RESEARCH ARTICLE

Regulation of flowering time using temperature, photoperiod and spermidine treatments in *Anoectochilus roxburghii*

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Abstract This study investigated the effects of different temperatures, photoperiods and spermidine concentrations on the flowering time regulation of *Anoectochilus roxburghii* by measuring changes in the soluble sugar, soluble protein, malondialdehyde and proline contents, and the peroxidase, superoxide dismutase and catalase activities in *A. roxburghii* flower buds. The flowering time could be advanced under 25/20 °C (day/night), 16/8-h (day/night) long day conditions or low spermidine concentrations. The plants grew more rapidly and flowering rates were greater. The flowering time could be delayed under a low temperature of 20/15 °C or 8/16-h short day conditions, resulting in a low flowering rate. Under a high temperature of 30/25 °C or high spermidine concentrations, the plants could not flower normally and even died. There were significant differences in the seven measured indices among the various treatments. Thus, different treatments had significant effects on the flowering time regulation and flowering quality of *A. roxburghii*, providing a reliable theoretical basis for further studies on the flowering-related regulatory mechanisms of *A. roxburghii*.

Keywords *Anoectochilus roxburghii* · Flowering regulation · Photoperiod · Spermidine · Temperature

Introduction

Anoectochilus roxburghii (Orchidaceae) is a rare perennial herb that was first recorded as an herbal medicine in ancient Chinese literature. The whole herb of *A. roxburghii* has unique curative effects that protect the liver and prevent diseases such as cancer (Zeng et al. 2016). Therefore, it is extremely economically valuable (Ye et al. 2017). It is not only one of the original plants used in Traditional Chinese Medicine for diabetes (Cui et al. 2012), tumors (Yu et al. 2017), hyperpiesia (An et al. 2014) and hepatitis (Zeng et al. 2017), but it also has ornamental, cosmological, culinary and other beneficial uses (Zhang et al. 2015; Shao et al. 2014). And aromatic and medicinal plants have been used for therapeutic, religious, cosmetic, nutritional, and beautification purposes since ancient times and humanity of all civilizations and culture are familiar with their usage (Ersoy et al. 2017; Abramovic et al. 2018; Tiwari et al. 2018). Therefore, it is popular with the general public. However, owing to its slow growth, environmental requirements and low reproductive rate, its wild natural resources are very scarce (Shao et al. 2016; Xu et al. 2017). Consequently, it is important to make rational use of *A. roxburghii* resources (Wu et al. 2014a, b; Hong et al. 2016). It is significant to master the flowering regulation technology of *A. roxburghii* for its rational utilization.

Many factors affect flowering (Ausín et al. 2005; Melzer 2017). Temperature is an essential ecological factor for plant growth (Vaz and Kerbauy 2004; Kim et al. 2013). Its influence is very complex and plays a decisive role in flowering time regulation. Controlling temperature to

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regulate the flowering period mainly includes two aspects: increasing the temperature to induce the flowering period and lower the temperature to promote flower bud differentiation (Balasubramanian et al. 2006; Qiu et al. 2016). Studies have shown that raising ambient temperature can promote the growth of *Tulipagesneriana*, increase plant height, advance flowering, flowering rate increase significantly (Yu and Liu 2015). Photoperiod is a biological clock rhythm in which plants perceive seasonal changes through periodic changes in the illumination time, thus coordinating their own metabolic processes and realizing growth and development (Wu et al. 2014a, b; Vaz and Kerbaui 2004). According to the different responses of plants to photoperiods, plants can be divided into long-day, day-neutral and short-day plants (Yeang 2013). After sensing the appropriate photoperiod, these plants will promote or inhibit the expression of flowering genes through the plant biological clock, which plays an important role in flower induction (Wang et al. 2018; Bai and Kawabata 2015). Studies have shown that in process of *Dendrobium nobile* cultivation, long day treatment could be used in the early growth stages and the short day treatment could be used in the flower bud differentiation stage, which is more conducive to its growth (Xue et al. 2018). Polyamines are low molecular weight aliphatic nitrogen-based compounds with high biological activities that are produced during biological metabolism, and they play important roles in regulating plant growth, morphogenesis and stress resistance (Chen et al. 2018). Spermidine (Spd) is a tribasic polyamine that is mainly involved in important physiological processes, such as plant growth and development, flower bud differentiation and stress resistance, and it influences the regulation of plant flowering (Tang et al. 2018). Studies have shown that low concentrations of Spd can significantly improve the physiological function and flower quality of the petal during the lotus senescence process (Li et al. 2017). In this experiment, we explored the effects of different temperatures, photoperiods and Spd concentrations on the flowering of *A. roxburghii*. Additionally, a preliminary study on the regulation of *A. roxburghii* flowering was conducted to provide a reliable theoretical basis.

Materials and methods

Plant materials and growth conditions

Anoectochilus roxburghii plants were selected from Yandang Mountain, Zhejiang Province, China. Disease-free and well-growing *A. roxburghii* plants were selected for ramet planting in Zhejiang Agricultural and Forestry University in July 2017. A 2:1:1 (volume ratio) mixture of organic fertilizer:peanut hull:sand was used. Ramets were

planted at intervals of 3 cm × 3 cm, and the Shao Qing-song cultivation management methods were applied (Shao et al. 2016). The *A. roxburghii* plants grown in a natural state were used as the control group (CK). The flower buds were collected every 7 days until flower bud differentiation was completed. All the plants were kept well-irrigated and were protected from bacterial pathogens and weeds. After 3 months of maintenance, the experimental plants were growing well.

Applications of different factors

Different temperatures

In this experiment, *A. roxburghii* plants of the same size were selected and divided into three groups of 150 plants each. In October 2017, the three groups of *A. roxburghii* seedlings were placed in an intelligent incubator at 20/15 °C (T1, day/night), 25/20 °C (T2) and 30/25 °C (T3), respectively. The light intensity was ~ 2500 LX, the relative humidity was about 70–80%, and the treatment time was 3 months. During this period, seedling nutrient solution and pest control drugs were sprayed once a week in 1/2 Murashige and Skoog. Plants grown under natural conditions served as the CK.

Different photoperiods

In this experiment, *A. roxburghii* plants of the same size were selected and divided into three groups of 150 plants each. In October 2017, the three groups of *A. roxburghii* seedlings were placed in 8/16-h (P1, day/night), 12/12-h (P2) and 16/8-h (P3) illumination environments, respectively. They were shaded by the same shading cloth using the same specifications every day. The 16-h illumination was supplemented using a 2500 LX fluorescent lamp, and the treatment time was 3 months. The management method during the growth period was the same as that for the temperature treatments. Plants grown under natural conditions served as the CK.

Different Spd concentrations

In this experiment, *A. roxburghii* plants of the same size were selected and divided into two groups of 150 plants each. In October 2017, the two groups of *A. roxburghii* seedlings were placed in their normal environment. During the growth process, the two groups were sprayed with 0.01 mol/L (A) and 0.1 mol/L (B) Spd, respectively, every 15 days, and the treatment time was 3 months. The management methods during the growth period were the same as those for the temperature and photoperiod treatments. Plants grown under natural conditions served as the CK.

Morphological indices

In this experiment, four morphological indices, the date of initial flowering, germination rate, pedicel length and flower bud size (length $\times$ width) of *A. roxburghii* were observed and recorded.

Physiological indices

In total, 0.5 g flower bud was cut into pieces, placed in phosphate buffer (0.1 mol/L, pH 7–7.4), and ground into a homogenate under ice water bath conditions. The samples were then diluted and labelled according to the requirement of each index. After all the samples were prepared, the samples were tested uniformly, and all the samples were measured three times. The physiological indicators to be measured were soluble sugar, soluble protein, peroxidase (POD), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and proline (PRO) levels. The test methods were as described in the appropriate Nanjing Jiancheng Bioengineering Institute (Nanjing, China) kits.

Statistical analysis

The test data were processed by Excel 2007, OriginPro 8.5 and other software, and the corresponding charts were produced. The average values of each group were compared using a one-way ANOVA with SPSS 19.0 software (SPSS, Chicago, IL, USA). The least significant difference method was used to detect the differences between the means ($P < 0.05$).

Results

Results of different temperature treatments

Flowering state of *A. roxburghii*

As shown in Table 1, compared with the CK, different temperature treatments had significant regulatory effects on the flowering time regulation of *A. roxburghii*, with the promotion and inhibition of flowering being more obvious. Using the initial flowering date of the CK group as the reference date, the *A. roxburghii* plants flowered well, with

the flowering rate reaching 34.87%, only in the incubator offset at T2, and the initial flowering period was advanced by 5 days. In the incubator set at T1, the process of inflorescence differentiation was not fully completed, resulting in dumb flowers and an 80% abortion rate of *A. roxburghii* plants. The flowering rate was only 10.50%, and the initial flowering period was delayed by 16 days. In the incubator set at T3, stem rot occurred in a large area owing to the excessive temperature, resulting in death, and the flowering rate was 0.

In addition, the flower bud sizes (length $\times$ width) and pedicel lengths of *A. roxburghii* treated at different temperatures were measured and compared periodically. As shown in Table 2, the flower bud sizes of *A. roxburghii* treated at the three temperatures were not significantly different during the vegetative growth period. However, at the later stage of flower bud differentiation, the flower bud size at T2 was significantly greater than those at T1 and T3. Additionally, the pedicel lengths of *A. roxburghii* grown at T2 were significantly longer than those of plants grown at T1 and T3. The pedicels grew from 7.20 cm to 14.02 cm during the whole flower bud differentiation period, suggesting that low and high temperatures were not conducive to the growth of the *A. roxburghii* flower buds or pedicels.

Physiological indicators of *A. roxburghii*

Soluble sugar and soluble protein contents Soluble sugar was consumed continuously during the differentiation of *A. roxburghii* flower buds (Fig. 1). The soluble sugar content in *A. roxburghii* flower buds increased from 0.42 mg/mL in the vegetative growth period to 0.59 mg/mL under the T1 treatment, and then decreased significantly. The T2 treatment conditions were similar to natural growth conditions, and the soluble sugar content decreased gradually during the whole flowering period. The soluble sugar content decreased slightly from the vegetative growth stage to the flower bud stage under T3 conditions, but after the flower bud stage, with the prolongation of the treatment time, the soluble sugar content showed an obvious upward trend. In contrast, the change in the soluble protein content in *A. roxburghii* flower buds was consistent with the change trend of the CK group, generally increasing at first and then decreasing with time. The soluble protein contents in flower buds of *A. roxburghii* under T1, T2 and T3

Table 1 Flowering of *A. roxburghii* after different temperature treatments

Temperature treatment	The initial flowering date	Florescence advance (days)	Flowering rate (%)
CK	12.29	0	35.48%
20/15 °C (T1)	01.15	– 16	10.50%
25/20 °C (T2)	12.24	+ 5	34.84%
30/25 °C (T3)	–	–	0

Table 2 Effects of different temperatures on the external morphology of *A. roxburghii*

Temperature treatment	Bud size (length × width) (mm)	Pedicle length (mm)	Determination time
CK	7.03 × 2.40	7.15 ± 0.04 ^a	2017.11.13.
T1	7.00 × 2.30	5.80 ± 0.12 ^b	2017.11.13.
T2	7.06 × 2.40	7.20 ± 0.09 ^a	2017.11.13.
T3	7.30 × 2.60	5.60 ± 0.13 ^b	2017.11.13.
CK	7.70 × 2.78	9.49 ± 0.12 ^a	2017.11.23.
T1	7.93 × 2.85	6.40 ± 0.11 ^c	2017.11.23.
T2	7.78 × 2.75	9.60 ± 0.14 ^a	2017.11.23.
T3	8.00 × 2.83	7.60 ± 0.08 ^b	2017.11.23.
CK	8.53 × 3.81	14.12 ± 0.11 ^a	2017.12.03.
T1	8.33 × 2.77	6.52 ± 0.11 ^c	2017.12.03.
T2	8.63 × 3.85	14.02 ± 0.15 ^a	2017.12.03.
T3	8.15 × 3.33	8.00 ± 0.08 ^b	2017.12.03.

The values are expressed as mean ± SE (n = 6). The mean values followed by different superscripts in the same row are significantly different ($P < 0.05$)

Fig. 1 Effect of temperature on soluble sugar and soluble protein content

conditions increased by 87.50%, 100% and 42.86%, respectively, in the middle stage of flower bud differentiation compared with in the early stage. From the middle stage of differentiation to the date of initial flowering, the soluble protein contents under the three temperature conditions decreased by 80.23%, 12.59% and 12.12%, respectively. The soluble sugar and soluble protein contents in *A. roxburghii* flower buds were statistically significantly different after 30 days under different temperature conditions ($P < 0.05$).

POD, SOD and CAT activity levels As shown in Fig. 2, the POD activity in *A. roxburghii* flower buds first decreased and then increased with time, and there were significant differences during the differentiation of flower buds under T1, T2 and T3 conditions. The increase in POD activity was greatest under T1 conditions, and the change

in POD activity was relatively small under T2 conditions. The SOD activity was more stable than those of POD and CAT, and there were no significant increases or decreases. There was no significant difference between the SOD activities of the T2 and CK group in each period. The SOD activity of the T1-treated group remained stable, and the activity in each period was significantly lower than that in the CK group. The SOD activity decreased slightly under T3 conditions. The CAT activity first showed a slow decline and then a sharp increase, which was consistent with the POD change trend. From the early stage of differentiation to the initial flowering stage, the changes in CAT activity levels under T1, T2 and T3 conditions were a 282.67 U/mg protein increase, a 29.53 U/mg protein decrease, and a 44.34 U/mg protein decrease, respectively, indicating that the greater increase in the CAT activity level occurred under low temperature conditions. The

Fig. 2 Effect of temperature on enzyme activity of POD.SOD.CAT

POD, SOD and CAT activities in *A. roxburghii* flower buds were statistically significantly different after 30 days under different temperature conditions ($P < 0.05$).

MDA and PRO contents As shown in Fig. 3, under T1, T2 and T3 conditions, the MDA contents in *A. roxburghii* flower buds increased compared with the CK at the initial stage of flower bud differentiation. The PRO content first increased and then decreased, and there were obvious differences among different temperature treatments. The MDA content showed an increasing trend with time, and compared with the early stage of flower bud differentiation, the MDA content decreased by 28.40% under T1 conditions but increased by 172.11% and 138.40% under T2 and T3 conditions, respectively. The increase in the MDA content showed the following trend: T2 > CK > T3 > T1. From the early stage of flower bud differentiation to the middle stage, the increases in the PRO contents in *A. roxburghii* flower buds under the three temperature conditions were significant, increasing by 40.77 $\mu\text{g/g}$, 11.83 $\mu\text{g/g}$ and 4.11 $\mu\text{g/g}$, respectively. The lower the

temperature, the greater the increase in the PRO content. Additionally, during the initial flowering stage, the PRO content was greatest under the T2 conditions. The stability levels of the changes in the PRO contents showed the following trend: T2 > CK > T1 > T3. The MDA and PRO contents in *A. roxburghii* flower buds were statistically significantly different after 30 days under different temperature conditions ($P < 0.05$).

Results of different photoperiod treatments

Flowering state of *A. roxburghii*

According to the photoperiod treatments, the flowering rate of *A. roxburghii* plants under P3 conditions was greater than those of the other groups (Table 3). The dates of initial flowering in P1, P2 and P3 were 8 days later, the same and 3 days earlier than that of the CK, respectively. Additionally, the pedicel length of *A. roxburghii* in P3 was significantly greater than those of the other groups. The flower bud sizes (length $\times$ width) of *A. roxburghii* growing

Fig. 3 Effect of temperature on MDA and PRO content

Table 3 Flowering of *A. roxburghii* after different photoperiod treatments

Photoperiod treatment	The initial flowering date	Florescence advance (days)	Flowering rate (%)
CK	12.29	0	35.48
08/16 h (P1)	01.07	− 8	10.26
12/12 h (P2)	12.29	0	14.65
16/08 h (P3)	12.26	+ 3	24.15

under P1 conditions were greater than those growing under P2 conditions (Table 4). There were significant differences in the growth and development of *A. roxburghii* under different photoperiod treatments. Short sunshine (8 h) was beneficial to vegetative growth, while long sunshine (16 h) significantly increased the number of flowers and significantly advanced the flowering period.

Physiological indicators of *A. roxburghii*

Soluble sugar and soluble protein contents The dynamic changes in soluble sugar and soluble protein contents in *A. roxburghii* flower buds subjected to different photoperiods were different (Fig. 4). After P1 and P3 treatments, the soluble sugar content first increased from the initial stage to the middle stage of flower bud differentiation, and then decreased sharply from the middle stage of flower bud differentiation to the initial flowering stage. The soluble sugar content under P2 conditions first decreased and then increased by 0.41 mg/mL. The *A. roxburghii* plants showed relatively good vegetative growth under P3 conditions. The soluble protein content of *A. roxburghii* plants grown under P1 and P3 conditions decreased slowly with time, and the soluble protein content under P2 conditions first increased and then decreased. The soluble protein content under P2 conditions was 0.03 g protein/L greater than under P3 conditions in the middle stage of flower bud differentiation, while under P1 conditions it was still 0.05 g

protein/L lower than under P3 conditions. The trend changes under P2 and P3 conditions were basically the same from the middle stage of flower bud differentiation to the initial flowering stage. The soluble sugar and soluble protein contents in *A. roxburghii* flower buds were statistically significantly different after 30 days under different photoperiod conditions ($P < 0.05$).

POD, SOD and CAT activity levels As shown in Fig. 5, the POD, SOD and CAT activities in *A. roxburghii* flower buds under different photoperiod conditions were measured. At the early stage of flower bud differentiation, the POD and SOD activities in the P3 treatment were significantly lower than those of other treatments, and the POD activities in the P1 and P2 treatments were about three times that in the P3 treatment. When growing to the initial flowering stage, the POD activity value of 486.59 U/mg protein under P3 conditions was significantly greater than under other conditions. However, the changes under the three photoperiod treatments were similar. With the prolongation of the treatment time, the POD activity levels first decreased and then increased. In contrast, the SOD activity under P1 conditions resulted in a gradual decreasing trend, P2 conditions resulted first in a decreasing trend and then an increase, and P3 conditions first resulted in an increasing trend and then a decrease. The changes in CAT activity in *A. roxburghii* flower or leaf under different photoperiod conditions were basically

Table 4 Effects of different photoperiod on the external morphology of *A. roxburghii*

Photoperiod treatment	Bud size (length × width) (mm)	Pedicle length (mm)	Determination time
CK	7.03 × 2.40	7.15 ± 0.04 ^a	2017.11.13.
P1	7.01 × 2.39	6.73 ± 0.07 ^b	2017.11.13.
P2	6.48 × 2.23	6.57 ± 0.09 ^b	2017.11.13.
P3	7.06 × 2.51	7.28 ± 0.12 ^a	2017.11.13.
CK	7.70 × 2.78	9.49 ± 0.12 ^a	2017.11.23.
P1	7.33 × 3.20	6.75 ± 0.11 ^c	2017.11.23.
P2	7.20 × 2.05	8.00 ± 0.10 ^b	2017.11.23.
P3	7.55 × 2.90	10.00 ± 0.08 ^a	2017.11.23.
CK	8.53 × 3.81	14.12 ± 0.11 ^a	2017.12.03.
P1	9.55 × 3.45	6.82 ± 0.09 ^c	2017.12.03.
P2	8.25 × 2.95	9.50 ± 0.15 ^b	2017.12.03.
P3	9.87 × 4.07	15.00 ± 0.12 ^a	2017.12.03.

The values are expressed as mean ± SE (n = 6). The mean values followed by different superscripts in the same row are significantly different ($P < 0.05$)

Fig. 4 Effect of photoperiod on soluble sugar and soluble protein content

Fig. 5 Effect of photoperiod on enzyme activity of POD.SOD.CAT

consistent with those of the POD activity in the flower buds, which indicated that the main role of CAT was to assist POD. At the initial flowering stage, the CAT activity of the T3 treatment was 328.88 U/mg protein, which was significantly higher than those of the other groups. The POD, SOD and CAT activities in *A. roxburghii* flower buds were statistically significantly different after 30 days under different photoperiod conditions ($P < 0.05$).

MDA and PRO contents As shown in Fig. 6, the MDA and PRO contents in *A. roxburghii* flower buds under different photoperiod conditions showed some differences with the prolongation of the treatment time. The MDA content in the CK, and P1 and P2 treatments showed a uniform upward trend, while in the P3 treatment it showed a slow downward trend, falling to $\sim 74.86\%$ in the early stage of flower bud differentiation. The PRO content in the CK group first increased and then fell. The PRO content in P1- and P3-treated *A. roxburghii* flower buds first decreased and then increased, while that in the P2 treatment showed a continuous increase. The MDA and PRO

contents in *A. roxburghii* flower buds were statistically significantly different after 30 days under different photoperiod conditions ($P < 0.05$).

Results of different Spd treatments

Flowering state of *A. roxburghii*

In this experiment, two Spd concentrations were used, and a water group was the CK. In the later stage, only the *A. roxburghii* plants treated with concentration A grew and blossomed well, with the flowering rate reaching 29.87%. However, the *A. roxburghii* plants treated with concentration B did not complete the inflorescence differentiation process, resulting in dumb flowers and the abortion of most *A. roxburghii* plants, resulting in a flowering rate of 0. As shown in Table 5, compared with the CK, the different Spd concentrations had significant regulatory effects on the flowering of *A. roxburghii*, and the flowering promotive effect was more obvious. Using the CK group as a reference, concentration A resulted in the advancement of initial

Fig. 6 Effect of photoperiod on MDA and PRO content

Table 5 Flowering of *A. roxburghii* after different Spd concentration treatments

Spd treatment	The initial flowering date	Florescence advance (days)	Flowering rate (%)
CK	12.29	0	35.48%
concentration A	12.22	+ 7	29.80%
concentration B	—	—	0

A. roxburghii flowering by 7 days, while concentration B resulted in *A. roxburghii*'s failure to blossom normally. In addition, as shown in Table 6, the pedicel lengths in plants treated with concentration A were significantly greater than that of B. The pedicel lengths of plants treated with A and B Spd concentrations increased by 9.28 cm and 0.65 cm, respectively, during the whole flower bud differentiation period, indicating that concentration B was not conducive to pedicel growth.

Physiological indicators of *A. roxburghii*

Soluble sugar and soluble protein contents The soluble sugar and soluble protein contents in *A. roxburghii* flower buds treated with different Spd concentrations were

determined. As shown in Fig. 7, the analysis of the soluble sugar and soluble protein contents in *A. roxburghii* flower buds treated with Spd revealed significant differences compared with the contents of the CK group. The soluble sugar contents in buds of plants treated with different Spd concentrations first increased and then decreased with the treatment time, reaching the greatest value in the middle of flower bud differentiation. There were no significant differences among soluble sugar contents in each period. The soluble sugar content in the CK group showed a tendency to continue to decline during this period. However, the soluble protein content of the CK group continued to rise, and the change trends of soluble protein contents under A and B treatments showed opposite trends. The A treatment resulted in a trend that first decreased and then increased,

Table 6 Effects of different Spd concentration treatments on the external morphology of *A. roxburghii*

Spd treatment	Bud size (length × width) (mm)	Pedicel length (mm)	Determination time
CK	7.03 × 2.40	7.15 ± 0.04 ^a	2017.11.13.
A	7.20 × 2.40	8.72 ± 0.10 ^a	2017.11.13.
B	6.50 × 2.25	5.87 ± 0.07 ^b	2017.11.13.
CK	7.70 × 2.78	9.49 ± 0.12 ^a	2017.11.23.
A	8.40 × 2.25	12.30 ± 0.16 ^a	2017.11.23.
B	7.25 × 2.29	6.10 ± 0.06 ^b	2017.11.23.
CK	8.53 × 3.81	14.12 ± 0.11 ^a	2017.12.03.
A	10.70 × 4.00	17.00 ± 0.13 ^a	2017.12.03.
B	7.43 × 2.35	6.52 ± 0.05 ^b	2017.12.03.

The values are expressed as mean ± SE (n = 6). The mean values followed by different superscripts in the same row are significantly different ($P < 0.05$)

Fig. 7 Effect of Spd treatments on soluble sugar and soluble protein content

while the B treatment resulted in a trend that first increased and then decreased. The turning point of the change occurred in the middle stage of flower bud differentiation, and the soluble protein content after the B treatment was much greater than after the A treatment. Additionally, the difference between the two contents widened. The soluble sugar and soluble protein contents in *A. roxburghii* flower buds were statistically significantly different after 30 days of different Spd treatments ($P < 0.05$).

POD, SOD and CAT activity levels After treatment with different Spd concentrations, the POD, SOD and CAT activities in *A. roxburghii* flower buds were measured. As shown in Fig. 8a, the POD activity first decreased and then increased with the prolongation of the treatment time. At the initial stage of flower bud differentiation, there were no significant differences between the CK group and activities in plants treated with A and B. From the initial stage of flower bud differentiation to the middle stage of flower bud differentiation, the POD activity levels in the three groups were similar. Subsequently, at the initial flowering stage,

the POD activities of the three groups increased to 480.9 U/mg protein, 812.05 U/mg protein and 541.31 U/mg protein, respectively. The early accumulation of POD is conducive to the smooth progression of the differentiation of *A. roxburghii* flower buds. As shown in Fig. 8b, there was a significant difference between the SOD activity levels in plants treated with Spd and that in the CK group. The SOD activity of the CK group continued to decrease, while in *A. roxburghii* subjected to the A and B treatments, the activity first increased and then decreased with the treatment time. At the initial stage of flower bud differentiation, there was no significant difference in the SOD activities between the A and B treatments. At the middle stage of flower bud differentiation, the SOD activity levels in A- and B-treated plants increased by 257.36 U/mg protein and 4.35 U/mg protein, respectively, and these activities were significantly different. At the initiation of flowering, the SOD activities in the three groups decreased to 102.15 U/mg protein, 182.56 U/mg protein and 114.37 U/mg protein, respectively, indicating that concentration A promoted the SOD activity. As shown in

Fig. 8 Effect of Spd treatments on enzyme activity of POD.SOD.CAT

Fig. 8c, the CAT activities in *A. roxburghii* after A and B treatments showed different trends with time. The CAT activity in A-treated plants first decreased and then increased, while that in B-treated plants decreased continuously. From the initial stage to the middle stage of flower bud differentiation, the CAT activities in the three groups decreased by 234.13 U/mg protein, 260.35 U/mg protein and 40.05 U/mg protein, respectively, indicating that concentration B inhibited the change in the CAT activity. The POD, SOD and CAT activity levels in *A. roxburghii* flower buds were statistically significantly different after 30 days of different Spd treatments ($P < 0.05$).

MDA and PRO contents As shown in Fig. 9, the MDA and PRO contents in *A. roxburghii* flower buds were determined. After A and B treatments, the MDA contents showed different patterns with time. The MDA content in plants treated with A showed a rising trend, which was consistent with the trend of the CK group. The MDA content in plants treated with B showed a continuous downward trend. At the initial stage of flower bud differentiation, the MDA content in the B treatment group was greater than in the A treatment group. At the flowering stage, the MDA content in the B treatment group was lower than that in the A treatment group, indicating that the Spd concentration in B inhibited the MDA content. The PRO content first decreased and then increased with time, contrary to the change trend of the CK group. At the initial stage of flower bud differentiation, the PRO content in the A treatment group was significantly greater than in the B treatment group, with a difference of 19.31 $\mu\text{g/g}$. At the middle stage of flower bud differentiation, there was no significant difference in the PRO content between the two Spd concentrations. At the initial stage of flowering, the PRO content in the B treatment

group was significantly greater than in the A treatment group, with a difference of 17.81 $\mu\text{g/g}$. This indicated that Spd functions in promoting the metabolism of PRO. The MDA and PRO contents in *A. roxburghii* flower buds were statistically significantly different after 30 days of different Spd treatments ($P < 0.05$).

Discussion

Effects of temperature on flowering regulation

The different temperature treatments had significant effects on the regulation and quality of *A. roxburghii* flowering. Different temperature treatments during flower bud differentiation caused significant differences in the initial flowering stage (Kurokura et al. 2013). The T1 treatment group after 30 days had the most change, with the flowering stage delayed by ~ 16 days. The T2 treatment group showed a significant advancement, by ~ 5 days, in the flowering stage. The temperature has a significant influence on the pedicel length. The lower the temperature, the shorter the pedicle. A low temperature during flower bud differentiation inhibits the elongation of the peduncle. The inflorescence differentiation process is not fully completed, flower development is inhibited and the flowering rate is reduced. If the temperature is too high, the damage to the plant itself will also increase, such as large-scale stem rot, which may lead to abortion or even plant death. This result is consistent with that of Yu and Liu (2015) in a study on the regulatory mechanisms of *Tulipa gesneriana*.

The soluble sugar and soluble protein contents in *A. roxburghii* flower buds of the CK, T1, T2 and T3 groups increased or decreased significantly with time. In the low temperature environment, the plants needed certain sugar

Fig. 9 Effect of Spd treatments on MDA and PRO content

and protein levels to protect against damage to the flower buds caused by low-temperature stress, and they also needed to maintain the required metabolic activities (Matsui et al. 2001). Therefore, the soluble sugar and soluble protein contents increased to a certain extent. When plants adapt to a low temperature environment, they need to strengthen the flower bud metabolism, which consumes nutrients during the flower bud differentiation stage. With the development of flower bud differentiation, nutrients are consumed continuously (Anderson et al. 2005). Here, with the prolongation of the high-temperature treatment time, the soluble sugar content showed an upward trend, while the soluble protein consumption changed little. Therefore, the higher temperature was not conducive to the consumption of nutrients and the growth of flower buds. This result is consistent with that of the flowering physiology of *Narcissus* spp (Liu et al. 2016).

During the middle stage of flower bud differentiation, the metabolism of reactive oxygen species (ROS) in plants is enhanced. The plants needed to increase the POD activity, start the enzymatic defense system to remove ROS in vivo, and avoid the peroxidation of cell membranes, thereby, enhancing the resistance of flower buds to stress. CAT is a main component of the plant ROS enzymatic scavenging system. Together with SOD and POD, it can scavenge excessive hydrogen peroxide and other ROS, protect plants from low temperature damage and maintain the vitality of plant flower buds (Xiao et al. 2018). From the initial stage of flower bud differentiation to the initial flowering stage, POD and CAT activity levels decreased significantly under T1 and T3 conditions, but slowly under T2 and CK conditions. The change in the SOD activity was contrary to that of the POD and CAT activities, which indicated that under the synergistic effects of the three factors, the flower bud differentiation process was successfully completed.

The MDA content is an important indicator of the damage caused by low temperatures to plants. It can reflect the degree of cell membrane lipid peroxidation and the degree of cell membrane damage. The PRO content is a main indicator for studying the tolerance and physiological status of plants against stress damage (Zhang et al. 2007). In this experiment, with the prolongation of the three temperature treatment times, membrane lipid peroxidation occurred in the cells of the plants, which led to an overall increase in the MDA contents of flower buds. The MDA increase was significant under T1 and T3 conditions, but lower under T2 and CK conditions. In addition, the PRO content first increased and then decreased with time, and it increased the most under T1 conditions. Wei (2015) found through a study of the regulation of the western rhododendron's flowering period that the MDA content increased with the prolongation of flowering time but that low

temperature inhibited the growth. Additionally, the PRO content in flower buds increased after a low temperature treatment, which is consistent with the results of our experiment. Thus, *A. roxburghii* could activate the enzymatic defense system to protect plant cells under T1 conditions, which alleviated the membrane lipid peroxidation damage to plant plasma membranes, and the increase in the PRO content under T1 conditions had a protective effect on plants during flower bud differentiation. The change trends in the MDA and PRO contents were stable at a suitable growth temperature.

Effects of photoperiod on flowering regulation

The different photoperiod treatments had significant effects on the regulation and quality of *A. roxburghii* flowering. With different photoperiod treatments, the florescence of *A. roxburghii* was significantly advanced or delayed, and there were also differences in flowering quality and flowering rate. Vaz and Kerbauy (2004) studied the effects of photoperiod and temperature on the in vitro growth and flowering of Orchidaceae plants and reasonably long exposures to sunshine could promote their growth and flower bud formation. In this experiment, the P3 treatment had the most promotive effect on flowering. The flowering date could be significantly advanced by 3 days, and the flowering rate was significantly greater than under the other treatments ($P < 0.05$). Under P1 conditions, the flowering delay effect was the greatest. The flowering period could be significantly delayed by 8 days, but there was no significant difference in flower bud size (length $\times$ width) between the other two groups. Thus, P1 conditions were beneficial to vegetative growth (promoting flower bud growth), and long exposures to sunshine significantly increased the flowering rate and significantly advanced the flowering period.

With the prolongation of the photoperiod treatment time, the physiological indicators in *A. roxburghii* flower buds changed significantly. The soluble sugar and soluble protein contents in *A. roxburghii* flower buds showed significant differences with time. Using the flowering conditions of plants treated with P1 and P2, we inferred that photoperiod treatments promoted vegetative growth by continuously increasing the soluble sugar content in *A. roxburghii* plants (Han et al. 2015). However, there were limited correlations between the regulation of flowering and florescence and changes in the soluble sugar content. The P1 treatment resulted in a low total soluble protein content, and the appropriate P3 treatment increased the total protein content. This phenomenon is similar to the result of Wang et al. (2018) when studying the effects of supplemental light quality on the secondary metabolites of *A. roxburghii*.

In addition, the POD and CAT activities shared a trend in which the activity first decreased and then increased with time. The differences among treatments were significant ($P < 0.05$). The number of flowers increased significantly and the flowering period advanced by 3 days under P3 conditions, suggesting that the change in the POD activity in *A. roxburghii* flower buds under P3 conditions might be correlated with the physiological activities that promote flowering. The SOD activity decreased with time, and its activity levels in the P1 and P2 treatment groups were much greater than that of the P3 treatment group at the flower bud differentiation stage. The short illumination time may affect the normal growth of plants, resulting in the plants' adaptive responses to avoid injury. Along with POD, SOD and CAT jointly help to form an antioxidant defense system, removing active oxygen free radicals in plants to protect them from the adversity caused by unfavorable growth factors (Yan et al. 2013). With the prolongation of the photoperiod treatment time, the MDA contents in the P1 and P2 treatment groups increased uniformly, while the content in the P3 treatment group continued to decrease. The PRO content showed an increasing trend with time, and there were significant differences among photoperiod treatments ($P < 0.05$). This indicated that under P3 conditions, *A. roxburghii* can activate the enzymatic defense system to protect its cells, relieve the damage caused by membrane lipid peroxidation on the plasma membrane, and play a protective role. This change is similar to the result of Xue et al. (2018) in which the photoperiod affected the photosynthesis and antioxidative enzyme activities of *Lonicera japonica*.

Thus, prolonging the illumination time can advance the initial flowering period of *A. roxburghii*. The initial flowering period of P3-treated plants occurred earlier than that of the control group. Because of the photoperiod induction, flower bud differentiation was accelerated and flowering occurred earlier. The photoperiod resulted in significant differences in florescence and the flower quality of *A. roxburghii*. This study is consistent with the results of Yeang (2013) in which the increased exposure time to light improved flower quality. However, some researchers believe that orchids are not affected by long or short illumination periods, making them day-neutral plants.

Effect of Spd on flowering regulation

The exogenous Spd treatments had significant effects on the florescence and flowering quality of *A. roxburghii*. The flowering period of *A. roxburghii* was significantly advanced or delayed when treated with different Spd concentrations, and there were differences in the flowering quality and rate of *A. roxburghii*. The A treatment had the greatest promotive effect on flowering, and the flowering

period could be significantly advanced by ~ 7 days. At this time, the pedicel length, flower bud ratio and flowering rate were significantly greater than those of the other treatment ($P < 0.05$).

The physiological indices after treatments with different exogenous Spd concentrations were measured. The higher concentration of exogenous Spd could significantly increase the soluble protein and proline contents, which was consistent with the evaluation of *Dendrobium* flower introduction and the effects of Spd on regulating the flowering period of *Dendrobium*. Compared with the CK group, the total soluble sugar content was greater and the consumption rate was lower. We hypothesized that the application of exogenous Spd delayed the decreases in soluble sugar and soluble protein contents in *A. roxburghii* and controlled the degrees of the reductions. Wu (2013) measured the POD activity in the leaves of *Dendrobium candidum* and found that the POD activity in plants treated with Spd first increased, then decreased and increased again, which was consistent with our experimental results. Yang and Li (2001) studied the growth process of broccoli and found that POD activity peaked at the stage of nutritional differentiation, then gradually decreased to the flower bud stage and rose again afterwards. Jin et al. (2007) studied the changes in POD activity levels in the bulbs of two kinds of *Lycoris* during growth and development. They found that the POD activity peaked at the early stage of flower bud differentiation and decreased slightly at the later stage. These results are consistent with our finding that the POD activity in the CK group first decreased and then increased.

The POD and SOD activities in *A. roxburghii* treated with two different Spd concentrations were greater than those in the CK, while the CAT activity in the initial flowering stage was lower than in the CK. This indicated that the three enzymes had synergistic effects and that exogenous Spd could increase the enzyme activity level. This phenomenon is similar to that found by Tang et al. (2018) when studying the effects of exogenous Spd on enzyme activity levels in rice. While a low Spd concentration could accelerate the process of flower bud differentiation, which not only promoted early flowering and prolonged the flowering period, but also improved the flower quality.

Conclusion

With the increasing demand for *A. roxburghii*, its quality and techniques to regulate flowering need to be improved. The whole flowering process of *A. roxburghii* requires 50 days. Different artificial treatment methods can affect its flower quality and some indicators, including the initial

flowering data, germination rate, flower bud size, pedicel length, the soluble sugar, soluble protein, MDA and PRO contents, and the POD, SOD and CAT activity levels. How to economically and efficiently induce and control the flowering period of *A. roxburghii* using its flowering characteristics and climatic conditions remains to be further studied.

Here, different temperature, photoperiod and Spd treatments had significant effects on the florescence and flowering quality of *A. roxburghii*. Appropriate cooling, long daylight conditions and Spd concentrations can advance the initial flowering stage of *A. roxburghii*. Low temperature and short daylight conditions can delay the initial flowering stage of *A. roxburghii*, while high temperature and high Spd concentrations can result in aborted flower buds and plant apoptosis. However, the most effective combinations could not be validated and further optimized. Therefore, further research is needed to find the most effective measures to promote and delay flowering in *A. roxburghii*.

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