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EFFECTS OF EXOGENOUS SPERMIDINE ON POLYAMINE METABOLISM IN LETTUCE (*LACTUCA SATIVA* L.) UNDER HIGH-TEMPERATURE STRESS

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Abstract

The physiological mechanism by which exogenous spermidine (Spd) alleviates high-temperature stress of lettuce (*Lactuca sativa* L.) was explored. The effects of exogenous Spd on the content of key enzymes of PA metabolism and endogenous polyamines (PAs) in lettuce under high-temperature stress were studied with G-S59 and P-S11 as test materials. The results showed that under high-temperature stress, the plant height, dry fresh weight of shoots and dry fresh weight of underground parts were all significantly reduced, and the growth of lettuce seedlings was inhibited. Exogenous Spd could significantly alleviate the inhibition of lettuce seedling growth by high-temperature stress. The contents of free, conjugate and bound putrescine (Put) were significantly decreased in leaves of lettuce seedlings under high-temperature stress, and in contrast, the contents of Spd and spermine (Spm) in free and bound states were significantly increased. Exogenous Spd effectively increased the Spd and Put contents but inhibited the increase in Spm content. Exogenous Spd could increase the plants Spd content by increasing the activity of diamine oxidase (DAO), polyamine oxidase (PAO), s-adenosylmethionine decarboxylase (SAMDC) and ornithine decarboxylase (ODC), and increasing the amount of upstream Spd, inhibiting the production of downstream Spm and maintaining higher endogenous Spd levels. The effects were more obvious in heat-resistant varieties such as P-S11. The results showed that exogenous Spd promoted the synthesis of endogenous Put and Spd, which might have great effects on the heat tolerance of lettuce seedlings.

Key words: Lettuce, High-temperature stress, Exogenous spermidine, Polyamine metabolism.

Introduction

In recent years, global temperature fluctuations have increased, and the frequency of extreme high-temperature weather events has increased (Piao *et al.*, 2010). High temperature is a common adversity encountered by plants, and it inhibits plant growth (Boyer, 1982). At extremely high temperatures, plant cell are damaged or cell death can occur within minutes (Ahuja *et al.*, 2010). High-temperature stress can affect all aspects of plants, such as development, growth, germination, reproduction and yield, and it also has disadvantageous effects on plant leaves, such as a decrease in leaf senescence, leaf area and leaf water potential (McClung & Davis, 2010), and on the total photosynthetic performance of plants (Yang *et al.*, 2006; Greer & Weedon, 2012). At present, by the traditional breeding method, there are few successful examples of transgenic plants with high-temperature resistance (Tian & Guo, 2012). In addition to modifying the genetic pathway, the application of exogenous growth regulators to plants under adverse conditions can also be used as an effective method to improve the high-temperature tolerance of crops (Wahid & Close, 2007).

Polyamines (PAs) are common polycations in other organisms and plants, and they function as important plant growth regulators (Hussain *et al.*, 2011) and in resistance to abiotic and biological stresses and plant growth and development (Majumdar *et al.*, 2019). The common PAs in plants are spermidine (Spd), putrescine (Put), spermine (Spm) and cadaverine (Cad), among others. Spm, Put and Spd are the three main PAs in plants, which mainly exist in the bound state, conjugate state and free state (Kakkar & Sawhney, 2002). Compared with other kinds of PAs, Spd has a more obvious effect on plant physiological function (Kasukabe *et al.*, 2006; Kasukabe *et al.*, 2004). The central product of the PA biosynthesis pathway is Put, and three key enzymes of the synthesis process, S-adenosylmethionine decarboxylase (SAMDC), arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), play a vital role (Mattoo & Handa, 2008).

In recent years, the effects of PAs on plant resistance to abiotic and biological stresses have been fully studied (Liu *et al.*, 2006). In these studies, the PA concentration in plants changed in response to various types of environmental stress, such as drought, high-temperature and salt. In stressed plants, PA biosynthesis ability increases with increasing stress, and levels of intracellular PAs increase by 2 times to 3 times more than in unstressed plants (Kasukabe *et al.*, 2004). Some research has revealed an increase in PA content in plants under low-temperature (Kushad & Yelenosky, 1987), salt (Chattopadhayay *et al.*, 2002), drought (Nayyar *et al.*, 2005) and other stresses that can improve the plant's ability to resist those stresses. PAs and temperature stress have always been a hot topic of research, and the alterations in Spm, Put and Spd in different plants under different stresses are also very different (Alcázar *et al.*, 2006). In a study of *Arabidopsis thaliana,* free Put content was observed to increase after 24 h of low temperature, but the content of Spd did not change significantly (Cuevas *et al.*, 2008). The contents of Spm, Spd and Put in loquat fruit changed continuously with time at chilling injury temperature, but the extent of the change in PA content was small when not at this temperature (Zheng *et al.*, 2000). When cold damage occurred in zucchini, the content of Put improved, but the contents of Spm and Spd declined. Heat treatment increases the content of Spd and Spm and reduces the content of Put, indicating that Spd and Spm are negatively related to the chilling injury index, while the content of Put is positively related to the chilling injury index (Kramer & Wang, 1990).

Lettuce (*Lactuca sativa* L.), which is native to the Mediterranean coast of Europe, belongs to the lettuce family Compositae. The species is native to cold environments and is not resistant to high temperatures, especially temperatures greater than 30°C, and such conditions result in poor growth, scorched hearts, bolting and flowering (Fan, 2018). In this experiment, a high-temperature tolerant lettuce variety (G-S59) and a high-temperature susceptible variety (P-S11) with different genetic backgrounds were used as experimental materials. The effects of spray application of exogenous Spd on physiological indexes and key enzymes of endogenous PAs and PA metabolism under high-temperature stress, and the alleviating effect of exogenous Spd on the high-temperature stress, were examined in lettuce, supporting a theoretical basis for the study of heat resistance of lettuce to potentially decrease losses caused by heat damage.

Materials and Methods

The experimental materials were high-temperature resistant (G-S59) and high-temperature susceptible (P-S11) lettuce varieties (*Lactuca sativa* L.) provided by the leaf vegetable research group of Beijing University of Agriculture.

Seeds with full grains, uniformity and disease free were selected for the experiment. First, 9-cm qualitative filter paper was soaked with distilled water and placed in the culture dish, which was placed in a light incubator to promote germination. The culture environment was set as follows: day-night temperature, 20°C/15°C; photoperiod, 12 h/12 h; relative humidity 70~75%. After the seeds were germinated, they were seeded in a plate well and cultured in a light culture box. After growing to the three-leaf stage, the lettuce seedlings were transplanted into an 11 × 12-cm culture dish and cultured in the above light incubator. Evenly growing plants were selected for subsequent experiments.

When the seedlings exhibited six leaves and one heart, half remained in the light incubator, and the other half were transferred to a light incubator with a day and night temperature of 30°C/25°C, relative humidity of 70~75% and photoperiod of 12 h/12 h. The plants in each light incubator were divided into two groups and sprayed with 1.0 mM of Spd and deionized water using a small atomizer with gloves at 5:00 PM each day (this is the optimum concentration for lettuce application to resist high-temperature stress) (Li *et al.*, 2019). The front and back of leaves were sprayed evenly until the entire leaf surface was covered and completely wet but without dripping. Spd was obtained from Sigma as a white solid. Four treatments were applied:

CK + Spd: control treatment with 1.0 mM Spd;
T + Spd: high-temperature stress treatment with 1.0 mM Spd
T: high-temperature stress treatment with distilled water
CK: control treatment with distilled water.

On the 7th day of the experiment, morphological indices such as aboveground fresh weight, plant height, water content and leaves were measured. PA metabolism-related enzymes and endogenous PAs were measured by sampling on days 0, 1, 3, 5 and 7 of treatment.

Determination of the growth index: Five lettuce seedlings were selected stochastically, and their plant height and root length were measured with tape. The selected lettuce seedlings were cleaned with deionized water, the surface impurities were removed, the water on the surface of the plants was dried with an absorbent paper and the fresh weight was determined with an electronic balance. Seedlings were then placed in an electric thermostatic air blowing drying box (DHG-9245, Shanghai Viheng Scientific Instrument Co., Ltd.) at 105°C for 30 min. After that, the temperature was adjusted to 75°C, and the dry weight was measured after baking to a constant weight.

The formulas for calculating the relative water and water content were as follows (Li, 2006):

$$\text{Relative water content} = (\text{fresh weight} - \text{dry weight})/(\text{saturated fresh weight} - \text{dry weight}) \times 100\%;$$

$$\text{Water content} = (\text{fresh weight} - \text{dry weight})/\text{fresh weight} \times 100\%.$$

The fourth leaf of lettuce seedling was selected on the 7th day of treatment to measure the leaf area, and an Epson Perfection V700 photo scanner and Win SEEDLE image analysis software were adopted for the measurements and processing.

Determination of polyamine content: Spd, Spm and Put in free and bound states were extracted according to Liu *et al.*, (2006), Flores & Galston (1982), Chen *et al.*, (2013) and Duan *et al.*, (2008).

Fresh lettuce leaves were washed and shredded, 0.5 g of leaves were placed in a mortar and 1.6 ml of 5% precooled PCA was added to the ice bath environment at 4°C for grinding. Seedlings were homogenized and transferred to centrifuge tubes. After incubation for 1 h in an ice bath at 4ºC, the samples were centrifuged at 12000 × g, 4°C for 20 min. Subsequently, the supernatant and precipitate were collected and used to determine the free and soluble bound PAs in perchloric acid and insoluble bound PAs in perchloric acid by precipitation.

Determination of free PA content: First, 0.7 ml of culture liquid was placed in a new centrifuge tube. Then, 1.4 ml of 2 M NaOH and 15 µl of benzoyl chloride were added to the mixture for 20 s and kept at 37°C for 30 min. Next, 2 ml of saturated NaCl was added, mixed well and supplemented with 2 ml of cold ether with oscillation, and the sample was centrifuged at 3000 × g for 5 min. Finally, 1 ml of ether was added for drying, and the samples were stored at -20°C in 100 µl methanol (60% W/V).

To determine the bound PA content, 0.7 ml of the above mentioned supernatant was placed in an amber bottle, and 5 ml of 6 mol/L HCl was added. Then, a butane spray gun was used for sealing. After the sealing was checked, the supernatant was placed at a temperature of 110°C for hydrolysis and then filtered after 18 h. After the solution was completely dried at 70℃, the remaining material was added to 1.6 ml of 5% PCA for re-suspension dissolution. The following procedure was consistent with the determination of the free PA content.

Determination of bound PA content: Five perfect PCAs were used to wash and precipitate the samples, followed by 4 rounds of centrifugation (1000 × g, 5 min), after which the supernatant was discarded. The precipitate was suspended in HCl (5 ml 6 mol/L) and transferred to ampoule bottles for sealing, hydrolysed at 110°C for 18 h, filtered, evaporated at 70°C and dissolved in 1.6 ml of 5% PCA.

The above samples were injected with 10 µl and analysed on an Agilent HPLC analyser with the following parameters: mobile phase, 64% methanol prepared with ultrapure water; detection wavelength, 230 nm; Kromasil reverse phase C18 column (250 mm×4.6 mm); column temperature, 25°C; flow rate, 0.7 ml/min. Standard curves were made from standard samples (Sigma Chemicals, USA) with Spd, Spm and Put.

Determination of polyamine synthase activity: The ODC activity was determined as previously described (Zhao *et al*., 2003). The reaction mixture included 0.3 ml of enzyme extract, 5 mM EDTA ≤ 50 µM pyridoxal phosphate and 100 mM Tris-HCL buffer (pH 7.5). The reaction lasted for 2 min at 37°C, after which 0.2 ml of 25 mM L-ornithine was added (ODC enzyme activity was determined). The reaction mixture was incubated at 37°C for 60 min, and PCA was then added to terminate the reaction (PCA in the control group was added at the beginning of the incubation). The mixture was centrifuged at 3000 g for 10 min, and 0.5 ml of the supernatant was collected. The supernatant was supplemented with 1 ml of 2 M NaOH and 7 µl of benzophenyl chloride with vortexing for 20 s and heated to 37°C for 30 min. Then, 2 ml of saturated NaCl and 2 ml of ether were added to extract benzoyl polyamine, followed by centrifugation at 1500 g for 5 min. Finally, 1 ml of the ether phase was collected and dried with forced air on an ultra-clean workbench, dissolved in 3 ml methanol and examined with an ultraviolet spectrophotometer at 254 nm.

As a reference for the determination of SAMDC activity (Kaur-Sawhney *et al*., 1982), the reaction substrates included 100 $\text{mmol}\cdot\text{L}^{-1}$ potassium phosphate buffer (1 $\text{mmol}\cdot\text{L}^{-1}$ PLP, pH 7.5, 5 $\text{mmol}\cdot\text{L}^{-1}$ EDTA, containing 5 $\text{mmol}\cdot\text{L}^{-1}$ DTT), 40 $\text{mmol}\cdot\text{L}^{-1}$ S-adenosine methionine and enzyme solution after dialysis and were kept at 37°C for 30 min with slight agitation to detect the change in CO_2 concentration before and after the reaction. SAMDC activity was expressed in mul µl $(CO_2)\cdot g\text{-}^{1}$ $\text{FW}\cdot\text{min}^{-1}$.

Determination of polyamine oxidase activity: Extraction and activity determination of diamine oxidase (DAO) and polyamine oxidase (PAO) were performed as previously described (Su *et al.,* 2005). Then, 0.5 g of fresh sample was added to 2 ml of 100 mmol·L-1 potassium phosphate buffer (pH 6.5), crushed in an ice bath and centrifuged at 10000 × g for 20 min (4°C). The supernatant was used as the crude extract for the determination of DAO and PAO activities.

The crude extract mixture consisted of 2.5 ml sodium phosphate buffer (0.1 $\text{mol}\cdot\text{L}^{-1}$, pH 6.5), 0.1 ml horseradish peroxide solution (250 $\text{U}\cdot\text{mL}^{-1}$) and 0.2 ml enzyme extract, and 0.2 ml of colour solution (100 ml containing 25 µg of N,N-dimethyl aniline and 10 mg of 4-aminopyrine) was added to 15 µl of Spd and Spm to initiate the reaction. To determine the activity, the reaction mixture was placed at 25°C for 30 min. Using 0.001 Δ $\text{OD550}\cdot\text{min}^{-1}$ as an enzyme activity unit (U), the enzyme activity was expressed as $\text{U}\cdot\text{g}^{-1}$ FW.

Statistical analysis

SPSS 19.0 and Excel 2007 were used for the variance and significance analyses. In the significance analysis, different letters indicated significant differences between different treatments in the same period ($p<0.05$).

Results

Effect of exogenous spd on morphological indexes of lettuce under high-temperature stress: Table 1 shows that the plant height, fresh weight and dry weight of the aboveground parts, fresh weight and dry weight of the underground parts, relative water content and water content of G-S59 and P-S11 all decreased significantly under high-temperature stress compared with the normal-temperature control. The above indices of G-S59 under high-temperature stress were decreased by 7.3%, 42.5%, 34.3%, 56.4%, 50.0%, 5.2% and 5.0%, respectively, compared with the normal-temperature control values. Spraying of exogenous Spd significantly increased the growth of lettuce under high-temperature stress, increasing the above indexes by 2.8%, 25.5%, 27.2%, 17.6%, 50.0%, 2.9% and 7.7%, respectively. The above indices of P-S11 under high-temperature stress decreased by 21.6%, 31.5%, 51.0%, 65.6%, 60.0%, 0.8% and 5.3%, respectively, compared with the normal-temperature control values. The application of exogenous Spd significantly increased the growth of lettuce under high-temperature stress, increasing the above indexes by 25.3%, 35.5%, 104%, 157%, 100%, 0.9% and 3.5%, respectively. In conclusion, spray application of exogenous Spd improved the growth indexes of lettuce at a high temperature.

As shown in Table 2, the leaf area of the normal-temperature control lettuce was significantly higher than that exposed to high-temperature stress. The average leaf area, average leaf length and average leaf width of G-S59 was decreased by 38.4%, 22.4% and 16.5%, respectively, compared with the control values under high-temperature stress. Under high-temperature stress, the average leaf area, average leaf length and average leaf width of exogenous Spd-treated plants were 29.9%, 11.7% and 26.4%, respectively, compared with the control. The corresponding values for P-S11 plants under high-temperature stress were 21.1%, 10.7% and 17.5% lower than the normal-temperature control. Under high-temperature stress, the average leaf area, average leaf length and average leaf width of exogenous Spd-treated plants were 39.4%, 22.1%, 22.8% higher compared with the high-temperature-treated plants, respectively.

The results shown in Tables 1 and 2 show that exogenous Spd could effectively relieve the effects of high-temperature stress on lettuce growth and that exogenous Spd had a reduced effect on the growth of G-S59 than P-S11 under high-temperature stress. Under high-temperature stress, exogenous Spd could maintain taller plant heights and larger leaf areas in lettuce.

Table 1. Effects of exogenous Spd on the growth of lettuce under high-temperature stress.

Variety	Treatment	Plant height (g·cm-1)	Shoot		Root		Water content (%)	Relative water content (%)
			Fresh weight (g·plant-1)	Dry weight (g·plant-1)	Fresh weight (g·plant-1)	Dry weight (g·plant-1)		
G-S59	CK	13.88 ± 0.25ab	8.85 ± 1.23a	0.67 ± 0.14a	0.39 ± 0.11a	0.04 ± 0.01a	92.44 ± 0.53a	88.30 ± 1.23ab
	CK+Spd	14.84 ± 0.93a	6.07 ± 0.59b	0.47 ± 0.02b	0.23 ± 0.05b	0.03 ± 0.01a	92.16 ± 0.65a	93.73 ± 2.31a
	T	12.87 ± 1.01b	4.51 ± 0.75b	0.44 ± 0.09b	0.17 ± 0.03b	0.02 ± 0.01b	87.62 ± 1.60b	83.88 ± 5.22b
	T+Spd	14.23 ± 0.15ab	4.66 ± 0.69b	0.56 ± 0.04ab	0.20 ± 0.05b	0.03 ± 0.01a	90.19 ± 0.26ab	90.34 ± 3.50ab
P-S11	CK	14.38 ± 0.70a	5.50 ± 1.09a	0.49 ± 0.14a	0.61 ± 0.20a	0.05 ± 0.02a	91.23 ± 1.18ab	89.12 ± 1.12ab
	CK+Spd	14.85 ± 0.12a	5.66 ± 0.178	0.43 ± 0.03ab	0.60 ± 0.10a	0.05 ± 0.01a	92.30 ± 0.40a	90.35 ± 1.64a
	T	11.27 ± 0.65b	2.77 ± 1.26b	0.24 ± 0.12b	0.21 ± 0.15b	0.02 ± 0.01b	90.45 ± 0.41b	84.40 ± 0.14c
	T+Spd	14.12 ± 0.47a	5.11 ± 1.14a	0.49 ± 0.10a	0.54 ± 0.17a	0.04 ± 0.02a	91.35 ± 4.11ab	87.34 ± 0.20b

CK, control treatment with distilled water; CK + Spd, control treatment with 1.0 mM spermidine; T, high-temperature stress treatment with distilled water; T + Spd, high-temperature stress treatment with 1.0 mM spermidine.

Note: different letters indicate significant differences among different treatments in the same period ($p<0.05$). The same scheme applies below.

Table 2. Effects of exogenous Spd on the growth of lettuce leaves under high-temperature stress.

Variety	Treatment	Average leaf length (cm)	Average leaf width (cm)	Leaf area ($cm^2 \cdot plant^{-1}$)
G-S59	CK	80.51 ± 4.31a	49.80 ± 3.71ab	26.97 ± 6.82ab
	CK+Spd	74.1 ± 2.98ab	56.80 ± 4.26a	27.88 ± 2.95a
	T	62.51 ± 6.54b	41.57 ± 3.90b	16.61 ± 2.73b
	T+Spd	64.80 ± 9.08b	52.55 ± 10.83ab	21.57 ± 7.43ab
P-S11	CK	72.39 ± 6.54a	53.98 ± 7.05a	24.62 ± 4.63ab
	CK+Spd	74.05 ± 5.68a	56.68 ± 5.22a	29.57 ± 7.24a
	T	57.12 ± 9.46b	48.18 ± 7.68a	20.32 ± 7.12b
	T+Spd	69.74 ± 4.27b	59.17 ± 6.88a	28.32 ± 6.24a

Effect of exogenous spd on the free polyamine content of lettuce under high-temperature stress: There was no significant difference in the free Spd, Spm and Put of G-S59 and P-S11 in the normal-temperature control (Fig. 1). In general, the free PA content of lettuce treated with exogenous Spd was higher than that of lettuce treated with deionized water, and the content remained relatively stable. Among the free PAs, the content of free Spd was the highest, showing a significantly higher value than the contents of free Spm and free Put.

Under high-temperature stress, the content of free Spd in G-S59 treated by spraying of exogenous Spd on the 1st, 3rd and 5th days was significantly higher than that of spraying deionized water, which increased by 52.8%, 21.9% and 19.9% respectively. There was no significant difference between day 0 and day 7. Under the high-temperature only treatment and exogenous Spd plus high-temperature treatment, the content of free Spd first increased and then decreased with the treatment time. After 1 day of high-temperature stress, the content of free Spd increased significantly and reached a peak on the 3rd day of treatment, and the content of free Spd in both treatments was higher than that of the control in each of the same periods. The free Spd content of P-S11, which was increased by 21.1%, 26.3% and 7.3% on the 1st, 3rd and 5th days, respectively, was significantly higher than the deionized water control under high-temperature stress. There was no significant difference between day 0 and day 7. Under high-temperature treatment alone and exogenous Spd plus high-temperature treatment, the free Spd content showed a trend toward slowly decreasing and maintained with the treatment time, and the peak value appeared on the 3rd day of treatment. From the 1st day of treatment, the free Spd content was significantly higher in lettuce sprayed with exogenous Spd at a high temperature. The free Spd content was lower in lettuce treated with deionized water than that treated with Spd and subjected to high temperature. The magnitude of the change in Spd content was slightly greater in P-S11 than G-S59.

The content of free-state Spm in G-S59 under high-temperature stress gradually increased with the treatment time and peaked on the 3rd day of treatment, after which the content was stabilized. The free-state Spm content of exogenous Spd with deionized water was significantly higher than the free-state Spm with treatment. The free-state Spm content of P-S11 increased gradually with the treatment time under high-temperature stress, and the content of free-state Spm was lower with the exogenous Spd spray treatment than the free-state Spm with deionized water treatment. The range of the quantitative change in P-S11 was slightly higher than that of the G-S59 content.

The free-state Put content of G-S59 was gradually decreased under high-temperature stress with the treatment time, and the content of free Put was higher in response to exogenous Spd spray than the free-state Put with deionized water treatment. The free-state Put content was gradually decreased in P-S11 with the treatment time, and the content of free Put was higher with the exogenous Spd spray treatment than the free-state Put with deionized water treatment. The magnitude of the change in free-state Put content was slightly higher in P-S11 than G-S59.

Under high-temperature stress, the ratio of (Spd+Spm)/Put was increased significantly between G-S59 and P-S11. There was no significant difference in the (Spd+Spm)/Put ratio of free PAs at room temperature. The ratio of exogenous Spd was lower under high-temperature stress compared with no high-temperature stress, but it was still significantly higher than the normal temperature control.

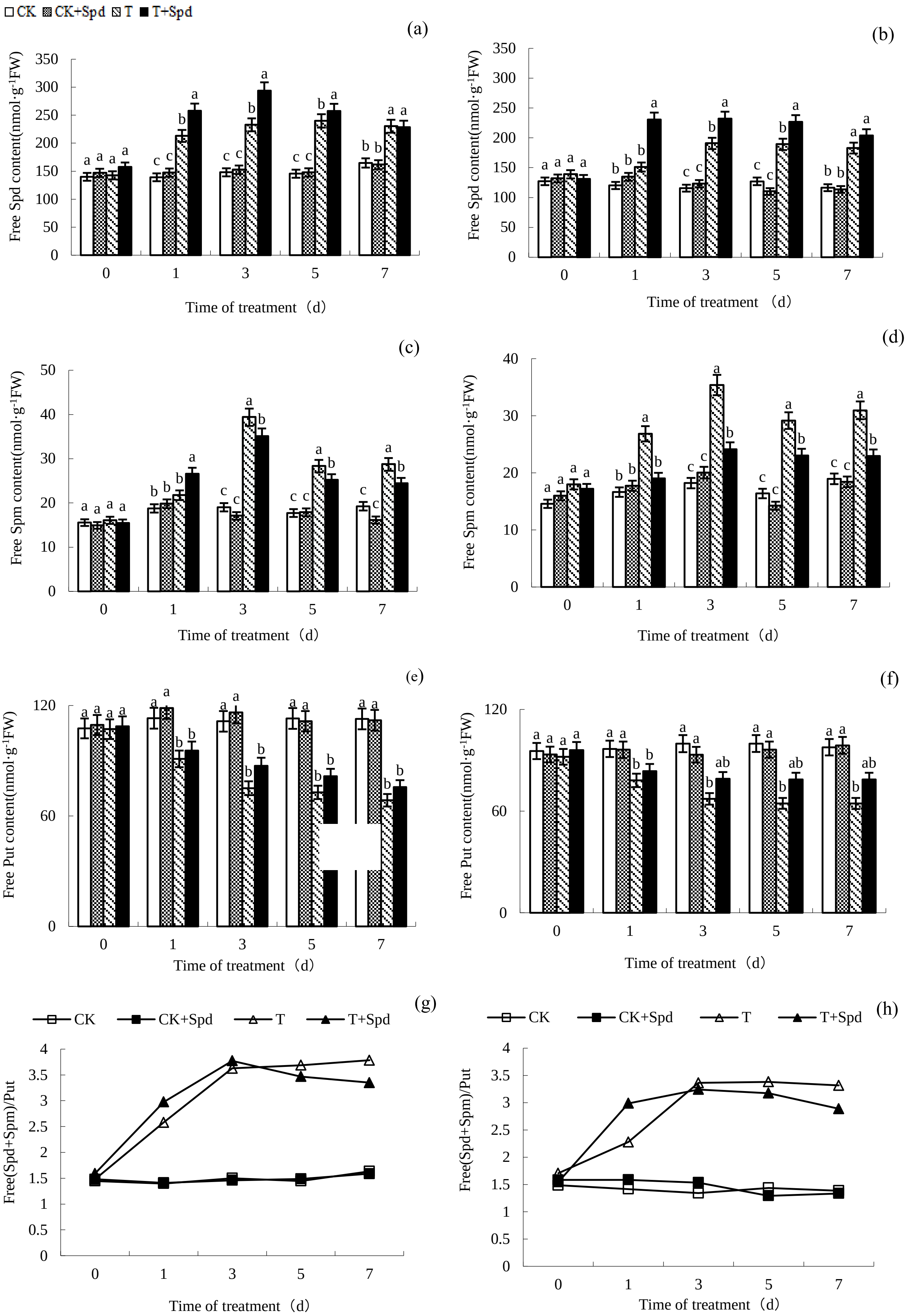

Fig. 1. Effect of exogenous Spd on free Put, Spd and Spm contents of lettuce under high-temperature stress. The lettuce variety of a, c, e, g was G-S59, and the lettuce variety of b, d, f, h was P-S11.

Effect of exogenous spd on the conjugated polyamine content of lettuce under high-temperature stress: The conjugate Spd, Spm and Put contents of G-S59 and P-S11 were not significantly different in the control at room temperature (Fig. 2). Overall, the content of conjugate PAs was higher in lettuce treated with exogenous Spd than with deionized water, and the content of conjugate PAs remained relatively stable. Among the conjugate PAs, the content of conjugate Spd was the highest, which was significantly higher than the conjugates Put and Spm.

The content of the conjugate Spd decreased in G-S59 with the treatment time, high-temperature stress with spray application of exogenous Spd and high-temperature stress only. The content of conjugate Spd increased significantly after 1 day of high-temperature stress, On days 5-7 of treatment, the content of conjugate Spd became stabilized, and the combined Spd content of the two treatments was higher than the corresponding level of their respective controls. Under high-temperature stress, the conjugate Spd content of P-S11 increased slightly at the beginning of the first day of treatment, and the content of the conjugate Spd was relatively stable over the next few days. Beginning on the first day of treatment, the content of the conjugate Spd clearly increased, peaked on the 3rd day of treatment and then tended to stabilize. During the treatment period, the content of the conjugate Spd was significantly elevated with high-temperature spraying than with the pure high-temperature treatment. The conjugate Spd content was lower in P-S11 than G-S59. The conjugate Spm content clearly increased in G-S59 in response to high-temperature treatment alone and high-temperature with exogenous Spd spray application at the beginning of the first day of treatment. On the first day of treatment, the conjugate Spm content was slightly elevated in response to high-temperature plus exogenous Spd treatment than high-temperature treatment alone. From the 3rd day to the end of treatment, the conjugate Spm content was lower with the high-temperature plus exogenous Spd treatment than the high-temperature treatment alone. The conjugate Spm content of P-S11 was determined under high-temperature stress. Under high-temperature only and high-temperature plus exogenous Spd treatments, the content of bound Spm clearly increased, and the content of the conjugate Spm was consistently significantly higher with the pure high temperature treatment. In response to high-temperature treatment alone, the content of conjugated SPM increased significantly. The difference in conjugate Spm contents between the two treatments under high-temperature stress was significantly higher when compared with G-S59.

The conjugate Put content decreased in G-S59 with the treatment time and was maintained at a certain high-temperature level together with exogenous Spd treatment and high-temperature treatment alone. The content of conjugate Put was lower in response to deionized water treatment than **e**xogenous Spd treatment, and it was lower than in the normal temperature control. Under high-temperature stress, the content of conjugate Put was decreased in P-S11 with the treatment time, and the content of free Put was higher with exogenous Spd than deionized water treatment. In the 3rd replicate, the difference was significant at 5 and 7 days. The variation in content was slightly greater than the combined Put contents of G-S59.

The (Spd+Spm)/Put ratios of G-S59 and P-S11 showed no significant difference at room temperature. Under high-temperature stress, the (Spd+Spm)/Put ratio of conjugate PAs was increased significantly. The (Spd+Spm)/Put ratio was lower in G-S59 sprayed with exogenous Spd under high-temperature stress than high-temperature only treatment on the 7th day of treatment, but it was higher than in plants exposed solely to high-temperature treatment prior to the 7th day of treatment. The (Spd+Spm)/Put ratio of P-S11 sprayed with exogenous Spd was consistently higher under high-temperature stress than high-temperature treatment alone.

Effect of exogenous spd on the bound polyamine content of lettuce under high-temperature stress: As shown in (Fig. 3) there was no significant difference between bound Spd and Spm of G-S59 and P-S11 in the normal-temperature control, and the Put content was increased slightly on day 1 of treatment and then stabilized. In general, the bound-state PA content was higher in response to the exogenous Spd spray than the deionized water spray treatment, and the content of the bound-state PA was relatively stable. Among the bound-state PAs, the bound-state Spd had the highest content and was obviously present at higher levels than the bound-state Put and bound-state Spm.

Under high-temperature stress, high-temperature only treatment and exogenous Spd spray treatment, the bound Spd content of G-S59 was clearly increased on the first day of treatment, peaked on the 3rd day of treatment and then decreased slightly but was relatively stable. On the 3rd and 5th days of treatment, the content of bound Spd was significantly higher with the exogenous Spd spray treatment than the pure high temperature treatment. The bound Spd content was significantly lower in the normal temperature control than the two high-temperature treatments. The bound Spd content of P-S11 under high-temperature stress, only high-temperature treatment and exogenous Spd treatment clearly increased from the 1st day of treatment and tended to stabilize after the 3rd day of treatment. On the 1st, 3rd, 5th and 7th days, the content was significantly lower in response to the high-temperature only treatment than the exogenous Spd spray treatment. The content of bound Spd was significantly lower in the control at room temperature than the two treatments at high temperature. During the treatment period, the bound Spd content was significantly lower in response to only high-temperature treatment than exogenous Spd spray treatment.

The bound Spm content was increased significantly in G-S59 at the beginning of the 1st day of treatment under high-temperature stress, and the bound Spm content increased significantly in response to both treatments after the 3rd day of treatment. On the 1st, 3rd, 5th and 7th days of treatment, the bound Spm content was significantly higher with the high-temperature only treatment than exogenous Spd treatment. The content of bound Spm was similar in P-S11 and G-S59 under high-temperature stress, and it was significantly higher in response to the high-temperature only treatment on the 3rd, 5th and 7th days of treatment than the exogenous Spd treatment. The bound Spm content was significantly lower in the control at room temperature than the two treatments at high temperature.

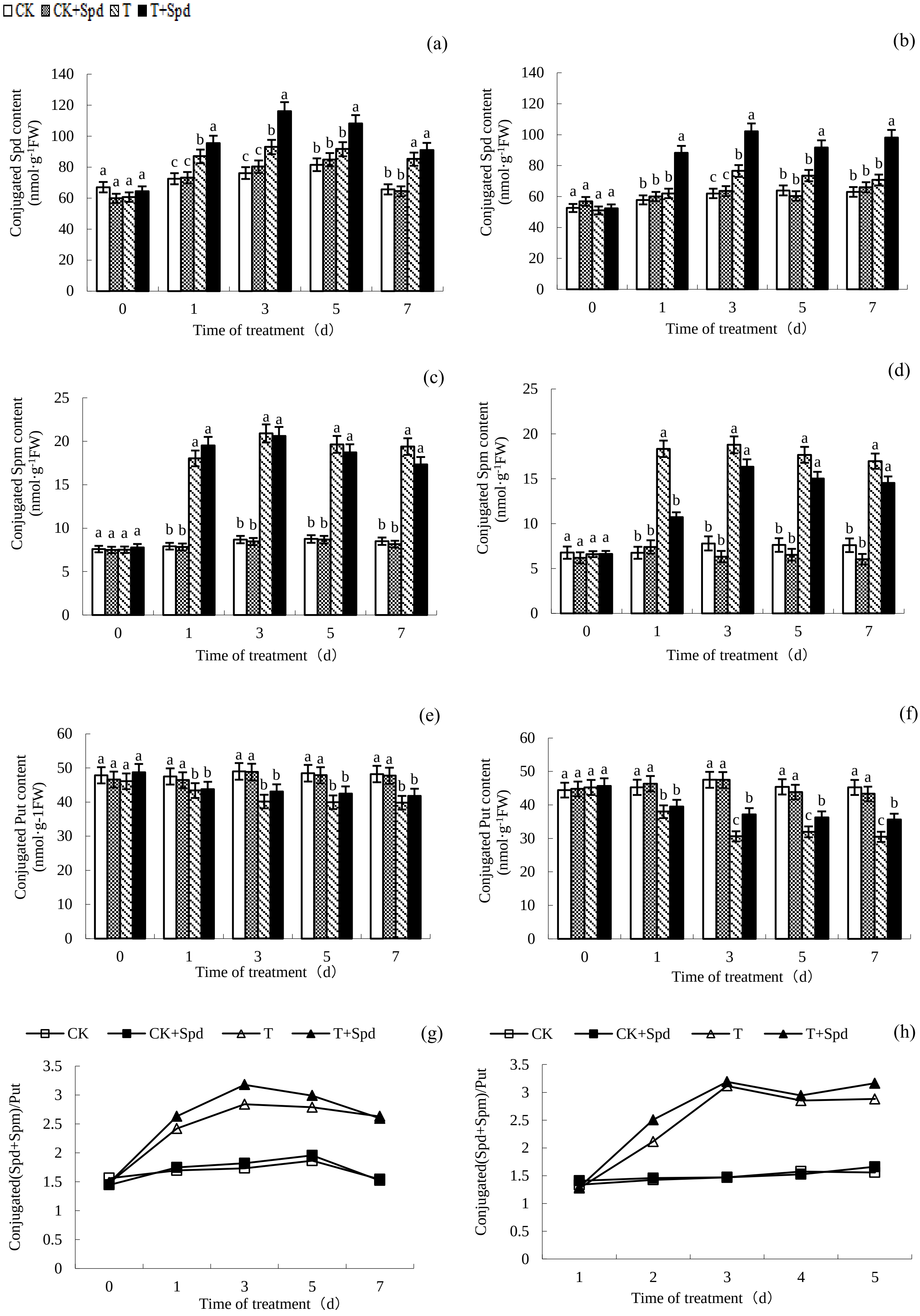

Fig. 2. Effect of exogenous Spd on conjugated Put, Spd and Spm contents of lettuce under high-temperature stress.The lettuce variety of a, c, e, g was G-S59, and the lettuce variety of b, d, f, h was P-S11.

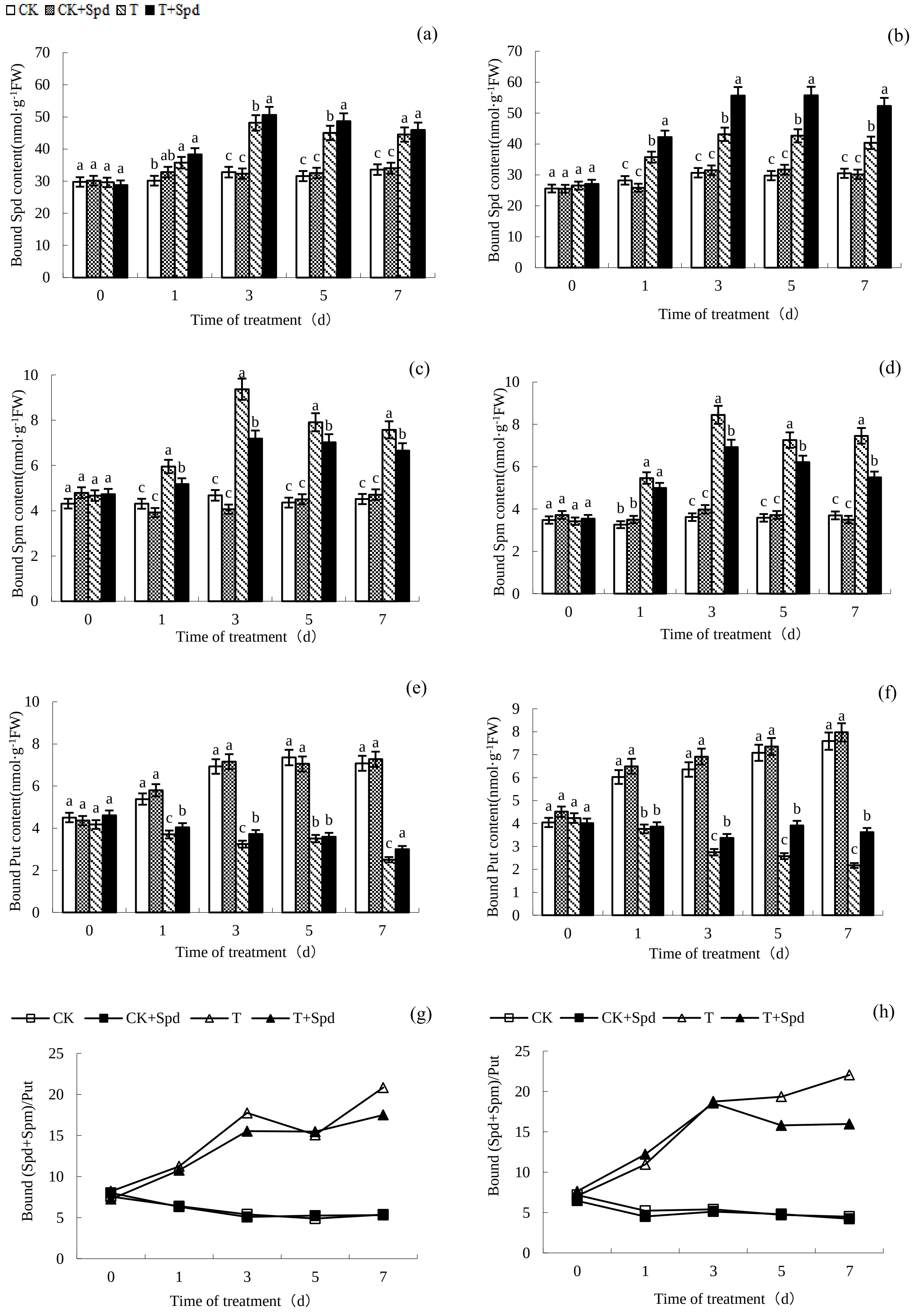

Fig. 3. Effect of exogenous Spd on bound Put, Spd and Spm contents of lettuce under high-temperature stress.The lettuce variety of a, c, e, g was G-S59, and the lettuce variety of b, d, f, h was P-S11.

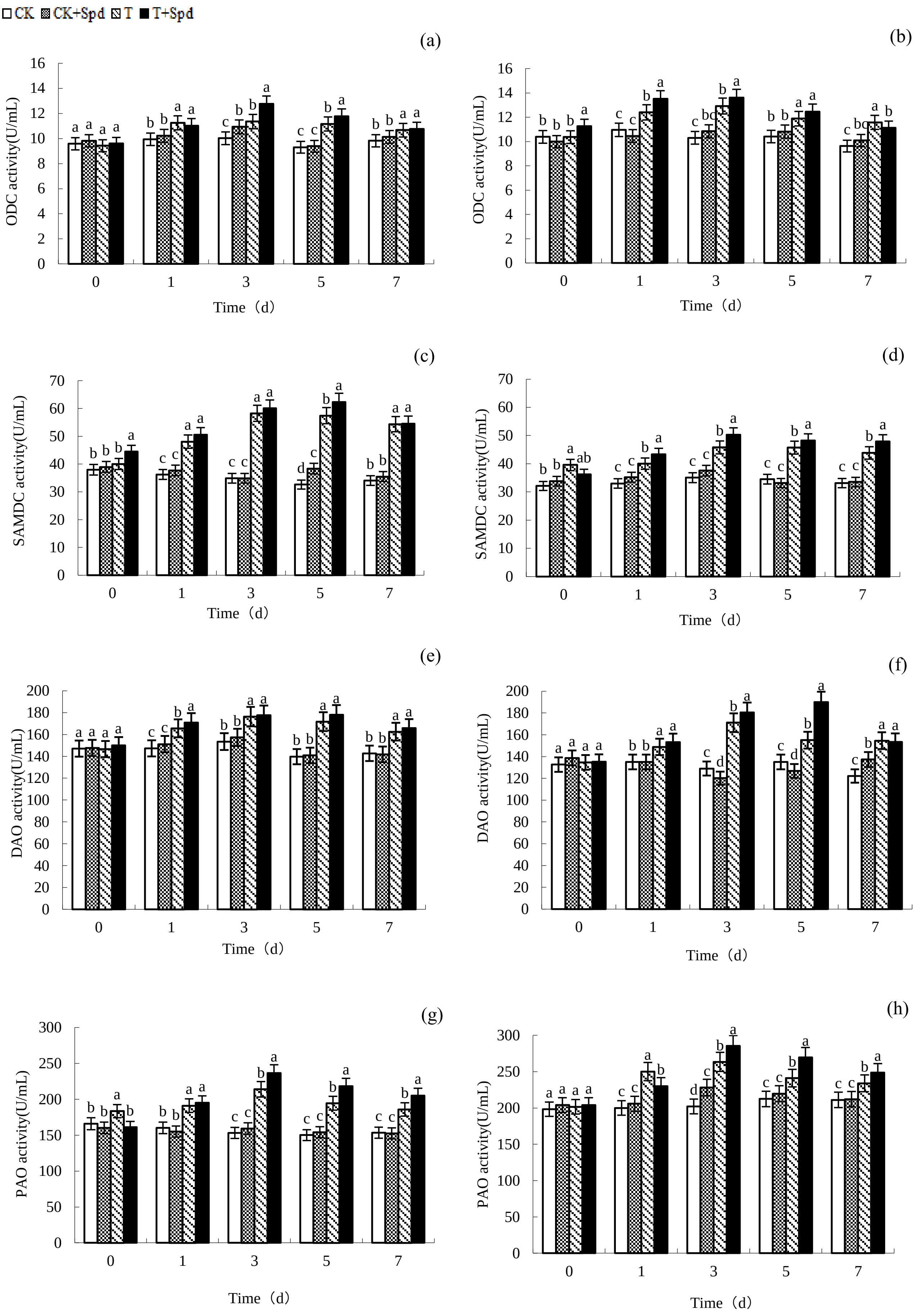

Fig. 4. Effect of exogenous Spd on polyamine synthase in lettuce under high-temperature stress. The lettuce variety of a, c, e, g was G-S59, and the lettuce variety of b, d, f, h was P-S11.

The bound Put content was significantly higher than control in the normal temperature. G-S59 when it was under high-temperature stress. On the 1st, 3rd and 7th days of treatment, the bound Put content was significantly lower with the high-temperature only than the exogenous Spd treatment. However, the bound Put content was consistently higher in the normal-temperature control than the two high-temperature treatments. The bound Put content of P-S11 under high-temperature stress was similar to that of G-S59. On the 3rd, 5th and 7th days of treatment, the bound Put content was significantly higher with exogenous Spd than high-temperature treatment alone.

When G-S59 and P-S11 were treated at room temperature, the (Spd+Spm)/Put ratio of bound PAs was relatively stable, and there was no significant difference between them. The normal temperature control, (Spd+Spm)/Put ratio of bound PAs was increased significantly and significantly lower compared with the value under high-temperature stress. Under high-temperature stress, the (Spd+Spm)/Put ratio was consistently lower in G-S59 and P-S11 sprayed with exogenous Spd than the high-temperature treatment.

Effect of exogenous spd on the activity of polyamine synthase in lettuce under high-temperature stress: As shown in Fig. 4a and b, the levels of ODC enzyme activity in both the G-S59 and P-S11 cultivars were relatively stable at room temperature. The effect of exogenous Spd on ODC activity was not significant. Under high-temperature stress, the ODC activity was clearly increased with exogenous Spd treatment in G-S59. On the 3rd and 5th day of treatment, it was significantly higher compared to high temperature treatment alone. The activity of the whole system showed a tendency to decrease, and the activity reached its peak on the 3rd day of treatment. The activity was close to that of the normal-temperature control, but it was still higher than the normal-temperature control. The activity of ODC in Spd treated with P-S11 increased significantly, which was significantly higher than in pure high-temperature treatment on days 0, 1 and 3, and significantly lower than with the high-temperature treatment alone on day 7; however, the ODC activity was consistently higher compared with the normal-temperature control.

As shown in Figure 4c and d, SAMDC enzyme activities were relatively stable in the G-S59 and P-S11 varieties under normothermic control. At room temperature, the effect of exogenous Spd spray application on SAMDC enzyme activity was not significant. On the 5th day of treatment, the SAMDC enzyme activity in G-S59 in response to exogenous Spd treatment at room temperature was significantly higher than in response to deionized water treatment at room temperature. The SAMDC enzyme activity was significantly lower in the normal-temperature control than the two treatments under high-temperature stress. The SAMDC enzyme activity was increased significantly in G-S59 from the beginning of the high-temperature stress treatment and on days 0 and 5 of treatment. The SAMDC enzyme activity was higher in response to exogenous Spd than deionized water spray treatment. The SAMDC enzyme activity was higher in P-S11 compared to deionized water spray treatment on days 1, 3, 5 and 7. Overall, the SAMDC enzyme activities were generally higher in the G-S59 and P-S11 varieties than the normal-temperature control.

Figure 4e and f show that the DAO activity remained stable in G-S59 and P-S11 normal-temperature controls. Under high-temperature stress, DAO activity was improved in response to the two treatments compared with the normal-temperature control. During the treatment period, DAO activity was higher in G-S59 exposed to exogenous Spd than deionized water spray treatment, and the difference was significant on the 1st day of treatment; the change trend in DAO activity was basically the same. Under high-temperature stress, DAO activity in P-S11 and its increasing trend were higher under exogenous Spd than deionized water spray treatment. On days 3 and 5, the activity of DAO was significantly lower in deionized water-treated than Spd-treated plants. Generally, the activity of DAO was higher in both the G-S59 and P-S11 varieties than the normal-temperature control under high-temperature stress treatment, and the activity of DAO was more significant in P-S11.

Figure 4g and h show that the activity levels of PAO were very stable in both G-S59 and P-S11 normal-temperature controls. At high temperature, PAO activity was significantly higher in response to the two treatments than in the normal temperature. PAO activity was significantly lower in G-S59 than the deionized water control on day 0 of treatment under high-temperature stress. On days 3, 5 and 7 of treatment, PAO activity was significantly higher in Spd-treated than deionized water-treated plants. PAO activity was significantly lower in P-S11 on day 1 of treatment with exogenous Spd spray compared with deionized water spray. On the 3rd, 5th and 7th days, PAO activity was higher in response to exogenous Spd than deionized water spray treatment. The PAO enzyme activity was significantly lower in G-S59 compared with P-S11.

Discussion

The change in PA content of plants is very sensitive to stress and closely related to the plant species, tissue type and timing of stress (Groppa & Benavides, 2007). According to a study reported by Krishnamurthy and Bhagwat, the content of free Put was relatively decreased under salt stress, while the content of free Spd/Spm increased rapidly in roots of salt-tolerant rice varieties; however, the PA content in salt-tolerant rice varieties was opposite to that of salt-resistant rice varieties (Krishnamurthy & Bhagwat, 1989). According to the studies of Duan *et al*., (2008), the contents of free Spm and Spd and bound PAs increased significantly in the roots of salt-tolerant cucumber varieties under salt stress, while the contents of free Put were accumulated significantly in the roots of cucumber varieties with weak salt tolerance. Nada *et al*. (2004)observed that the growth inhibition of tomato plants were decreased after Put pretreatment under low oxygen conditions. Exogenous application of Spd can improve the antioxidant system activity in rice and thus enhance heat tolerance (Mostofa *et al.*, 2014). In the present study, the contents of Spd and

Spm in three forms increased significantly in G-S59 and P-S11 (free state and bound state) under high-temperature stress, while the content of Put was decreased significantly, resulting in a significant increase in the (Spd+Spm)/Put ratio. The contents of free SPD and SPM were significantly higher in G-S59 seedlings with high-temperature tolerance than P-S11 seedlings without high-temperature tolerance. At a high temperature, the decrease in Put content and increase in Spm, Spd contents might be involved in the stress response to high-temperature stress. Spraying with exogenous Spd under high-temperature stress increased the contents of the three forms of Spd, and the increase in Put content decreased the contents of the three Spm forms. This phenomenon might be explained by the role of Spd as the intermediate substance of three PAs; the precursor substance was Put, and the downstream product was Spm. Exogenous Spd spraying increased the content of endogenous Spd in plants, which also promoted the increase in Put content as a precursor of Spd, suppressed the Spm content as the downstream product of Spd and maintained the higher endogenous Spd content. Therefore, it can be inferred that the higher Spd content in plants might reduce the damage caused by high-temperature stress to plants.

The key enzyme in polyamine synthesis in higher plants, regulated by development and the environment (Yoshida *et al.*, 1999). Salt stress increases ODC activity in cucumber seedlings, which indicates that ODC is involved in the synthesis of Put in cucumber under salt stress and that activation is increased in salt-tolerant varieties under salt stress than salt-tolerant varieties in the early stage of amine synthesis (Kaur-Sawhney *et al.*, 1982). ODC promotes Put synthesis, Put acts as a substrate to activate DAO to enhance its oxidation activity and the feedback adjusts Put levels. In this experiment, high-temperature stress induced an increase in ODC enzyme activity in lettuce, and exogenous spraying of Spd further increased the activity of ODC, especially in P-S11. With the increase in ODC activity, synthesis of Put was promoted, but the data showed that the content of Put clearly decreased, potentially due to the increase in Put, activation of DAO, decrease in Put in plants by oxidative decomposition of Put and decrease in Put by exogenous spraying of Spd. The Put content could also increase. Among them, DAO showed a marked change in P-S11. The results showed that exogenous spraying of Spd had an obvious effect on high-temperature tolerant varieties.

Some studies have shown that under high-temperature stress, the activities of SAMDC and PAO play an important role in regulating the content of SPD and SPM in plants. After application of NaCl stress to transgenic rice plants, the growth of non-transgenic rice was weaker than transgenic rice. Simultaneously, the contents of Spm and Spd increased by 3-4 times, indicating that the increase in Spd and Spm contents slowed down the damage caused by sodium salt to rice and enhanced the salt tolerance of rice (Roy & Wu, 2002). The SAMDC gene cloned from pepper was transferred into *Arabidopsis thaliana* mediated by *Agrobacterium tumefaciens*. The contents of Spm and Spd were higher in *Arabidopsis thaliana* under drought stress than in wild *Arabidopsis thaliana*, which indicated that the SAMDC gene from pepper could improve plant drought resistance (Wi *et al.*, 2014). The obtained results show that under high-temperature stress, SAMDC activity in plants increased significantly, promoting the synthesis of Put and its transformation to Spm and Spd; however, the activity of PAO increased slightly, which led to the slow oxidative decomposition of Spd and Spm and the significant increase in Spm and Spd contents. The contents of Spm and Spd in seedlings treated with Spd were significantly increased, which might be due to the increase in SAMDC activity and accelerated transformation of Put to Spm and Spd by exogenous Spd. Simultaneously, exogenous spraying of Spd decreased the PAO activity at the later stage of treatment, thus increasing the Spd and Spm contents. The contents of these PAs rose significantly, and the high level of Spd in plants was maintained.

Conclusions

Exogenous spray application of Spd can augment the content of Spd in plants by increasing the activities of DAO, SAMDC, ODC and PAO, increase the content of Put, an upstream precursor of Spd, inhibit the production of the downstream product, Spm, and thus maintain a high level of Spd in vivo. Under high-temperature stress, exogenous spraying of Spd could regulate the content of PAs in lettuce seedlings and regulate the activities of DAO, SAMDC, ODC and PAO to alleviate the damage caused by high-temperature stress to lettuce seedlings.

The results showed that water content and relative water content, plant height, dry weight and fresh weight of underground parts and dry and fresh weights of aerial parts of G-S59 and P-S11 were significantly inhibited under high-temperature stress, and the degree of inhibition was significantly higher in P-S11 than G-S59. The resistance of lettuce seedlings to high-temperature stress was improved by exogenous spraying of Spd, and the effect of exogenous Spd was more obvious in P-S11 than G-S59. The results showed that a high content of Spd might play a key role in the heat resistance of lettuce seedlings.

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