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Physiological responses to drought in three provenances of *Dioscorea nipponica* Makino

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Summary

Dioscorea nipponica Makino is an optimal candidate to develop the diosgenin industry in North China. Given the large fluctuations in soil water availability induced by global climate change, information on drought tolerance of this species is urgently needed. Thus, seedlings of three provenances, selected from Manghe, Pangquangou and Luyashan Nature Reserves in Shanxi Province, were exposed to 70%-85%, 55%-60%, 40%-45% and 20%-35% of water holding capacity, representing normal-watered, light drought stress, moderate drought stress and severe drought stress, respectively. Thirteen indices concerning plant water status, photosynthesis, antioxidants and osmotic regulation were recorded. Principal Component Analysis was applied to identify indices with a high contribution to drought tolerance, fulfilled by the average of subordinate function values (\bar{X}_{ij}) of drought tolerance index (X_{ij}).

We found that rhizome-propagated seedlings of *D. nipponica* Makino could survive eighty days of severe drought. The drought tolerance of this species is achieved mainly through physiological responses including decreased photosynthesis, increased activity of antioxidant enzymes, and accumulation of osmotic regulating compounds. The means of drought tolerance index for the provenances Manghe, Pangquangou and Luyashan were 0.29, 0.68 and 0.50, respectively. Pangquangou provenance showed higher drought tolerance than the other two, indicating that it might be a good candidate for cross breeding to combat the increasing drought climate in Shanxi Province.

Keywords: *Dioscorea nipponica* Makino; provenance; physiological response; drought tolerance

Introduction

The genus *Dioscorea* (commonly known as yam, family Dioscoreaceae) comprises over 600 species with about 70 sections. In Sect. *Stenophora* Uline, there are about 30 species, 2 varieties and 1 subspecies (GAO et al., 2008; JING et al., 2017). Most of them have well-developed rhizomes, which are generally used as an important raw material for the industrialized production of diosgenin (DU et al., 2017). Diosgenin (CAS number 512-04-9) is a typical initial intermediate to produce steroids and contraceptives. Currently, 60% of steroidal drugs and contraceptives are produced from diosgenin, and it is therefore in great demand (DIBY et al., 2009; REN et al., 2015). Moreover, it is also an effective medicine to treat leukemia, hypercholesterolemia, climacteric syndrome and colon cancer (PAN et al., 2014).

As one of the main factors that determine plant growth, vitality, and survival worldwide (MCDOWELL et al., 2011; LÉVESQUE et al., 2013), drought has been a predominant constraint for root/tuber crop production. Growth constraint and yam yield reduction have been reported for *D. alata* L. and *D. rotundata* Poir. when growing under water stress (DIBY et al., 2009). However, the extents of drought sen-

sitivity and yield reduction of yams are still unclear due to the limited experimental data (DARYSNTO et al., 2016). Given that large fluctuations in soil water availability may occur due to altered precipitation patterns induced by climate change, there is a great need for a better understanding of the resilience of rhizome-bearing species to water stress. *Dioscorea* spp. not only include plants with different sex and different ploidy levels, mating system and geographical distribution, but also has a high level of genetic diversity within populations based on its biological characteristics (NUR FATIHAH et al., 2017). Therefore, it is necessary and possible to select better varieties for sustainable production in sedentary cropping systems (DIBY et al., 2009; NUR FATIHAH et al., 2017).

In China, *D. zingiberensis* C. H. Wright and *D. nipponica* Makino are the two main plants that produce diosgenins (PAN et al., 2014; REN et al., 2015; LI et al., 2017). *D. zingiberensis* C. H. Wright is endemic to China (REN et al., 2015), and mainly distributed in the south of Qinling Mountain (GAO et al., 2008), its geographic range is 98°53'-121°50' E, 23°42'-32°10' N (Fig. 1). Given that the population of *D. zingiberensis* C. H. Wright has been decreasing for many years because of the overexploitation, *D. nipponica* Makino is becoming more popular for the production of diosgenin and medicinal material (YUAN et al., 2007). *D. nipponica* Makino is predominantly distributed in forest gaps and edges of mountainous areas in North China (GAO et al., 2008; CHO et al., 2013), its geographic range is about 105°-109°E, 26°34'-50°15' (Fig. 1). It is also found in Korea and Japan (WOO et al., 2013).

D. nipponica Makino is a perennial herb, its vine climbs in the clockwise direction and measures up to five meters. It has shiny green alternate leaves with a long petiole. The rhizome with cork layer is readily peeled or flaked off (GAO et al., 2008). Just like other yam plants, *D. nipponica* Makino possesses a vegetative cycle with two phases: vegetative growth from emergence (EM) to rhizome initiation (RI) and rhizome growth, from RI to harvest (HA) (MARCOS et al., 2009). During vegetative growth, leaves and stems are the dominant sinks for assimilates, while the storage parts are the dominant sinks once rhizomes are initiated. Changes in assimilate partitioning throughout the life cycle are controlled mainly by organ development. Thus, the vegetative growth affects assimilate partitioning and is detrimental to rhizome initiation and yield (MARCOS et al., 2009).

Increased drought has already occurred as a consequence of warmer temperatures and decreased precipitation in North China (ZHANG et al., 2014). Climate scenarios for the coming decades indicate even drier summers in regions such as Shanxi Province (Fig. 2). Plants in these areas are likely to experience more frequent water deficits (GAO et al., 2017). Careful attention must be paid to the selection of appropriate provenances because genetics appear to overwhelm biotic facilitation in such severe environmental conditions (BINGHAM and SIMARD, 2013). Little information is available on the drought tolerance of *D. nipponica* Makino from Shanxi Province. Furthermore, the traditionally extensive collection of its rhizomes in the wild is threatening the local populations of *D. nipponica* Makino.

In this study, we selected three provenances (Manghe, Pangquangou and Luyashan) of *D. nipponica* Makino from Shanxi Province,

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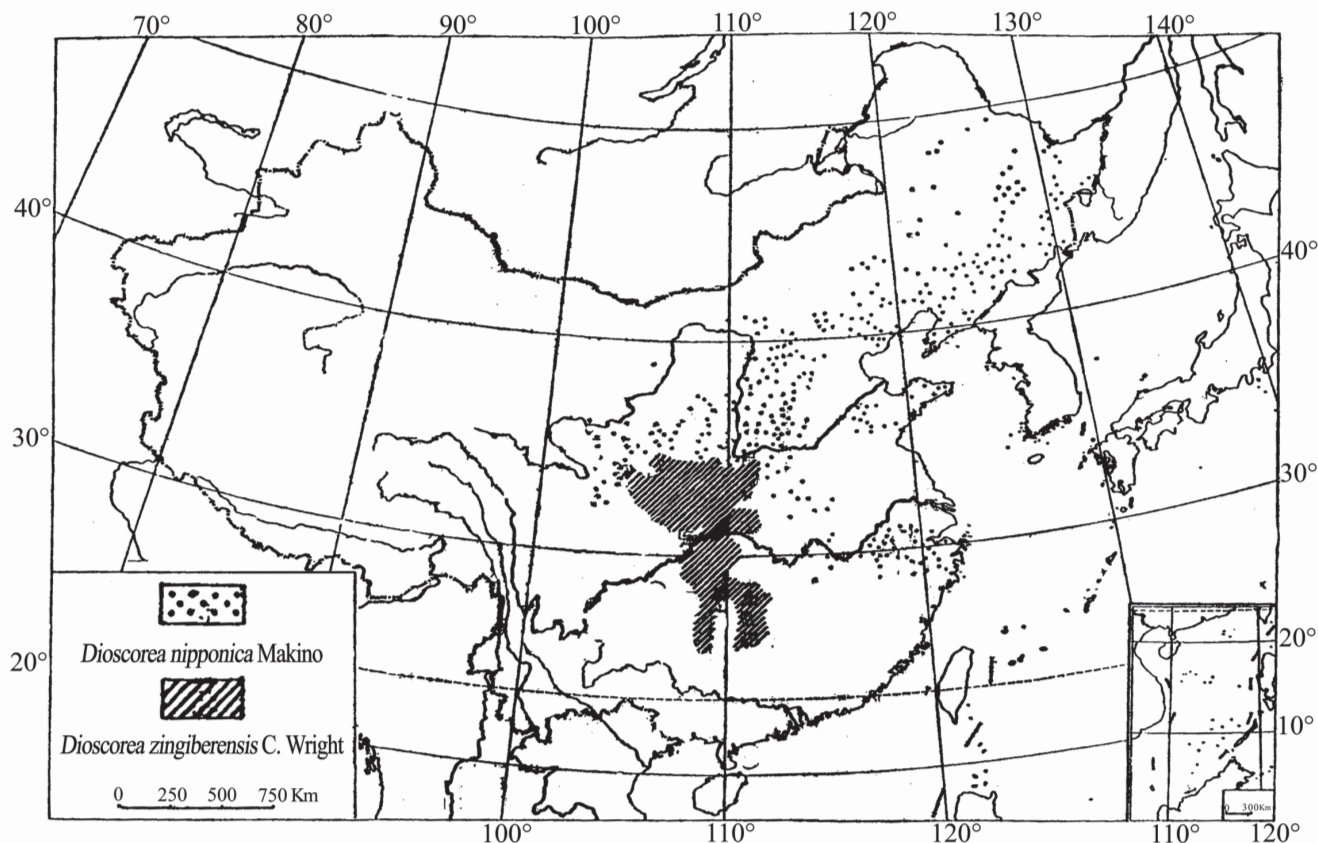


Fig. 1: Natural distribution of *Dioscorea zingiberensis* C. H. Wright and *Dioscorea nipponica* Makino in China.

China with the aim to (i) determine the variation of physiological responses to drought stress, (ii) identify the relationship of physiological responses with the drought stress, and (iii) select a suitable provenance to plant in Shanxi Province under future climate conditions. The finding of this study will contribute to a successful propagation and breeding program for *D. nipponica* Makino.

Materials and methods

Three provenances of *Dioscorea nipponica* Makino

From September to October 2014, rhizomes of *D. nipponica* Makino were selected from its three natural habitats in Shanxi Province: Manghe, Pangquangou and Luyashan Nature Reserves. The geographical sites are shown in Fig. 3, and environmental factors are presented in Tab. 1.

Manghe Nature Reserve (112°22'10"-112°31'35" E, 35°12'30"-35°17'20" N), locates in Yangcheng county, in the south of Shanxi Province. It is characterized by warm temperate semi-humid and continental monsoon climate. The typical vegetation is warm temperate deciduous forest, dominated by *Quercus* spp. and *Carpinus turczaninowii* Hance.

Pangquangou Nature Reserve (111°22'33"-111°32'22" E, 37°47'45"-37°55'50" N), locates in the junction of Jiaocheng and Fangshan county, the center of Shanxi Province. This region belongs to warm temperate continental mountain climate. 74% of this region is covered by forest dominated by coniferous and broadleaved species, such as *Picea wilsonii* Mast., *P. meyeri* Rehd. et Wils., *Larix gmelinii* (Rupr.) Kuzen. var. *principis-rupprechtii* (Mayr) Pilg., *Pinus tabulaeformis* Carr., *Betula albosinensis* Burk., *B. platyphylla* Suk., *Q. wutaishanica* Mayr, *Populus cathayana* Rehd..

Luyashan Nature Reserve (111°46' to 112°54' E, 38°36' to 39°02' N), locates in the junction of Ningwu county, Kelan County and Wu-

zhai county, where is the north of Shanxi Province. Similar to Pangquangou Nature Reserve, Luyashan belongs to the warm temperate continental mountain climate. About 50% of this region is covered by forest dominated by tree species similar to Pangquangou, such as *P. wilsonii* Mast., *P. meyeri* Rehd. et Wils. and *L. gmelinii* (Rupr.) Kuzen. var. *principis-rupprechtii* (Mayr) Pilg.

For each provenance, the rhizomes were placed in plastic drums and covered by moist sand in winter 2014 to prepare for planting in 2015.

Drought stress experimental design

In April 2015, rhizomes were taken out and cut to pieces of 8 to 10 cm length with 2 to 3 buds, and planted in the nursery of Forestry College, Shanxi Agricultural University. The site locates in Taigu County, Shanxi Province. It belongs to warm temperate continental climate, with a mean annual temperature of 10.1 °C, a mean annual precipitation of 410 mm, a mean frost-free period of 176 days. Each rhizome section was soaked and disinfected by the solution of carbendazim powder, 2-3 rhizome sections were then set in a container ($r = 25$ cm, $h = 21$ cm, with 4 water pores), filled with an equal weight of seedling substrate (pH 7.0, organic matter $\geq 50\%$). About 10 days later, new buds emerged and the seedlings were tended by general field management.

The experiment to evaluate the responses of the species origins to water deficit was conducted for 80 days in a greenhouse at Shanxi Agricultural University, Taigu, Shanxi Province. In late May 2015, healthy six-week-old seedlings of similar sizes were randomly selected. The factorial combination of four water regimes and three provenances was arranged in a completely randomized design with six replicates. Seedlings were exposed to water regimes by withholding water to 70%-85%, 55%-60%, 40%-45% and 20%-35% water holding capacity (WHC), respectively representing control (normal-

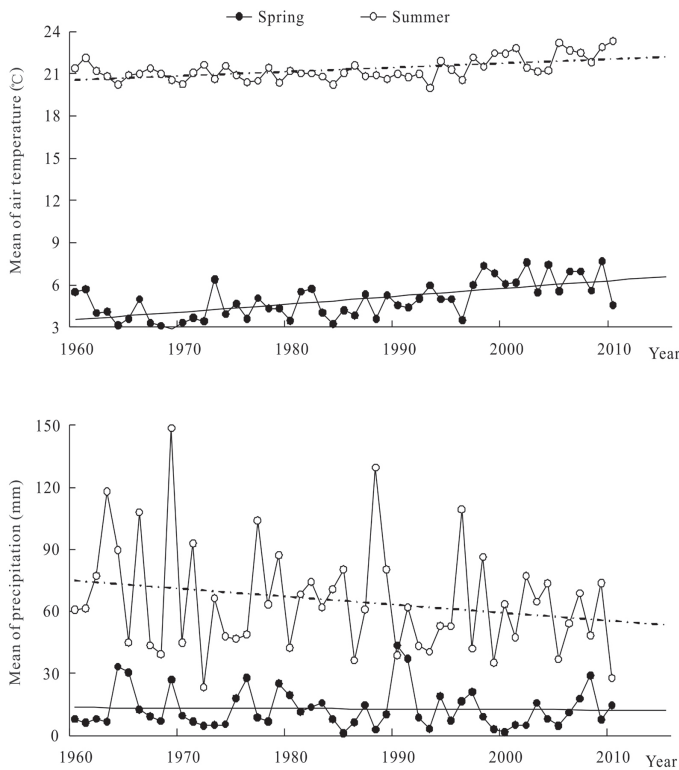


Fig. 2: Mean air temperature and precipitation of spring and summer in Shanxi province during 1960-2010. Data was collected from China Meteorological Information Center. The figure indicated the increasing drought in future in Shanxi Province. Temperature had been significantly increased about 2.5 °C and 1.5 °C in spring and summer, respectively ($P < 0.01$). Precipitation dramatically fluctuated ($P > 0.05$), but with a declining tendency during this period. The mean of decrease was about 20%.

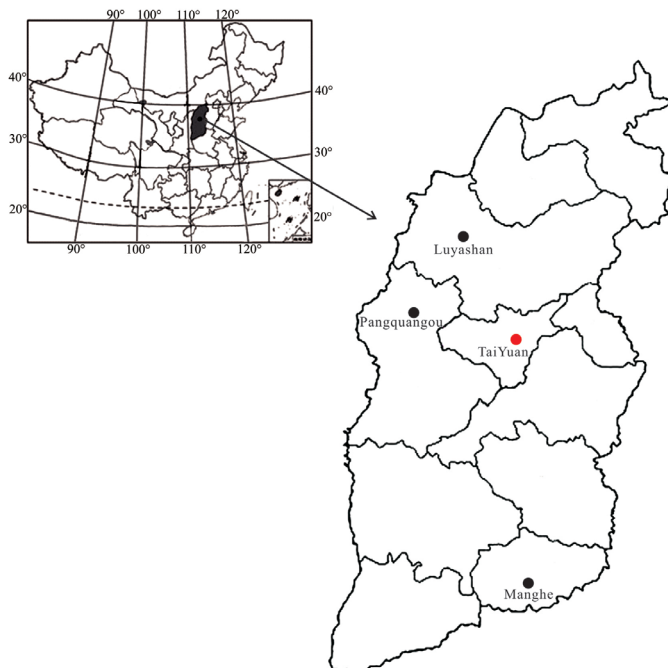


Fig. 3: Geographical sites of Manghe, Pangquangou and Luyashan Nature Reserves, showing relative location of three provenances in Shanxi Province.

Tab. 1: Environmental characteristics of Manghe, Pangquangou, and Luyashan Nature Reserves in Shanxi Province.

Factor	Manghe	Pangquangou	Luyashan
Altitude (m)	300-1572	780-2838	1260-2736
Annual temperature (°C)	14	4.3	4.0
Annual precipitation (mm)	600-800	450-700	500-600
Annual frost-free period (days)	180-240	180	90-120

watered, NW), light drought stress (LD), moderate drought stress (MD) and severe drought stress (SD) (OLAREWAJU et al., 2017; GAO et al., 2017; BISTGANI et al., 2017; OLAREWAJU et al., 2017). All the seedlings were watered with tap water using a 150 mL beaker to allow the water permeating into peat moss slowly and thus minimizing gravimetric water loss. Relative humidity was approximately 50% and temperature fluctuated from 20 °C (night) to 25 °C (day). Water content was controlled by determining the weight of each pot every other day and additional watering to replace the amount of transpired water. A mixed nutrient solution (50 mL, N:P:K, 15:15:10) at a concentration of 5 g·L⁻¹ was added to the water once every two weeks. The containers were moved randomly periodically to reduce edge effects.

Parameter measurements

120 days after planting, three plants of each treatment were randomly selected and three mature leaves in the middle of stem were used per plant for parameter measurements. Fresh weight (FW) and, after 24 h at 70 °C, dry weight (DW) were recorded and the leaf water content (LWC, FW%) was calculated using the equation:

$$\text{LWC (FW\%)} = (\text{FW} - \text{DW}) / \text{FW} \times 100$$

Content of chlorophyll (Chl) in leaves was measured as described by LI (2000). Fresh leaves (0.5 g) were cut and dipped in 80% acetone overnight at 4 °C. Then, the extract was centrifuged for 5 min at 10,000 r·min⁻¹. Absorbance of the supernatant was recorded at 645 nm and 663 nm using a spectrophotometer. Stomatal conductance (G_s), intercellular CO₂ concentration (C_i), net photosynthetic rate (P_n) and transpiration rate (T_r) were measured at 9:00-11:00 on five consecutive sunny days by an open gas-exchange system (LI-6400, Li-Cor, Inc., Lincoln, NE), a vapor pressure deficit of 1.5-1.9 KPa, an irradiation of 800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, an ambient CO₂ concentration of 370 $\mu\text{mol}\cdot\text{mol}^{-1}$ controlled by a CO₂ injector. The ambient environment was of 375±20 $\mu\text{mol}\cdot\text{mol}^{-1}$ (atmospheric concentration of CO₂), 35±8 % (relative humidity) and 25-30 °C (atmospheric temperature). Saturating light was used for all gas-exchange measurements to exclude bias by daily light fluctuations and limitation of photosynthetic capacity due to suboptimal light. Water use efficiency (WUE), defined as the ratio of net photosynthetic rate to transpiration rate (P_n / T_r), was calculated.

Fresh leaves (0.2 g each sample) were ground with pestle and mortar containing 1.6 mL phosphate buffer (0.05 M, pH 7.8). Then the extract was centrifuged at 12,000 r·min⁻¹ at 4 °C for 15 min. The supernatant was used for determining the activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). The enzyme activities were recorded on the basis of total soluble proteins. The method described by LI (2000) was followed for measuring SOD and CAT activities. 3 mL reaction mixture containing nitroblue tetrazolium (NBT, 10 μL), riboflavin (10 μL), methionine (Met, 2.7 mL), ethylene diamine tetraacetic acid disodium salt (EDTA-Na₂, 1 μL) and buffer (9 μL) were added to each test tube. All test tubes were kept under light for 20 min. Then absorbance of the mixture was recorded at 560 nm using a spectrophotometer. The reaction solution composed of leaf extract, phosphate buffer (0.15 M, pH 7.0) and hydrogen peroxide (30%) was used for the determination of CAT

activity. The reaction was initiated by adding the enzyme extract and changes in absorbance were recorded at 240 nm every 40 s.

According to the method of LI (2000), the thiobarbituric acid (TBA) assay was used for determining malondialdehyde (MDA) content in leaf samples. Fresh leaf material (1 g) was homogenized in 2 mL of 10% (w/v) TCA, then it was centrifuged at 4000 r·min⁻¹ for 10 min. To 2 mL of the supernatant, 2 mL of 0.6% (w/v) TBA in 10% TCA was added. The mixture was kept at 95 °C for 15 min and ice cooled, then mixture was centrifuged for 5 min at 4,000 r·min⁻¹, its absorbance was measured at 532 nm and 600 nm using a spectrophotometer.

Three regulating compounds, proline (Pro), soluble sugars (SS) and soluble protein (SP), were measured by methods of LI (2000). Pro: Fresh leaf (0.5 g) was ground in 5 mL of 3% sulfosalicylic acid (w/v) and the extract was filtered. 2 mL of the filtrate was taken in a test tube, along with 2 mL of acid ninhydrin solution and 2 mL of glacial acetic acid. Then the test tubes were heated in a water bath at 100 °C for 0.5 h. Thereafter, the test tubes were ice-cooled and 4 mL of toluene was added. The supernatant was centrifuged at 3,000 r·min⁻¹ for 5 min. The optical density of the mixture was recorded at 520 nm. SS: Homogenized pulp with pericarp (0.1 g) was extracted with 5 mL of distilled water. Extraction was repeated washed with distilled water and supernatants were collected into 25 mL volumetric flasks. The extract was pipetted from each treatment into separate test tubes, and then tubes were placed into a boiling water bath for 10 min. 1 mL of extract, 1 mL of distilled water and 0.5 mL of anthrone reagent (10 mg in 5 mL H₂SO₄) were added in each test tube and placed in ice cold water. The intensity of color was read at 620 nm on spectrophotometer. SP: Fresh leaf material (0.5 g) was homogenized with 5 mL of distilled water in an ice bath. The aliquot was centrifuged at 10,000 r·min⁻¹ for 10 min at 4 °C. 5 mL solution of coomassie brilliant blue (CBB) was added in 0.2 mL of supernatant. The absorbance was measured at 595 nm.

Statistical analysis

Data are represented as mean value for each treatment. Differences among the treatments for each provenance of *D. nipponica* Makino were determined using analysis of variance (ANOVA). All statistical analyses were performed using SPSS 22.0 software (SPSS, Chicago, IL, USA) and post-hoc testing was carried out using Duncan's Multiple Range Test. The values of $P < 0.05$ was considered as being significantly different.

Drought tolerance evaluation of provenances

Given the potential correlation between the selected thirteen parameters, we used Principal Component Analysis (PCA) to select the principal components for further evaluation (LIU et al., 2015; ZHENG et al., 2017). Drought tolerance index (X_{ij}) (LIU et al., 2015) of each parameter was calculated as follows,

$$X_{ij} = \text{value of drought stress} / \text{value of control}$$

Fuzzy comprehensive evaluation was used to achieve the drought tolerance assessment of each provenance (CHENG and TAO, 2010). The formulas of subordinate function values (\hat{X}_{ij}) are as follows,

$$\hat{X}_{ij} = (X_{ij} - X_{jmin}) / (X_{jmax} - X_{jmin})$$

\hat{X}_{ij} means subordinate function value of the j parameter for the provenance i ; X_{ij} means the measured value of the j parameter for the provenance i ; X_{jmax} and X_{jmin} means the maximum and minimum value of the j parameter for three provenances.

$$\bar{X}_i = \frac{1}{n} \sum_{j=1}^n \hat{X}_{ij}$$

Results

Leaf water content

FW of leaves showed no significant difference by the effects of provenances, drought regimes and their combinations ($P > 0.05$, Tab. 2). DW of leaves was influenced by the water regimes in three provenances ($P < 0.05$, Tab. 2). LWC showed significant variations by the effects of provenance, drought level and their combination ($P < 0.01$) (Tab. 2). As expected, the highest value of LWC was recorded under NW condition and as drought stress increased, it showed a reduced trend (Tab. 3). Compared to seedlings of NW condition, the LWC of seedlings under SD condition reduced by 13.57%, 15.78% and 6.11% in samples from Manghe, Pangquangou and Luyashan, respectively (Tab. 3).

Chlorophyll content and photosynthesis

The results indicated that content of Chl was affected by drought levels, provenances and their combinations ($P < 0.001$, Tab. 2). Within each water regime, samples from Manghe had the highest Chl followed by that of Pangquang and Luyashan provenances (Fig. 4, A). Chl was influenced by drought in all provenances, but the influences were different between the provenances (Fig. 4, A). Manghe and Luyashan provenances showed a similar increase of Chl by water deficit. It was constantly raised with the increasing of drought stress

Tab. 2: F values derived from analysis of variance (ANOVA) of data for physiological attributes on three provenances of *Dioscorea nipponica* Makino exposed to varying water regimes.

Source of variations	df	FW		DW		LWC(FW%)	
Water regime (W)	3	0.195 ns		4.41 *		57.93 ***	
Provenance (P)	2	1.484 ns		0.35 ns		11.12 ***	
W × P	6	1.060 ns		1.90 ns		5.95 **	
Source of variations	df	Chl	G_s	C_i	P_n	T_r	WUE
Water regime (W)	3	69.52 ***	88.51 ***	69.16 ***	154.57 ***	156.11 ***	23.64 ***
Provenance (P)	2	2149.92 ***	6.04 **	3.64 *	17.30 ***	26.52 ***	6.78 **
W × P	6	47.54 ***	0.61 ns	4.70 **	2.87 *	7.26 ***	1.64 ns
Source of variations	df	SOD	CAT	MDA	Pro	SS	SP
Water regime (W)	3	28.39 ***	41.09 ***	47.83 ***	12.02 ***	21.67 ***	12.32 ***
Provenance (P)	2	39.41 ***	1.92 ns	248.94 ***	43.68 ***	8.74 **	282.44 ***
W × P	6	3.14 *	1.99 ns	25.95 ***	19.41 ***	8.94 ***	31.07 **

FW, fresh weight of leaf; DW, dry weight of leaf; LWC (FW%), leaf water content of fresh weight; Chl, chlorophyll concentration; G_s , stomatal conductance; C_i , intercellular CO₂ concentration; P_n , net photosynthetic rate; T_r , transpiration rate; WUE, water use efficiency; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; Pro, proline; SS, soluble sugar; SP, soluble protein. *, ** and *** indicate significant difference at level of 0.05, 0.01 and 0.001, respectively; ns indicates no significance at level of 0.05.

Tab. 3: The effect of drought on leaf fresh weight (FW), leaf dry weight (DW) and leaf water content (LWC, FW%) of three provenances for *Dioscorea nipponica* Makino.

Provenance	Drought	FW (g)	DW (g)	LWC (FW%)
Manghe	NW	0.54±0.04 a	0.19±0.01 c	65.61±2.04 a
	LD	0.52±0.03 a	0.20±0.00 abc	61.52±1.80 cd
	MD	0.59±0.02 a	0.25±0.01 a	58.08±0.45 ef
	SD	0.54±0.01 a	0.24±0.01 abc	56.71±0.62 f
Pangquangou	NW	0.53±0.09 a	0.19±0.03 c	64.47±1.09 ab
	LD	0.53±0.09 a	0.20±0.03 bc	62.91±1.51 bc
	MD	0.51±0.02 a	0.22±0.01 abc	57.25±0.84 f
	SD	0.51±0.08 a	0.23±0.04 abc	54.29±0.51 g
Luyashan	NW	0.59±0.14 a	0.22±0.05 abc	63.52±0.91 abc
	LD	0.57±0.04 a	0.21±0.02 abc	63.22±1.54 abc
	MD	0.50±0.05 a	0.19±0.03 c	62.59±1.66 bc
	SD	0.60±0.02 a	0.24±0.00 ab	59.64±1.77 de

Values are means±SE of three replicates. Bars with different letters show significant differences at $P < 0.05$ (LSD).

and mounted to the highest value of 477.50 $\text{mg} \cdot \text{g}^{-1}$ FW (Manghe) and 235.26 $\text{mg} \cdot \text{g}^{-1}$ FW (Luyashan) under MD condition, increased by 30.00% and 67.55%, respectively. Conversely, content of Chl was constantly dropped by increasing drought stress in samples from Pangquangou, the decrease of LD, MD and SD condition was 5.77%, 7.56% and 14.92%, respectively.

In our study, based on ANOVA results (Tab. 2), significant differences were found between drought treatments and provenances in the level of G_s ($P < 0.01$, Tab. 2). Regardless of provenances, the leaf G_s constantly decreased by increasing drought stress (Fig. 4, B). Under SD condition, seedlings of Manghe, Pangquangou and Luyashan reduced G_s to the minimums of 4.83 $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 6.17 $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and 6.53 $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, respectively. Compared to NW seedlings, the decreases of above provenances were 79.81%, 72.54% and 74.49%, respectively. G_s was insignificantly different between three provenances under NW and SD conditions; While under LD and MD conditions, G_s of samples from Luyashan was higher than that of the other two provenances.

According to ANOVA results (Tab. 2), C_i was significantly affected by drought stress, provenances and their combinations. C_i of Manghe and Pangquangou provenances kept no change under LD and MD

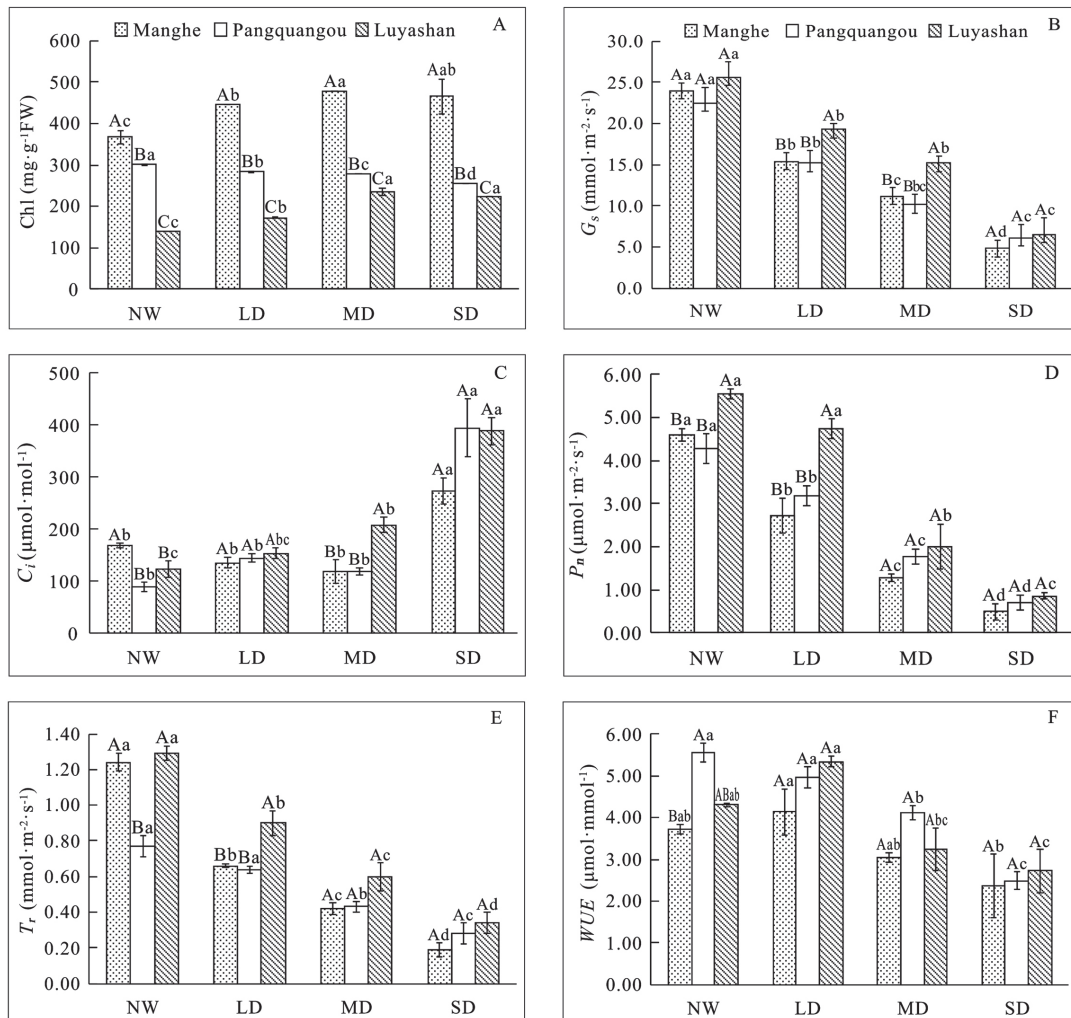


Fig. 4: Photosynthesis indexes of three provenances of *Dioscorea nipponica* Makino treated by four water regimes. Six indexes included Chlorophyll concentration (Chl, $\text{mg} \cdot \text{g}^{-1}$ FW), stomatal conductance (G_s , $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$); intercellular CO_2 concentration (C_i , $\mu\text{mol} \cdot \text{mol}^{-1}$); net photosynthetic rate (P_n , $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), transpiration rate (T_r , $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and water use efficiency (WUE, $\mu\text{mol} \cdot \text{mmol}^{-1}$). Three provenances involved Manghe, Pangquangou and Luyashan. Four water regimes involved normal-watered (NW, 70%-85% water holding capacity, WHC), light drought stress (LD, 55%-60% WHC), moderate drought stress (MD, 40%-45% WHC) and severe drought stress (SD, 20%-35% WHC). Different capital letters indicate significant difference among three provenances at $P < 0.05$; Different lower-case letters indicate significant difference among four water regimes at $P < 0.05$.

conditions, but were sharply increased by SD; while C_i of samples from Luyashan rose significantly by MD and SD (Fig. 4, C). Under SD condition, C_i of Manghe, Pangquangou and Luyashan provenances rose to the highest value of $274.33 \mu\text{mol}\cdot\text{mol}^{-1}$, $395.00 \mu\text{mol}\cdot\text{mol}^{-1}$ and $388.67 \mu\text{mol}\cdot\text{mol}^{-1}$, respectively. Contrasted to NW seedlings, the maximum increase of C_i occurred in Pangquangou provenance by about 342.18%.

Both P_n and T_r showed significant difference between drought levels, provenances and their combinations (Tab. 2). As expected, P_n and T_r followed a similar reduction trend in three provenances (Fig. 4, D, E). Under NW and LD conditions, samples from Luyashan had the highest P_n and this difference disappeared under MD and SD conditions. Under SD condition, P_n of samples from Manghe, Pangquangou and Luyashan decreased to the least of $0.49 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, $0.70 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $0.86 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the decreases were 89.35%, 83.64% and 84.50%, respectively, compared to NW seedlings (Fig. 4, D). T_r of Manghe and Luyashan provenances was significantly higher than that of Pangquangou in NW condition. T_r showed no difference between provenances but under MD and SD conditions. The maximum decrease of T_r occurred under SD condition by about 84.68%, 63.64% and 73.64% in samples from Manghe, Pangquangou and Luyashan, respectively. (Fig. 4, E).

WUE of sampled leaves was affected by drought stress ($P < 0.01$, Tab. 2). In contrast to expect, WUE had shown a decline trend by increasing drought stress in three provenances (Fig. 4, F). Under SD condition, WUE of samples from Manghe, Pangquangou and Luyashan dropped to $2.37 \mu\text{mol}\cdot\text{mmol}^{-1}$, $2.49 \mu\text{mol}\cdot\text{mmol}^{-1}$ and $2.73 \mu\text{mol}\cdot\text{mmol}^{-1}$, decreased by 36.29%, 55.22% and 36.51%, respectively. WUE was only found different between three provenances under NW condition: samples from Pangquangou had the highest value, followed by that of Luyashan and Manghe, successively.

Antioxidant activity

It was found that activity of SOD in leaves was significantly affected by drought stress, provenances and their combinations (Tab. 2). Activity of SOD increased considerably under water deficit conditions (Fig. 5, A). The highest values of three provenances presented under LD condition, activities of SOD in samples from Manghe, Pangquangou and Luyashan were mounted to 6.20, 1.67 and 5.60 times of that of NW seedlings, respectively. SOD of Manghe and Pangquangou provenances were then consecutively declined by MD and SD, but even under SD condition, activities of SOD for Manghe and Pangquangou provenances were 69.91% and 52.74% higher than that of NW seedlings. Activity of SOD showed a fluctuating trend by the increasing drought stress in Luyashan provenance, the increases of LD, MD and SD condition were 560.3%, 302.9% and 535.0%, respectively.

Increasing drought stress caused a significant increment in activity of CAT, but with a gradual trend (Fig. 5, B). Under NW condition, seedlings had the least value, $1.34 \text{U}\cdot\text{g}\cdot\text{min}^{-1}$ FW, $0.99 \text{U}\cdot\text{g}\cdot\text{min}^{-1}$ FW and $1.16 \text{U}\cdot\text{g}\cdot\text{min}^{-1}$ FW in samples from Manghe, Pangquangou and Luyashan, respectively. While under SD condition, the increments of activity of CAT in Manghe, Pangquangou and Luyashan provenances were 39.39%, 100% and 69.83%, respectively. Within each water regime, no difference was observed in activity of CAT between provenances and their combinations (Tab. 2, Fig. 5, B).

Tab. 2 also showed that content of MDA was significantly affected by water regimes, provenances and their combinations (Tab. 2, $P < 0.001$). Generally, content of MDA raised with the increasing drought stress. The least content of MDA was observed under NW condition in three provenances. The maximum increase of MDA for Manghe and Pangquangou provenances both occurred under LD condition, increased by 56.29% and 73.89%, respectively. The most increment of MDA for Luyashan provenance (182.07%) was observed under

SD condition (Figure 5, C). Within each water regime, samples from Manghe had the highest content of MDA among the three provenances (Fig. 5, C).

Osmotic regulation

Leaf content of Pro, SS and SP were all significantly different between water regimes, provenances and their combinations ($P < 0.01$, Tab. 2).

Seedlings grown under drought stress accumulated considerable amounts of Pro (Fig. 5, D). A linear increase was found in Pro between two provenances (Manghe and Pangquangou) and drought severity. The least values of both provenances were recorded under NW condition. Under SD condition, seedlings of Manghe and Pangquangou provenances accumulated 140.09% and 122.22% more Pro than that of NW condition. No stable trend of Pro was observed in Luyashan provenance, the maximum value occurred under LD condition, increased by 129.50%; content of Pro then declined with the increasing drought stress, to the minimum of $28.43 \mu\text{g}\cdot\text{mL}^{-1}$ FW under SD condition. Under MD and SD conditions, content of Pro for Pangquangou provenance was significantly higher than the other two provenances.

Similar to Pro, content of SS in leaves from Pangquangou and Luyashan was also increased by drought stress (Fig. 5, E). However, the increase was not as great as that of Pro. The increase of SS for samples from Pangquangou was 53.08% under LD, 52.74% under MD and 23.63% under SD, respectively. The increase of SS for Luyashan provenance was 37.08% under LD, 43.27% under MD and 31.64% under SD, respectively. It indicated that increasing drought stress had no further impacts on SS. Content of SS was detected stable in Manghe provenance (Fig. 5, E).

As to Pangquangou and Luyashan provenances, content of SP took on a raising trend under LD condition, then declined to the minimums of $8.88 \text{mg}\cdot\text{g}^{-1}$ FW and $6.50 \text{mg}\cdot\text{g}^{-1}$ FW under SD condition, decreased by 32.68% and 17.72% than that of NW condition, respectively. Surprisingly, content of SP displayed an undulate trend with water deficiency in Manghe provenance: declined 14.97% by LD, increased 33.56% by MD, fell down gradually under SD condition. Regardless of water regimes, samples from Pangquangou had the highest content of SP among the three provenances.

Drought tolerance evaluation of provenances

The different contribution of thirteen parameters could be seen in Tab. 4. The contribution of the first principal component was 44.06%, mainly composed of P_n , G_s , T_r , CAT and LWC; The contribution of the second principal component was 21.26%, mainly composed of SP and Pro. The total contribution rate of the first two principal components amounted to 65.32%, including a large number of information on drought responses of *D. nipponica* Makino. Therefore, these seven parameters were chosen to the evaluation of drought tolerance. Subordinate function values of the drought tolerance indexes were shown in Tab. 5. The mean of subordinate function value of MD and SD for Manghe, Pangquangou and Luyashan provenances was 0.29, 0.68 and 0.50, respectively. The order of drought tolerance of three provenances was, Pangquangou > Luyashan > Manghe.

Discussion

Physiological responses of *Dioscorea nipponica* Makino to drought

It is much easier for pot experiments to control water content and other environmental factors that interact with water availability (KOLENC et al., 2016). For this purpose, physiological responses of *D. nipponica* Makino have been screened under well-controlled conditions in which the watering regime was defined on the basis of the water holding capacity (WHC). In this study, we found that drought

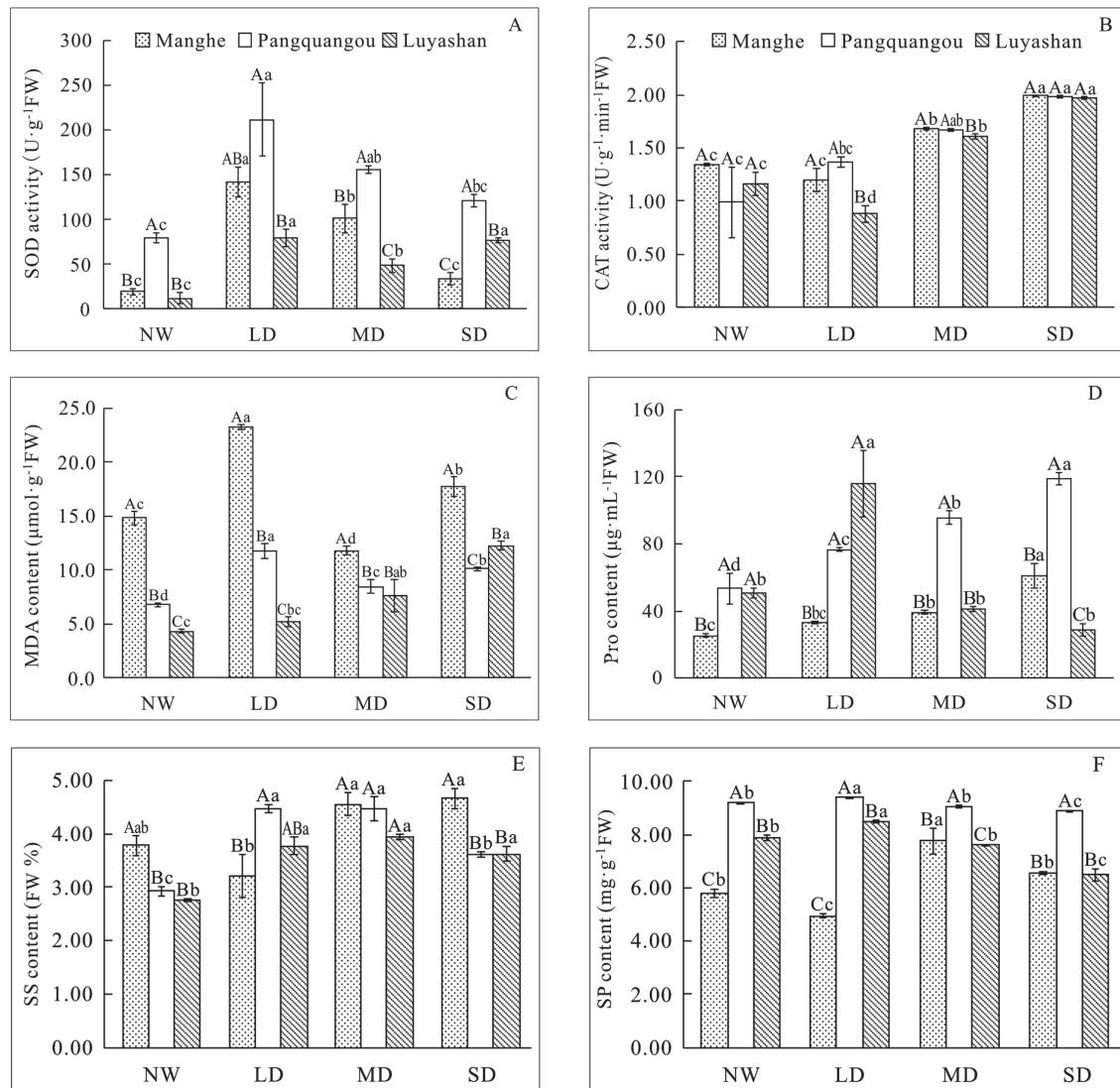


Fig. 5: Antioxidant-response and osmotic-regulation indexes of three provenances of *Dioscorea nipponica* Makino treated by four water regimes. Three antioxidant-response indexes included superoxide dismutase (SOD, $U \cdot g^{-1} \cdot FW$), catalase (CAT, $U \cdot g^{-1} \cdot min^{-1} \cdot FW$) and malondialdehyde (MDA, $\mu mol \cdot g^{-1} \cdot FW$). Three osmotic substances included proline (Pro, $\mu g \cdot mL^{-1} \cdot FW$), soluble sugar (SS, FW%) and soluble protein (SP, $mg \cdot g^{-1} \cdot FW$). Three provenances involved Manghe, Pangquangou and Luyashan. Four water regimes involved normal-watered (NW, 70%-85% water holding capacity, WHC), light drought stress (LD, 55%-60% WHC), moderate drought stress (MD, 40%-45% WHC) and severe drought stress (SD, 20%-35% WHC). Different capital letters indicate significant difference among three provenances at $P < 0.05$; Different lower-case letters indicate significant difference among four water regimes at $P < 0.05$.

stress affected plant water status, chlorophyll content, gas exchange parameters, activities of antioxidant enzymes and osmotic regulating compounds in three provenances of *D. nipponica* Makino.

During drought stress, plant water content is one of the most important physiological and reliable indices to evaluate the tolerance or resistance of plants. It has been indicated that plant water content was declined by drought stress in several species (KHOYERDI et al., 2016; KOLENC et al., 2016). As expected, LWC reduced with increasing drought stress in three provenances of *D. nipponica* Makino.

Photosynthesis is the primary process affected by drought stress, as a consequence of pigments changes, stomatal closure or low cellular water potential (MARCINSKA et al., 2013; KOLENC et al., 2016; BISTGANI et al., 2017). In our study, content of Chl showed a significant decrease with increasing drought stress in Pangquangou provenance. Such a response of reduced Chl is also known from other studies (KHOYERDI et al., 2016; BISTGANI et al., 2017; RAZZAQ et al., 2017), which may be due to perturbation in biosynthesis and degradation by chlorophyllase (KHOYERDI et al., 2016). During drier periods, re-

duction in available soil water resulted in a decrease of G_s to limit evaporative losses, thereby both P_n and T_r reduced (RUIZ et al., 2013; KOLENC et al., 2016; MAHALINGAM, 2017; TRENTACOSTEA et al., 2018). In this paper, the reduction of water status induced a significant decrease in G_s , P_n and T_r , but an increase in C_i of *D. nipponica* Makino. The increase of C_i was mainly because that the decreasing G_s limited the exchange of intercellular CO_2 and air O_2 , or the reduction of P_n resulted in inefficient utilization of intercellular CO_2 . It indicated that the decrease of P_n in *D. nipponica* Makino was not caused by insufficient supply of CO_2 , but by lower assimilation capacity. Besides the stomata inhibition, the significant increasing C_i was also a limit factor to photosynthesis. Both T_r and WUE of *D. nipponica* Makino was significantly affected by drought, but all seedlings survived during severe drought of long duration for eighty days. It implied that this species had a conservative water-use strategy to higher drought tolerance.

Since membrane destruction and H_2O_2 increase are consequences of active oxygen production induced by drought stress, plants damage

Tab. 4: Principal component analysis (PCA) results of thirteen parameters on seedlings treated by drought stress.

Parameter	Principal component 1	Principal component 2	Principal component 3
LWC	0.84	-0.27	0.04
Chl	-0.45	-0.59	0.55
G_s	0.95	-0.15	0.01
C_i	-0.66	0.04	-0.57
P_n	0.97	-0.02	0.03
T_r	0.88	-0.21	-0.16
WUE	0.62	0.27	0.44
SOD	-0.23	0.43	0.68
CAT	-0.87	0.00	-0.26
MDA	-0.46	-0.70	0.38
Pro	-0.11	0.82	0.05
SS	-0.57	0.15	0.39
SP	0.14	0.91	0.09
Characteristic value	5.73	2.76	1.69
Contribution rate (%)	44.06	21.26	13.02
Total contribution rate (%)	44.06	65.32	78.34

The value indicates the loading score of each parameter. The total contribution rate of the first two principal components amounted to 65.32%, including a large number of information. These font-weighted seven parameters were chosen to evaluate the drought tolerance (Tab. 5).

LWC, leaf water content of fresh weight; Chl, chlorophyll concentration; G_s, stomatal conductance; C_i, intercellular CO₂ concentration; P_n, net photosynthetic rate; T_r, transpiration rate; WUE, water use efficiency; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; Pro, proline; SS, soluble sugar; SP, soluble protein.

Tab. 5: Subordinate function values of drought tolerance (\hat{X}_{ij}) for seven selected parameters and the drought tolerance order of three provenances.

Parameter	Manghe		Pangquangou		Luyashan	
	MD	SD	MD	SD	MD	SD
LWC	0.00	0.23	0.03	0.00	1.00	1.00
G _s	0.11	0.00	0.00	1.00	1.00	0.73
P _n	0.00	0.00	1.00	1.00	0.62	0.85
T _r	0.00	0.00	1.00	1.00	0.58	0.52
CAT	0.00	0.00	1.00	1.00	0.31	0.41
Pro	0.75	1.00	1.00	0.90	0.00	0.00
SP	1.00	1.00	0.07	0.47	0.00	0.00
Means of subordinate function value (\bar{X}_i)	0.27	0.32	0.59	0.77	0.50	0.50
	0.29		0.68		0.50	
Drought tolerance order	3		1		2	

LWC, leaf water content of fresh weight; G_s, stomatal conductance; P_n, net photosynthetic rate; T_r, transpiration rate; CAT, catalase; Pro, proline; SP, soluble protein. MD, moderate drought stress (40%-45% water holding capacity, WHC) and SD, severe drought stress (20%-35% WHC).

could be detected by estimating activities of antioxidant enzymes and measuring content of MDA, which serve as indicators of the importance of free radical reactions in the stressed tissue (KHOYERDI

et al., 2016; OLAREWAJU et al., 2017). In most plants, activities of antioxidant enzymes usually increase in response to stress-induced oxidation (ASHRAF, 2009; ANJUM et al., 2011; CAVERZAN et al., 2016; RAZZAQ et al., 2017). SOD and CAT are the most important antioxidant enzymes (ANJUM et al., 2011; NOUMAN et al., 2018). Our study showed that SOD activity significantly increased by drought stress in three provenances of *D. nipponica* Makino to eliminate O₂⁻ and form H₂O₂ by the antioxidant defense system. CAT activity slightly fluctuated and showed an obvious upward trend with the increasing drought stress, mainly to protect the cell membrane by the elimination of excess H₂O₂ (DE CARVALHO, 2008). Under SD condition, content of MDA increased by 49.26% and 182.07% for samples from Pangquangou and Luyashan, respectively. It indicated that *D. nipponica* Makino suffered from considerable cell membrane damage under SD condition (KHOYERDI et al., 2016; BISTGANI et al., 2017). Osmoregulation has been widely reported as the important response mechanism to deal with water stress, let the retention of cellular turgor and favor the absorption of water (MARCINSKA et al., 2013; KHOYERDI et al., 2016). Pro and SS are the common solutes that play major roles in osmotic adjustment. They may also protect membranes from damage and stabilize the structures and activities of proteins and enzymes (BISTGANI et al., 2017). Under SD condition, the content of Pro in samples from Manghe and Pangquangou was 2.40 and 2.22 times more than that of NW seedlings, respectively. The content of SS did not increase as much as Pro, that increased by 23.28%, 23.63% and 31.64% in Manghe, Pangquangou and Luyashan provenances, respectively. Inhibition of protein synthesis is one of the changes that befall during stress condition (ANJUM et al., 2011). We detected a decrease in content of SP under SD condition for Pangquangou and Luyashan provenances. It might be due to a severe decrease in photosynthesis, as a consequence of reduction of protein synthesis.

Drought sensitivity and regional variation of *D. nipponica* Makino

Plants adapt to drought stress mainly by two types of mechanisms, namely, drought avoidance and drought tolerance (RUIZ et al., 2013; KOLENC et al., 2016; ZHENG et al., 2017). Studies focused on drought effects on root/tuber species have proven that potatoes, sweet potatoes and taros are highly sensitive to water deficit after planting, whereas yam is probably the only kind with high drought-tolerance within the root/tuber group (LEBOT, 2009; DARYSNTO et al., 2016). A higher amount of water (approximately 1500 mm) is essential for yams to ensure high yield during the total growth cycle (LEBOT, 2009), but yams can survive in areas with annual rainfall as low as 500 mm in African drylands (e.g., south Madagascar). Yams could be planted during dry season due to their tolerance to drought, although with low yield potentials in these regions (LEBOT, 2009). Consistent with studies from other yams, *D. nipponica* Makino survive in its three natural ranges with low annual rainfall (i.e., between 450 mm and 800 mm), imply this species has high drought-tolerance, which would be closely correlated with the initial fresh mass of parent rhizomes. If water stress occurs, seedlings can tap most of its early moisture needs from the 'mother' rhizomes. However, if drought stress continues, the rhizome initiation can be delayed (LEBOT, 2009). Some of xerophytic features were observed in the young plants of yams. The young plant is devoid of leaves (and therefore has very low transpiration). After surviving a dry period, the new yam plants emerged with considerable vine length expansion (sometimes exceeding two meters) without forming new leaves. These vines, which initially obtain moisture and nutrients from the parent tuber or rhizome, were also covered with a waxy bloom that reduces moisture loss as the plant continues to develop (DARYANTO et al., 2016). Furthermore, the rhizomes of *D. nipponica* Makino could be less sensitive, due to its partially wooded-tissue and high

accumulation of carbohydrate content in the store organ (PŁAZEK et al., 2014). Physiological adaptations of drought avoidance include reduction of stomatal conductance and low cuticular transpiration to reduce the water loss; Drought tolerance is achieved through stomatal regulation, osmotic adjustment and by increasing tissue elasticity (RUIZ et al., 2013). *D. nipponica* Makino seemed to use the two strategies to cope with drought, including stomatal closure and transpiration reduction of drought avoidance type, and osmotic adjustment of drought tolerance type.

The responses of plants to drought is heritable and shows a wide regional variation (EILMANN et al., 2013; LÉVESQUE et al., 2013; GAO et al., 2017). Many reports demonstrated that the effect of drought stress is genotype-specific, where genotypes showed different responses in the same stress condition (CAVERZAN et al., 2016; KHOYERDI et al., 2016; KHODADADI et al., 2017; ZHENG et al., 2017; TRENTACOSTE et al., 2018). Under stress condition, tolerant genotypes of pistachio (*Pistacia vera* L.) could maintain longer water potential and gain more dry weight than the susceptible ones (KHOYERDI et al., 2016); Tolerant genotypes of wheat generally maintained a higher antioxidant capacity resulting in lower oxidative damage (CAVERZAN et al., 2016). As to fennel (*Foeniculum vulgare* Mill.), the relative water content was increased in drought-tolerant genotype and proline content was decreased in drought-sensitive ones (KHODADADI et al., 2017). It has been confirmed that region-specific variations exist in yam tolerance of biotic stress (MARCOS et al., 2009; CORNET et al., 2014; NUR FATIHAH et al., 2017), which was also proved by our results of *D. nipponica* Makino. In Shanxi Province, climatic drought stress is severe or is increasing with climate change, the drought tolerance evaluation of *D. nipponica* Makino provenances could be the best strategy to cope with future climate conditions in this region. Generally, plant responses to water deficit that are out of their natural environments depend on their native habitats. In a common drought stress environment, seedlings originating from drier areas tend to display better performance than those from wetter areas (RUIZ et al., 2013; LÉVESQUE et al., 2013). Consistent with this conclusion, drought tolerance of *D. nipponica* Makino from Pangquangou and Luyashan were obviously higher than that of Manghe because the latter is from a slightly wetter environment than the former two regions, which were with similar climate and vegetation. Pangquangou provenance showed higher antioxidant capacity and considerable proline accumulation, which indicated it might be a more drought-tolerant genotype, and is most suitable to the future climate change in Shanxi Province.

Conclusions

Our findings of physiological responses of *D. nipponica* Makino demonstrates that this species achieves a high drought tolerance by decreased photosynthesis, increased activity of antioxidant enzymes, and accumulation of osmotic regulating compounds. Three provenances presented regional specific drought responses. In severe drought stress, Manghe and Pangquangou provenances exhibited a high accumulation of Pro. Further on Pangquangou provenance considerably increased CAT activity and Luyashan provenance displayed a great increase of MDA content. Consistent with the common opinion, Pangquangou provenance possessed higher drought tolerance to moderate and severe drought stress, which indicates that it might be more suitable to combat the increasing drought climate in Shanxi Province.

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