq 0.05$) were shown for the main factors of drought and low temperature and the interaction between drought and low temperature. Different letters (a-c) in the same column indicate significant differences among data within the same factor or interaction

Photosynthetic chlorophyll is involved in the process of light absorption, energy transfer and electron transfer reaction. Researches in many plants showed that chlorophyll content generally decreased or remained unchanged under different stresses (Akcin & Yalcin, 2016; Guo *et al*., 2016; Kalisz *et al*., 2016). Independent of temperature, chlorophyll a, chlorophyll b, total chlorophyll did not change under moderate drought (after 5 d of withholding water) but increased significantly under severe drought (after 9 d of withholding water). It seemed that the chlorophyll content of *N. insignis* was dependent on drought severity. The increase of chlorophyll content under severe drought might be due to the loosening of combination between chlorophyll and proteins which enable chlorophyll to be more easily extracted (Strogonov, 1973). The increase of chlorophyll content under severe drought might also be related to the increase of chlorophyll concentration resulting from water loss of leaves. The increase of chlorophyll content under stresses was also found in some other plants (Wang *et al*., 2006; Fu *et al*., 2010). The adjustment of chlorophyll a/b was one of the adaptive mechanisms to various environmental factors. The change of chlorophyll a/b could reflect the change of the number of peripheral light-harvesting complexes and it vary with plant species and stress types (Utriainen & Holopainen, 2001; Wang, 2014; Schoefs *et al*., 1998; Kouril *et al*., 1999). Chlorophyll a/b of *N. insignis* decreased significantly after 5 d of withholding water. It could be seen that the number of peripheral light-harvesting complexes increased which was conducive to light absorption under moderate drought. However, to avoid photodamage by absorbing excessive light, chlorophyll a/b increased to the control level after 9 d of withholding water. Carotenoids are involved in light absorption, antioxidation, phytohormone biosynthesis and other biological process (Strzałka *et al*., 2003; Cao *et al*., 2015). Carotenoid content of *N. insignis* increased significantly after 9 d of withholding water. The increase of carotenoid content was also reported in some other plants under certain types of stresses which could protect plants from oxidative stress (Wang & Zhang, 2012; Zhang *et al*., 2015). Low temperature had no significant effect on pigment content and composition of *N. insignis*. Kalisz *et al.* (2016) reported that although photosynthetic activity was decreased significantly, chlorophyll concentration of basil was not affected by low temperature. Sofronova *et al*., (2016) reported that the content of chlorophyll was decreased and total content of carotenoids did not change upon cold treatment, however, the composition of carotenoids was considerably changed. It can be seen that the variation of pigment content and composition under stresses might reflect the different adaptive mechanisms of plants.

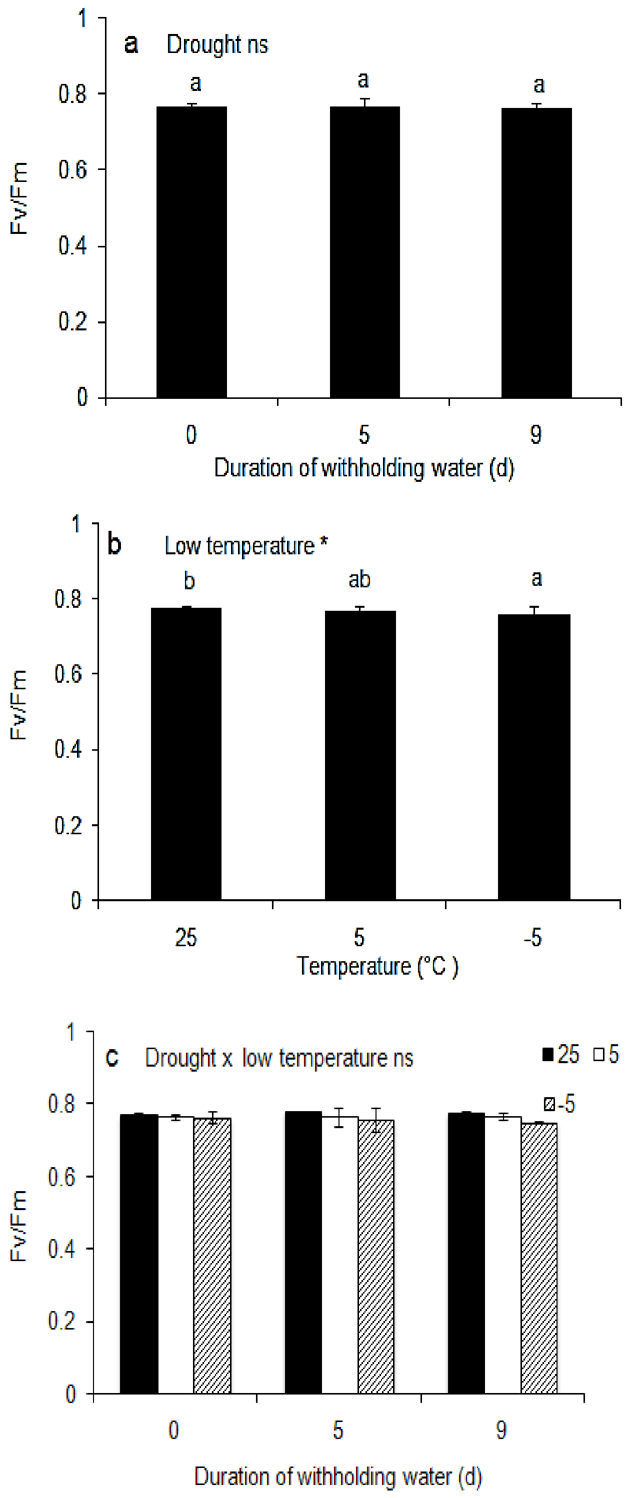

Fig. 2. Maximum quantum yield of photosystem II (Fv/Fm) of *Nouelia insignis* seedlings after rewatering for 2 d at 25°C following drought, low temperature and combination of drought and low temperature. Two-way ANOVA analysis was performed in the general linear model. **a** Drought effect, drought was achieved by withholding water for 0, 5 and 9 d, respectively. **b** Low temperature effect, low temperature was conducted by treating seedlings at 25, 5 and -5°C for 1.5 h, respectively. **c** The interactive effect between drought and low temperature. The data represent means for each factor and means ± SD for each treatment. Comparison of the means by LSD ($p \leq 0.05$) were shown for the significant interaction between drought and low temperature. Different letters above the bars indicate significant differences among data within the same factor or interaction.

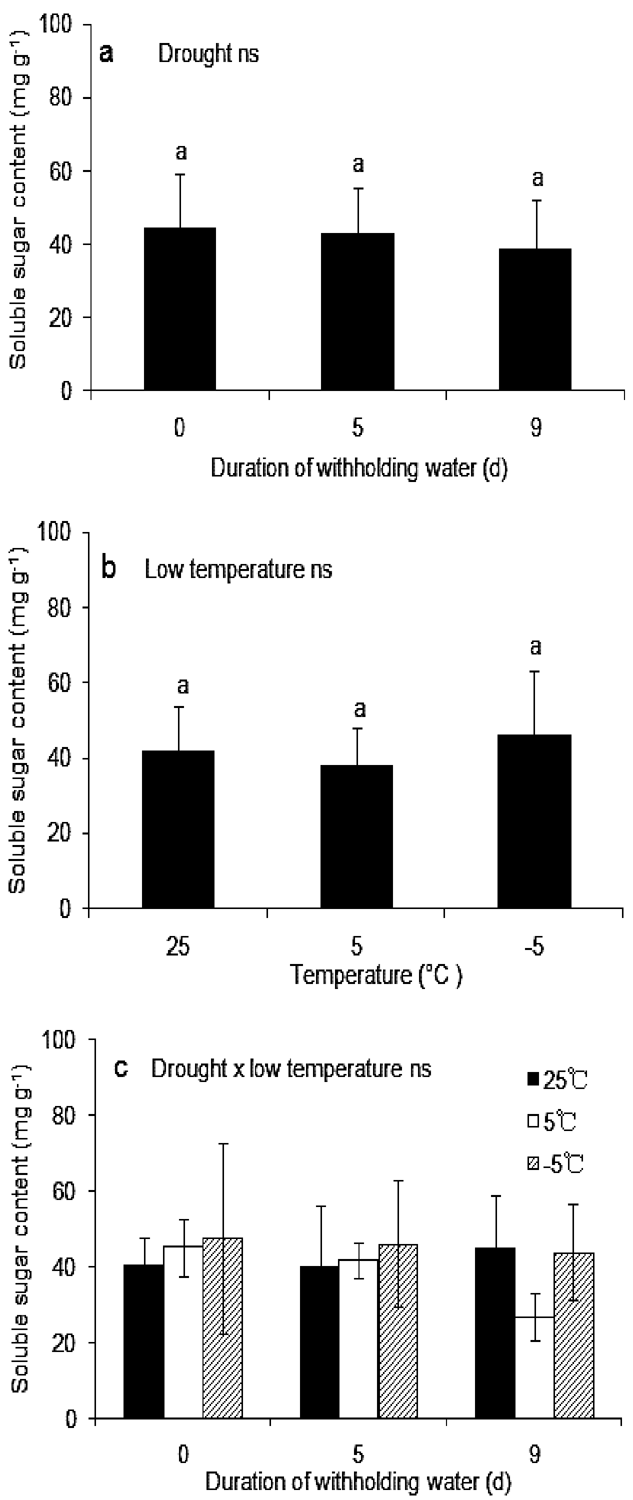

Fig. 3. Soluble sugar content of *Nouelia insignis* seedlings under drought, low temperature and combination of drought and low temperature. Two-way ANOVA analysis was performed in the general linear model. **a** Drought effect, drought was achieved by withholding water for 0, 5 and 9 d, respectively. **b** Low temperature effect, low temperature was conducted by treating seedlings at 25, 5 and -5°C for 1.5 h, respectively. **c** The interactive effect between drought and low temperature. The data represent means for each factor and means ± SD for each treatment. Comparison of the means by LSD ($p \leq 0.05$) were shown for the significant interaction between drought and low temperature. Different letters above the bars indicate significant differences among data within the same factor or interaction.

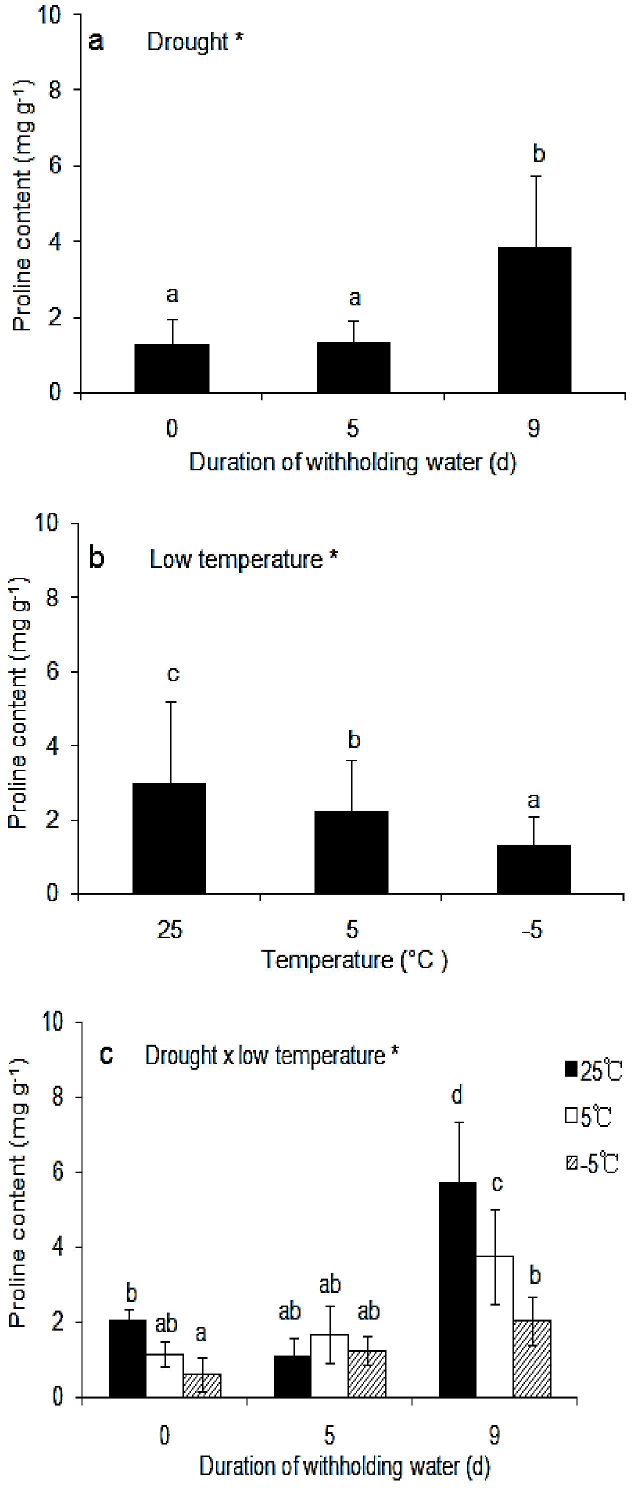

Fig. 4. Proline content of *Nouelia insignis* seedlings under drought, low temperature and combination of drought and low temperature. Two-way ANOVA analysis was performed in the general linear model. **a** Drought effect, drought was achieved by withholding water for 0, 5 and 9 d, respectively. **b** Low temperature effect, low temperature was conducted by treating seedlings at 25, 5 and -5°C for 1.5 h, respectively. **c** The interactive effect between drought and low temperature. The data represent means for each factor and means ± SD for each treatment. Comparison of the means by LSD ($p \leq 0.05$) were shown for the significant interaction between drought and low temperature. Different letters above the bars indicate significant differences among data within the same factor or interaction.

Any damage of the components of photosynthetic process caused by stresses would reduce the photosynthetic capacity (Ashraf & Harris, 2013). Chlorophyll fluorescence could sensitively reflect the physiological status of plants. Fv/Fm determines potential efficiency of PSII, Y(II) indicates the relative activity of linear electron transport, qp measures the openness of PSII centers and ETR reflects the relative rate of electron transport through PSII (Krall & Edwards, 1990; Ninemets & Kull, 2001). Y(II), qP and ETR began to decrease significantly from 5 d of withholding water and Fv/Fm decreased significantly after 9 d of withholding water. The results suggested that photosynthetic activity was decreased under drought. The increase of Y(NPQ) and NPQ after 5 d of withholding water showed that plants of *N. insignis* could dissipate the excessively absorbed light and protect the photosynthetic apparatus from photodamage under stresses (Demmig-Adams & Adams III, 2006; Wang, 2014). However, the two variables decreased to the control level after 9 d of withholding water which suggested that seedlings of *N. insignis* could not avoid photodamage through heat dissipation under severe drought. It was confirmed by the increase of Y(NO) after 9 d of withholding water demonstrating that the energy fluxes could not be adequately controlled by seedlings of *N. insignis* (Demmig-Adams & Adams III, 2006). All the tested chlorophyll fluorescence parameters did not change in seedlings treated at 5°C. The results showed that seedlings of *N. insignis* were little affected at the temperature. However, Fv/Fm, Y(II), Y(NPQ), qP, NPQ and ETR were decreased significantly and Y(NO) increased significantly in seedlings treated at -5°C. These suggested that photosynthetic activity of *N. insignis* was decreased significantly and seedlings could not dissipate the excessively absorbed light to avoid oxidative stress at -5°C. The decrease of photosynthetic activity at freezing temperature might be due to the photooxidative damage of photosynthetic components and the decreased activity of some key enzymes such as Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco). Y(II), qP and ETR were decreased and Y(NO) was increased with the duration of withholding water for seedlings treated at 5 and -5°C. This suggested that pretreatment with drought had more injurious effects on the photosynthetic process of *N. insignis* seedlings than individual low temperature applied. Fv/Fm of drought-stressed seedlings recovered to the control level after 2 d of recovery. However, Fv/Fm of seedlings treated at -5°C did not recover which decreased with the duration of withholding water. It showed that although both severe drought and freezing had deleterious effects on photosynthetic activity and membrane. Seedlings of *N. insignis* were more affected by freezing treatment, particularly by the combination of severe drought and freezing.

Under various stresses, excessive reactive oxygen species (ROS) could initiate the oxidation of biomoleculars such as lipids and proteins and ultimately lead to the inactivation of cellular functions (Anjum *et al.*, 2015). MDA is the lipid-oxidation product and generally used as an important biomarker of oxidative stress. MDA content of *N. insignis* increased significantly in seedlings subjected to 9 d of withholding water or -5°C than those in seedlings treated by other water conditions or temperatures, respectively. This demonstrated that seedlings of *N. insignis* were exposed to oxidative stress after severe drought or freezing treatments. The results were coincident with the chlorophyll fluorescence parameters. Antioxidant enzymes play key roles as ROS scavengers in plant adaptation to various stresses (Hasanuzzaman *et al*., 2012). However, different enzymes might present different responses to various stresses and the specific enzymes that participate in the resistance of stresses might depend on some factors such as plant species and stress types (Zhou *et al*., 2015; Yuan *et al*., 2016). In the present study, CAT, POD and SOD of *N. insignis* also showed different change pattern after drought treatments. CAT activity did not vary, POD activity increased only after severe drought but SOD activity increased after both of mild and severe drought. It can be seen that POD and SOD were involved in the acclimation of *N. insignis* to drought. Except that CAT activity was decreased significantly in seedlings treated at 5°C compared to the control, POD and SOD activities did not vary with the decrease of temperatures. It suggesting that the three enzymes did not contribute to the tolerance of *N. insignis* to low temperature.

The accumulation of osmotic substances contributes to the tolerance of plants to some types of stresses (Kaushal *et al*., 2011; Suprasanna *et al*., 2016). Besides their roles in an osmotic adjustment under conditions causing cellular dehydration, osmolytes could also act as osmoprotectants in protecting cellular structure and function (Bhandari & Nayyar, 2014). However, the relevance and relative contribution of specific osmolytes to stress tolerance vary with plant species, cultivars and stress intensity (Ruelland *et al*., 2009; Wu *et al*., 2013; Bhandari & Nayyar, 2014). Independent of temperatures, soluble sugar content did not vary with the duration of withholding water but proline content increased significantly after 9 d of withholding water. The results indicated that proline might be involved in the adaptation of *N. insignis* to severe drought. The content of soluble sugar was not affected by low temperature but proline content decreased significantly with the decrease of temperature. It can be seen that neither soluble sugar nor proline played roles in the tolerance of *N. insignis* to low temperatures. Many studies showed that proline accumulated under low temperatures. However, its role in the improvement of freezing tolerance is controversial (Ruelland *et al*., 2009). The decrease of proline content under low temperature was also found in other plants such as grapes and *Clematis heracleifolia* (Ai *et al*., 2004; Ma & Yue, 2010). However, the mechanism of this phenomenon is not clear and should be studied in future. Based on the results of pigment, chlorophyll fluorescence parameters and MDA, seedlings of *N. insignis* were not significantly affected at 5°C. As none of the antioxidant enzymes and osmotic substances determined in the present study was involved in the tolerance of *N. insignis* to low temperature, it could be inferred that seedlings *N. insignis* might adopt some other pathways against moderate low temperature.

Conclusions

Both severe drought and low temperature adversely affected plant photosynthetic activities and membrane integrity of *N. insignis*. Particularly, the effects were aggravated when severe drought and freezing temperature occurred simultaneously. Seedlings of *N. insignis* were more tolerant to drought than low temperature which was due to the improvement of some antioxidant enzyme activities and accumulation of some osmotic substances. In the natural habitat of *N. insignis*, severe drought is sometimes accompanied by extremely low temperature which could lead to plant injury or even the death of some plant species. Such extreme weather events might be one of the factors limiting the natural regeneration of *N. insignis*. During the vegetation restoration of *N. insignis*, control of water deficit is practicable to alleviate the damaging effects of these stresses mentioned above. Studies have shown that application of hydrogel could improve the water status of seedlings exposed to water deficit stress (Mudhanganyi *et al*., 2018). Therefore, hydrogel might be used to ensure that seedlings of *N. insignis* could survive the severe water drought stress, thereby alleviating the combined stress of water deficit and heat.

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