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EFFECTS OF ENVIRONMENTAL STRESSES ON THE GROWTH OF *ARABIDOPSIS THALIANA* ROSETTE LEAVES

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ABSTRACT

Plant growth is constantly affected by biotic and abiotic stresses, which are especially expressed in plant leaves. Therefore, leaf phenotype is considered to be an important indicator of phenotypic plasticity in plants. The effects of various growth environmental factors on the final size of *Arabidopsis thaliana* rosette leaves and the number of leaves were analyzed in orthogonal tests using image analysis, and growth curves were estimated statistically. Finally, the optimum growth environment for *A. thaliana* Col-0 was determined. In this study, temperature, humidity, and light intensity were chosen as factors and studied at the three levels each (temperature: 22 °C, 25 °C, 28 °C; humidity: 50%, 65%, 80%; light intensity: 92 μmol·m^{-2}·s^{-1}; 184 μmol·m^{-2}·s^{-1}; 278 μmol·m^{-2}·s^{-1}). The results showed that light intensity was a major factor in the final leaf size, whereas for the number of plant leaves the most important was temperature. According to the major and minor order of environmental factors, the following scheme appeared to be optimal for *A. thaliana* growth: temperature 22 °C, humidity 50%, illumination intensity 184 μmol·m^{-2}·s^{-1}.

Key words: *Arabidopsis thaliana*, humidity, image analysis, light intensity, rosette leaves, temperature

INTRODUCTION

Plant phenotype is the result of the interaction between genotype and growth environments. Studying the phenotype of plants under various growth environments is the core of understanding plant function. Recent research indicates that in the context of rapid climate change, phenotypes play a key role in plant adaptation to various growth factors (Hu et al. 2008; de Jong et al. 2012; Gratani et al. 2014; Scharr et al. 2016), and biotic and abiotic stresses lead to phenotypic changes in plants. Therefore, identifying the plant phenotypic signatures under biotic or abiotic stress conditions is helpful for the early detection of biotic or abiotic stresses, which have great economic benefits for plant production (Pauli et al. 2016).

Leaves are the energy factories of plants and play an important role in the process of plant survival and growth. Through photosynthesis, leaves convert solar into chemical energy, which can then be used for further metabolism and ultimately in the production of food, feed, and fuel (Xu et al. 2009; Rodriguez et al. 2014). Since most photosynthesis occurs in leaves, it is important to characterize them in terms of size, shape, and number, which are regulated by the growth environment and genetic factors (Gonzalez et al. 2010, 2012; Mishra et al. 2012; Weraduwage et al. 2015). Many functional genomic studies have been carried out to improve agricultural and forestry crops using high-throughput genomic tools. However, there are relatively few studies on the phenotypic characteristics (e.g., the size and morphological structure of organs) of plants with specific genotypes under various growth conditions (Rahaman et al. 2015). Therefore, the study of the relationships between phenotypes, genes, and environments should be expanded. It will deepen our understanding of the relationships between the observable plant phenotypes and their physiological status, and the effects of different growth environments on plant growth, yield, and quality (Ke 2014; Orgogozo et al. 2015).

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Studying the effects of environmental factors on plant phenotype is helpful in understanding the biological functions of plant development, serving in breeding to develop cultivars with ideal phenotypes. However, the majority of experiments testing the response of plants to changes in environmental conditions have focused on a single stress treatment applied to plants under controlled conditions (Wang & Zhou 2021). In contrast, in practice, a number of different stresses can occur simultaneously. These may include irradiance, temperature, humidity, or water availability and may alter plant metabolism in a novel manner that may be different from that caused by each of the different stresses applied individually. *A. thaliana* is an ideal model for studying those problems and has been widely used for this purpose (Minervini et al. 2014). As a model organism, *A. thaliana* has several advantages, such as its small size, short life cycle, and known gene sequence, which make it easy to conduct experiments in the laboratory. By cultivating *A. thaliana* under a controllable environment, the change regulation of its phenotypes can be used to guide the studies of other plants, and provide references for outdoor experiments, where the environmental conditions and other factors are difficult to control. Moreover, analyzing the phenotypic characteristics of *A. thaliana* under various growth environments can provide a theoretical framework for quantifying and understanding plant phenotypic differences caused by environmental stresses (Boyes et al. 2001).

Earlier studies did not analyze the effect of different growth environments (temperature, humidity, light intensity) on *A. thaliana* phenotypes, which could not adequately explain the relationship between *A. thaliana* phenotypes and environments, and the effect of different growth environments on *A. thaliana* growth. Moreover, different environmental factors and phenotypic characteristics have rarely been integrated into a single model, and predicting and analyzing the influence of environmental factors on phenotypic characteristics has been challenging.

Using image analysis to nondestructively analyze plant leaf size in greenhouses, a number of studies have been conducted (Fahlgren et al. 2015; Ge et al. 2016). Compared to destructive sampling, image analysis enables the measurements of leaf size multiple times during the plant growth cycle, and also allows the quantification of dynamic traits such as growth rate and leaf expansion rate (Liang et al. 2018).

The aim of this paper was to study the effect of environmental factors of temperature, humidity, and light intensity on the phenotypic characteristics of *A. thaliana* through a multi-factor orthogonal test, which may be helpful in optimizing the parameters for phenotyping and selecting new improved genotypes with the desired traits in cultivation under specific climatic conditions.

MATERIALS AND METHODS

Cultivation of *A. thaliana* and leaf image acquisition under various growth environments

From November 2015 to December 2016, the area of individual leaves and the number of leaves of *A. thaliana* in nine different growth environments were measured at Nanjing Forestry University, China. Temperature, humidity, and light intensity were selected as environmental factors for the experiments, and orthogonal tests of three factors at three levels were carried out. Seeds of *A. thaliana* Col-0 were sown into nursery substrate of peat fibers: 0–10 mm, and cultured in a climate chamber (RXZ-500B, Ningbo Jiangnan Instrument Factory, Zhejiang, China). The environmental parameters (temperature; humidity; light intensity) could be individually configured in the chamber, so various combinations of these parameters could be simulated. Nine sets of experiments were conducted by setting the environmental parameters of the climate chamber and 50 samples were taken in each experiment (Table 1). After *A. thaliana* leaf emerged, the growth image of *A. thaliana* was collected every day by a CCD camera (JAI, model: GO-5000C-PGE). A total of 20,200 original images were obtained, which were used to extract the leaf area of *A. thaliana*. Figure 1 shows the growth images of *A. thaliana* on the 20th day after the leaf emerged in the 38th pot in all nine sets of experiments.

Table 1. Trial environment parameters of each group

Trial number	Trial factors			Trial factors
	T	*H*	*I*	
	temperature (°C)	humidity (%)	light intensity (μmol·m^{-2}·s^{-1})	
1	22	50	278	22 °C; 50%; 278 μmol·m^{-2}·s^{-1}
2	22	65	92	22 °C; 65%; 92 μmol·m^{-2}·s^{-1}
3	22	80	184	22 °C; 80%; 184 μmol·m^{-2}·s^{-1}
4	25	50	184	25 °C; 50%; 184 μmol·m^{-2}·s^{-1}
5	25	65	278	25 °C; 65%; 278 μmol·m^{-2}·s^{-1}
6	25	80	92	25 °C; 80%; 92 μmol·m^{-2}·s^{-1}
7	28	50	92	28 °C; 50%; 92 μmol·m^{-2}·s^{-1}
8	28	65	184	28 °C; 65%; 184 μmol·m^{-2}·s^{-1}
9	28	80	278	28 °C; 80%; 278 μmol·m^{-2}·s^{-1}

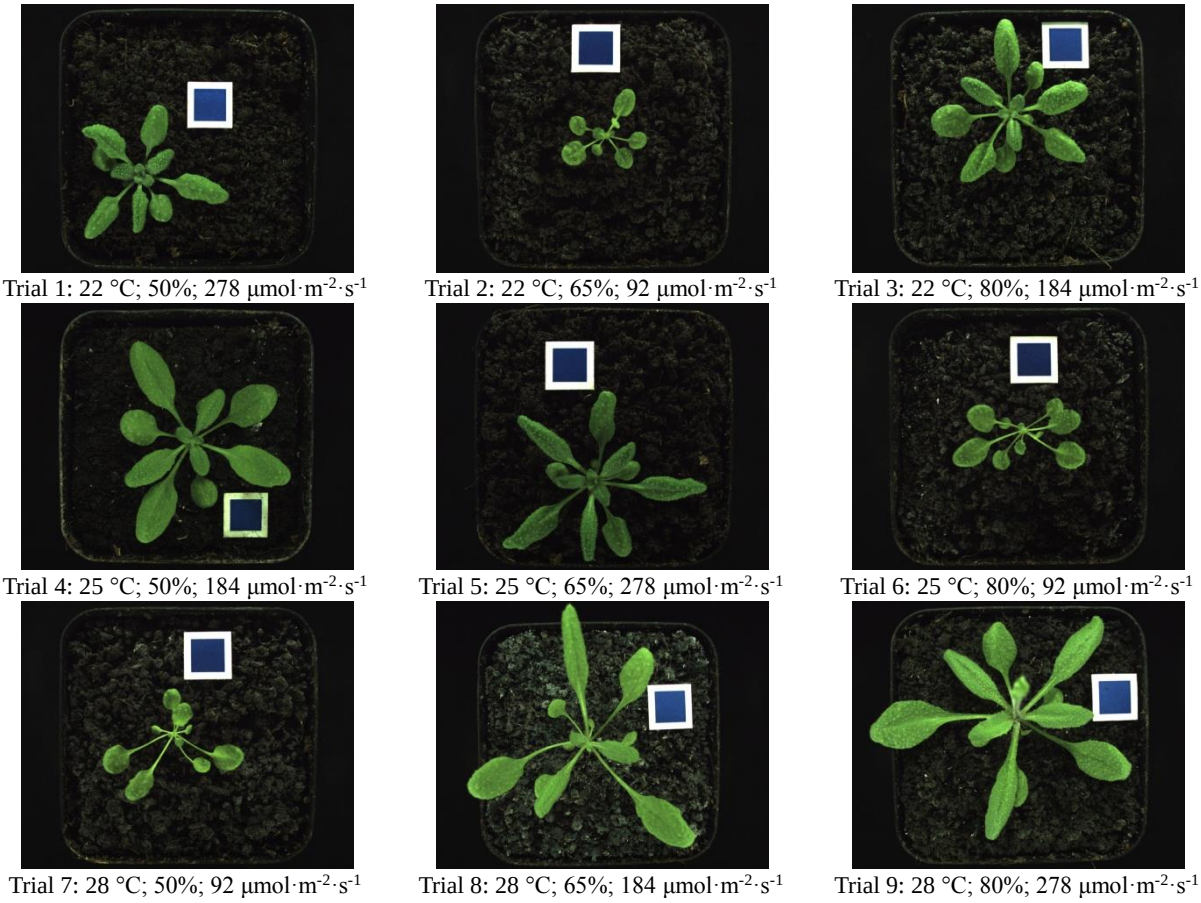

Figure 1. Example images of *A. thaliana* leaves at 20th day after emergence under various growth environments (temperature; humidity; light intensity). Note: the blue squares in each picture represent calibration plate

Image analysis and feature extraction

MATLAB (version R2010, MathWorks) was used for image analysis. Rosette leaves of *A. thaliana* were measured at regular intervals during the whole life cycle using image analysis. Firstly, individual leaves were segmented from the plant using an interactive segmentation process. Then, the total number of pixels in the region of the individual leaf area was extracted using the image region property measurement function (regionprops) in MATLAB's Image Processing Toolbox. Finally, the size of the individual leaf was obtained by the proportional relationship calculated from a blue square standard plate (10 mm × 10 mm) with the following equation:

$$A = P_L \times A_B / P_B \qquad (1),$$

where A is the *A. thaliana* leaf area (mm^2); P_L is the number of pixels of the *A. thaliana* leaf area (pixel); A_B is the area of the standard plate (mm^2); and P_B is the number of pixels of the standard plate (pixel).

The smallest leaf was visible when the individual leaf area was approximately 0.5 mm^2 (Cookson et al. 2010). In this paper, the number of leaves was calculated every 2 days during the period from two visible leaves to bolting.

Growth equation of rosette leaves of *A. thaliana*

Individual leaf area and leaf numbers were analyzed and fitted using a logistic model and a linear regression model. The regression equations between leaf area, leaf number, and growth time were established by SPSS software (IBM SPSS statistics 19.0). The equations of the models were as follows (Cookson et al. 2010; Karadavut et al. 2010; Jiao et al. 2018):

logistic model $$Area = \frac{a}{1 + b \cdot e^{-k \cdot t}} \quad (2);$$

linear regression model $$N = k_1 \cdot t + k_2 \quad (3),$$

where *Area* is the fitted values of leaf area (mm^2); *t* is the growth time (d); *k* is the growth rate (mm^2·d^{-1}); *a* is the final leaf size (mm^2); *b* is a constant scale; *N* is the number of leaves; k_1 is a slope, which represents leaf emergence rate; and k_2 is the intercept term.

The final sizes of the leaf area under different growth environments were determined according to the established growth equations. Then, the effects of various growth environments on the final leaf size and the leaf number were analyzed.

RESULTS AND DISCUSSION

Curve fitting to the final *A. thaliana* leaf size and the number of leaves in the rosette in the various growth environments

Leaf 6 (the leaf number was determined by the order of the emergence) of each plant was taken as the target to analyze the effects of growth environments on the final leaf size of *A. thaliana*. Scatter plots of leaf 6 for each experiment under various growth conditions are shown in Figure 2.

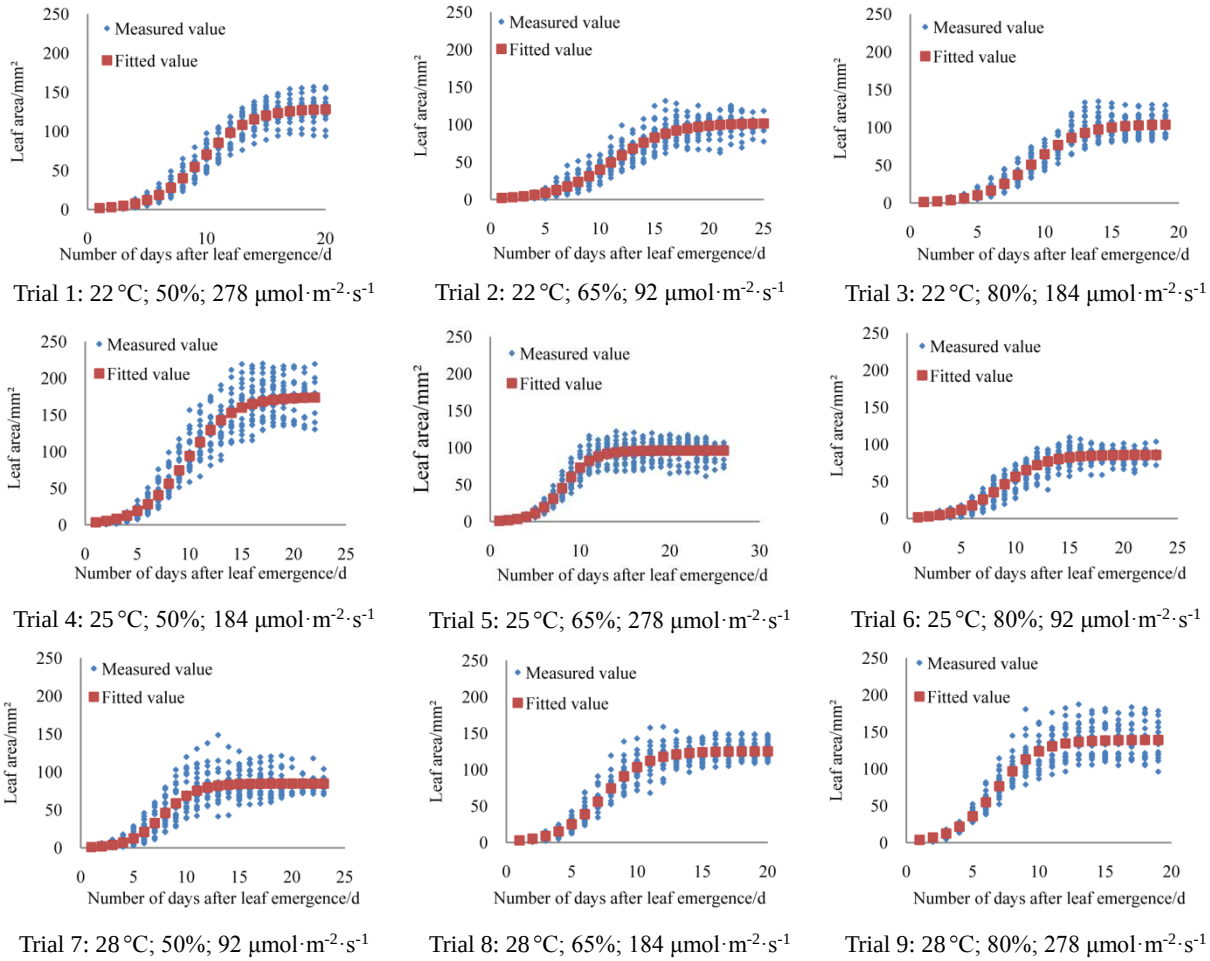

Figure 2. Scatter plots of leaf 6 areas for each experiment under various growth environments (temperature; humidity; light intensity)

It could be learned from Figure 2 that in the early stage, the growth and increase of leaf area were faster than in the maturation stage, producing a sigmoid curve. Moreover, the fitted values of leaf 6 agreed well with the measured values, with no obvious outliers. The results of regression analysis between leaf area and growth time (equation 2) were given in Table 2. The final size of leaf 6 for each experiment was determined by the parameter *a* in the regression equations. The minimum final size of leaf 6 was 84.75 mm^2 under the condition of temperature 28 °C, humidity 50%, and light intensity 92 μmol·m^{-2}·s^{-1}. The maximum final size of leaf 6 was 174.31 mm^2 under the condition of temperature 25 °C, humidity 50%, and light intensity 184 μmol·m^{-2}·s^{-1}. Therefore, the final leaf size was greatly affected by its growth environments. Studying the effect of growth environment factors on the final leaf size could provide basic data for breeding the ideal plant type.

The regression equations of the number of leaves per rosette with time were established using equation (3). The trends of the leaf number of *A. thaliana* under various growth environments with time are shown in Figure 3. It could be learned that there was a linear correlation between the leaf number and the growth time. The leaf emergence rate (the slope of the fitted line) under various growth conditions is shown in Table 2. The minimum leaf emergence rate was 0.38 leaves per day under the temperature of 28 °C, humidity 50%, and light intensity 92 μmol·m^{-2}·s^{-1}, while the maximum leaf emergence rate was 0.71 leaves per day under the temperature of 28 °C, humidity 80%, and light intensity 278 μmol·m^{-2}·s^{-1}. Therefore, the leaf emergence rate was also greatly affected by its growth environments.

Impact of growth conditions on the final leaf size of *A. thaliana* and the number of rosette leaves

Range analysis and variance analysis of the orthogonal test were conducted to evaluate the effect of various environmental factors (temperature, humidity, and light intensity) on the individual leaf final size and the number of rosette leaves of *A. thaliana*. The results are shown in Tables 3–5.

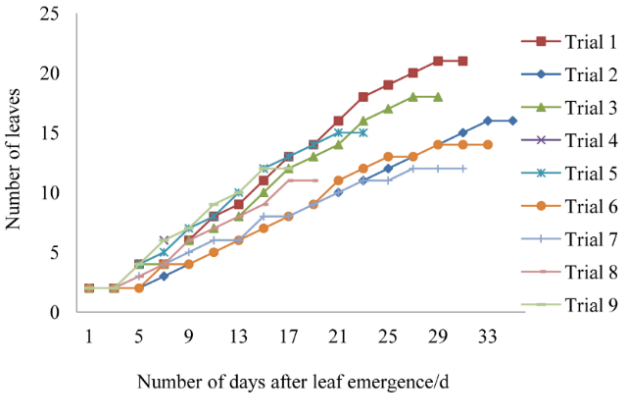

Figure 3. The variation trends of the number of leaves with time under various growth environments (temperature; humidity; light intensity)

Table 2. Regression equations of leaf number under various growth environments (temperature; humidity; light intensity)

Trial factors	Growth regression equation	Trial factors	Growth regression equation
22 °C; 50%; 278 μmol·m^{-2}·s^{-1}	N = 0.68*t+0.17	22 °C; 80%; 184 μmol·m^{-2}·s^{-1}	N = 0.65*t+0.40
22 °C; 65%; 92 μmol·m^{-2}·s^{-1}	N = 0.47*t+0.22	25 °C; 65%; 278 μmol·m^{-2}·s^{-1}	N = 0.68*t+0.80
25 °C; 50%; 184 μmol·m^{-2}·s^{-1}	N = 0.67*t+0.99	25 °C; 80%; 92 μmol·m^{-2}·s^{-1}	N = 0.45*t+0.61
28 °C; 80%; 278 μmol·m^{-2}·s^{-1}	N = 0.71*t+0.74	28 °C; 65%; 184 μmol·m^{-2}·s^{-1}	N = 0.57*t+0.63
28 °C; 50%; 92 μmol·m^{-2}·s^{-1}	N = 0.38*t+1.53		

Table 3. Range analysis results of the final size and the number of the rosette leaves of *A. thaliana*

Trial number		Trial factors			Estimated results	
		T	*H*	*I*	Final leaf size per mm^2	Leaf number
		temperature (°C)	humidity (%)	light intensity (μmol·m^{-2}·s^{-1})		
Trial design	1	22	50	278	129.03	21
	2	22	65	92	102.06	16
	3	22	80	184	104.26	18
	4	25	50	184	174.31	15
	5	25	65	278	95.99	15
	6	25	80	92	85.90	14
	7	28	50	92	84.75	12
	8	28	65	184	125.30	11
	9	28	80	278	139.18	12
Final leaf size	K_1	335.35	388.09	272.71		
	K_2	356.2	323.35	403.87		
	K_3	349.23	329.34	364.21		
	$\bar{K}_1$	111.78	129.36	90.90		
	$\bar{K}_2$	118.73	107.78	134.62		
	$\bar{K}_3$	116.41	109.78	121.40		
	Range *R*	6.95	21.58	43.72		
Leaf number analysis	K_1	55	48	42		
	K_2	44	42	44		
	K_3	35	44	48		
	$\bar{K}_1$	18	16	14		
	$\bar{K}_2$	15	14	15		
	$\bar{K}_3$	12	15	16		
	Range *R*	6	2	2		

Table 4. Analysis of variance for the final rosette leaf size of *A. thaliana*

Source	SS	DF	MS	*F*-value	F_α	*Sig.*
T	75.11	2	37.56	0.026		
H	853.19	2	426.60	0.30		
I	3018.77	2	1509.39	1.06	$F_{0.05}(2,2) = 19.0$ $F_{0.1}(2,2) = 9.0$	
Error	2860.54	2	1430.27			
Total	6807.61	8				

Table 5. Analysis of variance for the number of rosette leaves of *A. thaliana*

Source	SS	DF	MS	*F*-value	F_α	*Sig.*
T	66.89	2	33.45	42.88		**
H	6.22	2	3.11	3.99		
I	6.22	2	3.11	3.99	$F_{0.05}(2,2) = 19.0$ $F_{0.1}(2,2) = 9.0$	
Error	1.56	2	0.78			
Total	80.89	8				

The increase in the individual leaf area resulted in the increase in the rosette area, which intensified the interception and utilization of light energy and provided higher above-ground biomass. It could be concluded by range analysis (Table 3) that the order of environmental factors affecting the final size of *A. thaliana* leaves was: light intensity (I) > humidity (H) > temperature (T). So the light intensity was the main factor affecting the final size of the individual leaf. Compared with the light intensity of 184 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the final size of leaf 6 decreased by 32.84% and 9.82% at 92 and 278 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively. This was consistent with the study results of Cookson et al. (2005), where low light condition caused a significant reduction in the final leaf area. Under the light intensity of 92 and 278 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the duration of leaf 6 expansion increased by 2 days and by 1 day, respectively, compared with that under the condition of 184 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Under the condition of 92 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, the leaf 6 expansion rate decreased by 5.99 $mm^2 \cdot d^{-1}$ compared with that under 184 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity. These indicated that the reduction in individual leaf area was associated with a reduction in leaf expansion rate and an increase in the duration of leaf expansion (Cookson & Granier 2006). However, under the condition of 278 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, the leaf 6 expansion rate increased by 0.21 $mm^2 \cdot d^{-1}$ compared with that under 184 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity. This may be caused by temperature and humidity, which need to be further studied. Light intensity also significantly affected the leaf emergence rate of *A. thaliana*. Compared with 278 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the *A. thaliana* leaf emergence rates were reduced by 0.26 and 0.06 leaves per day under the condition of 92 and 184 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, respectively. Analysis of variance (Table 4) showed that the effect of light intensity (I) on the final leaf size was greater. However, the effect of temperature, humidity, and light intensity was not significant, which was quite different from the growth image in Figure 1, mainly because it represents the image on day 20, and the emergence time and growth rate of leaves under different conditions was different, moreover, the analysis concern the final size of the leaf. It could be concluded, that the order of environmental factors affecting the final size of *A. thaliana* leaves was: light intensity (I) > humidity (H) > temperature (T), which was consistent with that obtained by range analysis.

The increase in the leaf number caused an increase in the rosette area. It could be concluded by range analysis that the order of environmental factors affecting the leaf number of *A. thaliana* was: temperature (T) > humidity (H) = light intensity (I). Compared with 22 °C, the number of rosette leaves was reduced by 19.97% and 38.19% under the conditions of 25 °C and 28 °C, respectively. This was consistent with the study results of Crawford et al. (2012), where the leaf number of *A. thaliana* grown at high temperature (28 °C) was lower than that at low temperature (22 °C). Compared with 22 °C and 25 °C, *A. thaliana* had the shortest growth cycle at 28 °C, indicating that as the temperature increased, the life cycle of *A. thaliana* was shortened. Moreover, the reduction in the duration of the phase of leaf initiation could result in a reduction in leaf number (Cookson & Granier 2006). Therefore, as the temperature increased, the leaf number had a declining trend. Analysis of the variance (Table 5) showed that the effect of temperature on the leaf number was highly significant. This was because low light intensity inhibited growth and development, especially when the humidity was too high. It could be concluded, by F-value, that the order of environmental factors affecting the leaf number of *A. thaliana* was: temperature > humidity = light intensity, which was consistent with that obtained by the range analysis.

In order to intuitively analyze the rules and trends of the effect of environmental factors on the individual leaf size of *A. thaliana* and the leaf number, the environmental factor level was taken as the abscissa and the average value $\bar{K}$ of the final size of the individual leaf, and leaf number at each environmental factor level was taken as the ordinate. In this way, the environmental factors and the test results were obtained, as shown in Figure 4.

In Figure 4, the final size of leaf 6 reached its maximum value at 25 °C, and increased by 5.85%, and 1.95% compared with that at 22 °C and 28 °C, respectively. With the increase in temperature, the final leaf size exhibited little difference. This means that the effect of temperature on the final leaf size was not significant. When the relative humidity increased from 50% to 65%, the final size of leaf 6 decreased by 16.68%, and when the relative humidity increased from 65% to 80%, the final size of this leaf increased by 1.82%. Therefore, a low humidity (50%) was beneficial to the growth and development of leaf 6,

and high humidity (80%) inhibited the growth. Compared with 184 μmol·m^{-2}·s^{-1}, the final size of leaf 6 was reduced by 32.84% and 9.82% at 92 and 278 μmol·m^{-2}·s^{-1}, respectively, which indicated that the final size of this leaf decreased significantly under low-light conditions.

In Figure 4, the number of leaves was reduced by 19.97% and 38.19% at 25 °C and 28 °C, respectively, compared with 22 °C. At the relative humidity of 65%, the number of leaves increased by 12.5% and 6.67% compared with 50%, and 80%, respectively. When the light intensity increased from 92 μmol·m^{-2}·s^{-1} to 184 μmol·m^{-2}·s^{-1} and 278 μmol·m^{-2}·s^{-1}, the number of leaves increased by 6.67% and 6.25%, respectively. The analysis of the number of leaves under the influence of various environmental factors showed that the influence of temperature was significant, while the influence of humidity and light intensity was small.

The optimum combination of environmental factors could be determined by analyzing the above test results, as shown in Table 6. The optimization conditions of the final leaf size and leaf number obtained by individual leaf analysis were inconsistent. Therefore, the optimum environmental parameters for the growth of *A. thaliana* rosette leaves must be determined according to the major and minor order of environmental factors.

The temperature was the major factor influencing the leaf number, but for the final leaf size, it was a secondary factor. Therefore, the temperature of 22 °C was selected as the optimum growth temperature according to the test result of leaf number. The effects of humidity on the final leaf size and leaf number were both the secondary factor, and the value of 50% was selected as the optimum growth humidity. Light intensity was the major factor affecting the final leaf size, but for the leaf number, it was a secondary factor. Therefore, the light intensity 184 μmol·m^{-2}·s^{-1} was selected as the optimum growth according to the test result of the final leaf size.

Finally, the optimum growth environments for the rosette leaves growth of *A. thaliana* were determined, which were temperature 22 °C, relative humidity 50%, and light intensity 184 μmol·m^{-2}·s^{-1}.

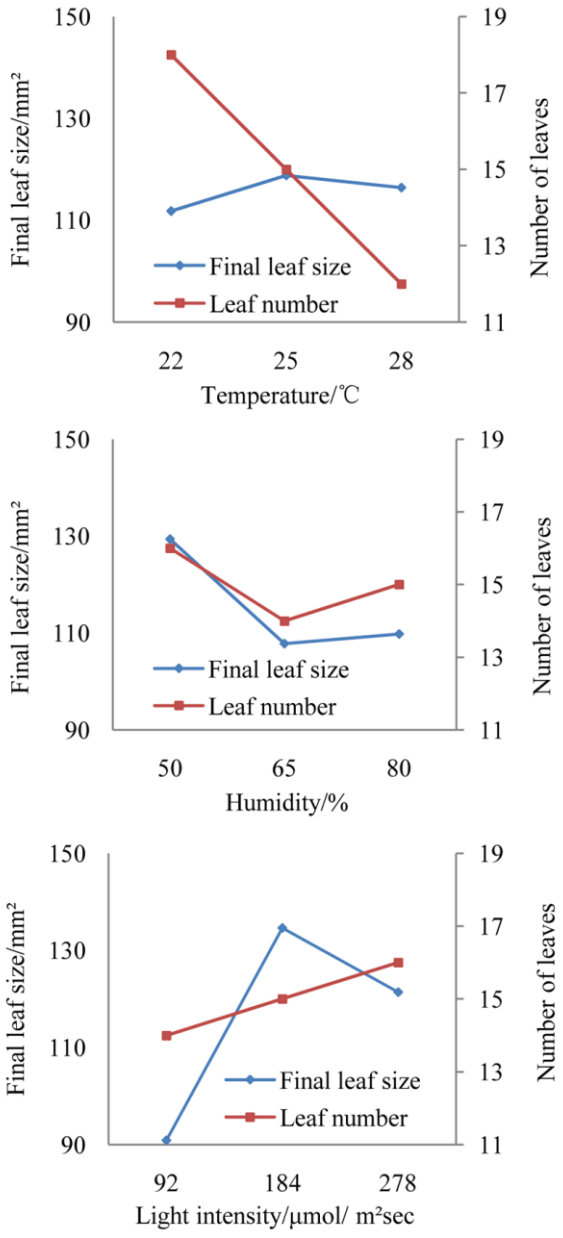

Figure 4. Trend charts of environmental factors level

Table 6. Optimization table for the environmental factors

Characteristic	Combination of optimal growth conditions
Final leaf size	25 °C, 50% humidity, 184 μmol·m^{-2}·s^{-1}
Leaf number	22 °C, 50% humidity, 278 μmol·m^{-2}·s^{-1}

CONCLUSIONS

The main motivation for the present experiments was to study how the temperature, relative humidity, and light intensity affected the final leaf size and leaf number of *A. thaliana*, which was a notable advantage of our research analyzing the effects of multi environmental factors on the phenotypes of *A. thaliana*, since previous studies had mostly focused on the impact of single environmental factors on the plant growth. The effects of various environmental factors on the final size and the number of leaves of *A. thaliana* were determined, the cognitive scope of its physiological function was expanded, as well as the optimum growth environment for specific genotype *A. thaliana* was validated.

The dynamics of individual leaf area and leaf number as a function of time were analyzed and fitted by the statistical analysis method.

In this paper, an effective method of phenotypic assessment was developed, which can be helpful in optimizing phenotyping procedures applicable for breeding new cultivars adapted to given climatic conditions, or to select genotypes with the desired traits suitable for cultivation under specific conditions.

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