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The alleviating effect of exogenous polyamines on heat stress susceptibility of different heat resistant wheat (*Triticum aestivum* L.) varieties

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High temperature inhibits wheat grain filling. Polyamines (PAs) are closely associated with plant resistance caused by abiotic stress. However, little is known about the effect of PAs on the grain filling of wheat under heat stress. Two wheat varieties differing in heat resistance were used, and endogenous PAs levels were measured during grain filling under normal growth conditions outside the greenhouse (CK), artificially simulated high temperature (HT), artificially simulated high temperature plus exogenous application of spermine (HT + Spm) and artificially simulated high temperature plus spermidine (HT + Spd) treatments. Additionally, the variation of antioxidant enzymatic activities and osmotic adjustable substances content in grains was measured during grain filling. The results showed that compared with HT, HT + Spm and HT + Spd significantly increased grain weight of XC 6 (heat-resistant variety) by 19% and 5%, and XC 31 (heat-sensitive variety) by 31% and 34%, activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and content of Spm, Spd, and proline (Pro) increased significantly, while putrescine (Put), malondialdehyde (MDA) and soluble sugar (SS) content decreased during grain filling; The correlation analysis showed that grain weight was negatively correlated with the content of PUT, MDA, Pro and activity of SOD and CAT and positively correlated with the content of Spd and activity of POD in grains. Our results indicated that exogenous Spm and Spd could alleviate the heat injury of grain filling.

Wheat (*Triticum aestivum* L.) is one of the main food crops all over the world, and its production is directly related to the issue of food security. The optimum temperature of wheat at filling stage was 17–23 °C. When the daily maximum temperature is above 30 °C, the growth and development of wheat will be adversely affected. In severe cases, the yield decreased about 20% compared with the normal temperature¹. In recent years, the global average temperature has been on the rise, and the frequency of high temperature is increasing, which poses a serious threat to wheat yield and quality^{2,3}. Heat stress induces various biochemical and physiological responses in plants because of alteration of water content within the plant tissue and oxidative stress such as protein denaturation, lipid peroxidation, MDA accumulation and pigment degradation due to produced reactive oxygen species⁴. In the long-term evolution process, plants have formed an effective mechanism to cope with environmental stress. Accumulation of amino acids (such as Pro), sugars (such as sucrose, trehalose and sorbitol), sugar alcohols (such as manitol), and amines (such as glycine betaine and PAs) are for osmotic protection in plant to preserve water^{5–7}. Meanwhile, enzymatic and non-enzymatic antioxidants were up-regulated to overcome oxidative stress⁸. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). SOD converts ROS into H₂O₂ and O₂, while CAT and POD convert H₂O₂ into H₂O^{4,9}. Nevertheless, growth and development will be threatened when the stress exceeds its regulatory range^{10,11}. At present, in the field of production the main way to enhance the tolerance of wheat to high temperature stress is to select heat-resistant wheat varieties through the accumulation of heat-resistant genes^{12,13}, but this process takes a long time and is greatly affected by the environment¹⁴.

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Year	Varieties	Treatment	Weight per panicle (g)	Grains number per panicle	Thousand grains weight (g)	Grain yield (kg hm ⁻²)
2018	XC6	CK	2.29 ± 0.036 ^a	45.07 ± 0.46 ^a	45.92 ± 0.20 ^a	5277.66 ± 76.81 ^a
		HT	1.72 ± 0.017 ^d	37.00 ± 0.20 ^d	39.81 ± 0.29 ^d	3756.17 ± 47.68 ^d
		HT + Spm	2.04 ± 0.004 ^b	43.33 ± 0.12 ^b	42.51 ± 0.15 ^b	4697.74 ± 26.94 ^b
		HT + Spd	1.80 ± 0.012 ^c	39.00 ± 0.20 ^c	41.81 ± 0.37 ^c	4158.38 ± 48.54 ^c
	XC31	CK	2.05 ± 0.087 ^a	48.33 ± 0.31 ^a	46.04 ± 0.25 ^a	5674.07 ± 54.98 ^a
		HT	1.40 ± 0.003 ^c	35.87 ± 0.42 ^c	39.18 ± 0.34 ^d	3583.80 ± 61.07 ^d
		HT + Spm	1.83 ± 0.030 ^b	44.33 ± 0.42 ^b	43.08 ± 0.13 ^c	4869.91 ± 59.85 ^b
		HT + Spd	1.87 ± 0.005 ^b	42.07 ± 0.12 ^b	43.77 ± 0.40 ^b	4694.93 ± 55.33 ^b
2019	XC6	CK	2.37 ± 0.014 ^c	47.07 ± 0.76 ^a	50.30 ± 0.54 ^b	6036.73 ± 39.18 ^b
		HT	1.89 ± 0.016 ^f	39.33 ± 0.61 ^c	45.17 ± 0.15 ^d	4530.89 ± 72.96 ^d
		CK + Spm	2.52 ± 0.012 ^a	47.93 ± 0.83 ^a	52.43 ± 0.85 ^a	6408.27 ± 103.01 ^a
		CK + Spd	2.46 ± 0.009 ^b	47.60 ± 0.20 ^a	50.60 ± 0.24 ^b	6141.51 ± 55.03 ^b
		HT + Spm	2.24 ± 0.041 ^d	41.80 ± 0.87 ^b	48.84 ± 0.29 ^c	5205.49 ± 83.68 ^c
		HT + Spd	2.15 ± 0.021 ^e	41.73 ± 0.58 ^b	48.31 ± 0.07 ^c	5141.22 ± 78.58 ^c
	XC31	CK	2.05 ± 0.037 ^b	43.07 ± 0.42 ^c	45.80 ± 0.15 ^a	5029.73 ± 46.44 ^b
		HT	1.44 ± 0.005 ^d	38.60 ± 0.20 ^c	42.97 ± 0.29 ^c	4229.24 ± 40.88 ^d
		CK + Spm	2.23 ± 0.022 ^a	45.87 ± 0.23 ^a	46.11 ± 0.30 ^a	5393.46 ± 51.83 ^a
		CK + Spd	2.17 ± 0.088 ^a	43.73 ± 0.12 ^b	45.89 ± 0.69 ^a	5117.16 ± 65.33 ^b
		HT + Spm	1.82 ± 0.004 ^c	41.73 ± 0.46 ^d	43.90 ± 0.73 ^b	4672.03 ± 127.38 ^c
		HT + Spd	1.74 ± 0.101 ^c	41.67 ± 0.42 ^d	43.54 ± 0.33 ^{bc}	4625.89 ± 11.18 ^c

Table 1. Effects of polyamines on yield and yield components of two varieties (XC 6 and XC 31) under high temperature stress in 2018 and 2019. Values (means ± SE, n = 3) followed by different letters among different treatments are significantly different according to the Duncan's multiple range tests (P < 0.05).

Polyamines (PAs), such as putrescine (Put), spermidine (Spd), and spermine (Spm), can be found in relatively high amounts in all living cells. They have been described as endogenous plant growth regulators or intracellular messengers that regulate plant growth, development, and responses to abiotic stresses^{5,15–18}. Zhang *et al.* (2010) suggested that application of spermidine (Spd, 0.1 mM L⁻¹) under condition of abiotic stress can increase the soluble protein content of leaves and reduce the relative conductivity and malondialdehyde (MDA) content;¹⁹ Many researches have reported that polyamine treatment under adverse conditions can maintain high chlorophyll content, promote the balance of O₂⁻ content, reduce plasma membrane permeability, and maintain the integrity of cell plasma membrane in seedling leaves (1 mM L⁻¹)^{20,21}. In addition, PAs were thought to be involved in the regulation of grain development.

The polyamine concentration of aborting maize grains (*Zea mays* L.) was significantly lower than that of normal grains, and the polyamine concentration was positively correlated with the endosperm nuclei number²². Yang *et al.* (2008) found that higher levels of spermine (Spm, 240 nM g⁻¹ FW) and Spd (300 nM g⁻¹ FW) could promote grain filling and increase the grain weight of rice (*Oryza sativa* L.)²³. Tan *et al.* (2009) showed that the low grain filling rate and low grain weight of inferior grains in super rice may be related to the fact that there are low concentrations of Spd (220 nM g⁻¹ FW) and Spm (180 nM g⁻¹ FW) and low Spd/Put and Spm/Put ratios in grains²⁴. These studies suggest that the PAs are related to regulate the grain development in plants. However, little is known about the relationship among PAs, antioxidant enzymatic activities and osmotic adjustable substances content in the regulation of wheat grain filling under heat stress. The main objective of this study was to investigate the effect of heat stress on grain filling of two wheat varieties and we also tried to determine whether exogenous PAs could regulate the grain filling of wheat by regulating the changes of endogenous PAs under high temperature stress.

Results

Yield and yield components. Compared with CK, HT treatment significantly reduced grain number per panicle (GNP), thousand grain weight (TGW), grain weight per panicle (GWP) and yield of XC 6 (reduced by 18%, 13%, 25% and 29% respectively in 2018 and by 16%, 10%, 20% and 25% respectively in 2019); and HT treatment also significantly reduced GNP, TGW, GWP and yield of XC 31 by 26%, 15%, 32% and 37% respectively in 2018 (Table 1, Year, 2018) and by 10%, 6%, 30% and 16% respectively in 2019 (Table 1, Year, 2019), indicating that the responses of XC6 and XC31 to high temperature stress were different in two years, XC 6 was superior to XC 31 in 2018, while XC 31 was superior to XC 6 in 2019. Moreover, the decrease of GWP and TGW caused by HT treatment in the early stage of grain-filling was the main reason for the decrease of wheat yield. Compared with HT, exogenous spraying Spm under high temperature treatment (HT + Spm) significantly increased GNP and TGW, GWP and yield of XC 6 by 17%, 7%, 19% and 25%, respectively, and XC 31 by 24%, 10%, 31% and 36%, respectively in 2018 (Table 1, Year, 2018), and XC 6 by 6%, 8%, 19% and 15%, respectively, and XC 31 by 8%, 2%, 26% and 10%, respectively in 2019 (Table 1, Year, 2019). Compared with HT, exogenous spraying Spd under high temperature treatment (HT + Spd) significantly increased GNP, TGW, GWP and yield of XC 6 by 5%, 5%, 5% and 11%, and XC 31 by 17%, 12%, 34% and 31% in 2018 (Table 1, Year, 2018), and XC 6 by 6%, 7%, 14% and 13%, respectively, and XC 31 by 8%, 1%, 21% and 9%, respectively in 2019 (Table 1, Year, 2019), indicating that

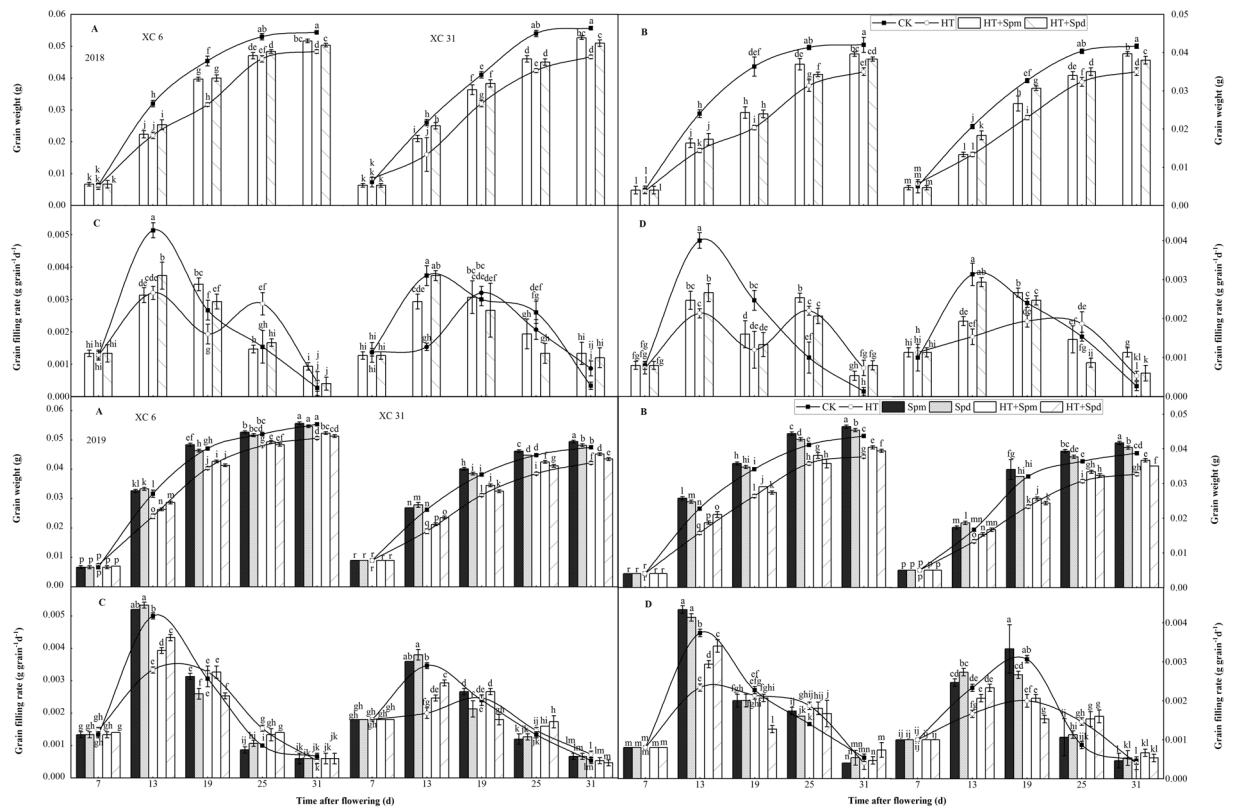


Figure 1. Changes of grain weights and grain filling rates (A–C: superior grain and B–D: inferior grain) of two varieties (XC 6 and XC 31) at normal temperature (CK), normal temperature plus spermine (CK + Spm), normal temperature plus Spermidine (CK + Spd), high temperature (HT), high temperature plus spermine (HT + Spm), and high temperature plus Spermidine (HT + Spd) with the days after flowering in 2018 and 2019. Bars indicate SD (n = 3). The same letters within each panel imply no statistically significant differences (P < 0.05).

exogenous Spm and Spd could significantly alleviate the damage of high temperature to wheat grain-filling under HT treatment, and the alleviating effects of two PAs were different in two years.

Grain filling. Under high temperature stress, the grain filling of XC 6 and XC 31 could be significantly inhibited, (Fig. 1, Year, 2018). From 13 day after flowering (DAF) to 31 DAF, the grain weight of two varieties in HT treatment was always significantly lower than that in CK treatment. From the perspective of superior and inferior grain weight of the two varieties, the effect of HT treatment on inferior grain (reduced by 17% and 16%) was greater than that on superior grain (reduced by 11% and 16%), compared with CK, the quality of superior grain of XC 31 (reduced by 16%) decreased more than that of XC 6 (decreased by 11%), this indicates that there are differences in the tolerance of different varieties to high temperature stress, XC 6 is better than XC 31, and maintaining the grain filling ability of superior grains under high temperature stress is the main reason for the variety difference; From the 19 DAF, the quality of superior and inferior grains of two varieties under HT + Spm and HT + Spd was significantly higher than that under HT, but significantly lower than that under CK, indicating that exogenous Spm and Spd significantly alleviated the inhibiting effect of high temperature stress on wheat grain filling. Moreover, from the final weight of superior and inferior grains of the two varieties, Spm had a better alleviating effect than Spd. From the curve of grain filling rate, it can be seen that, except the HT treatment of XC 6, the grain filling of superior and inferior grains of other treatments basically followed the grain filling process showing a single peak change trend of increasing at first (the peak at about 13 DAF) and then decreasing.

The maximum grain filling rate, final grain weight and average grain filling rate of the two varieties under HT treatment were significantly lower than CK (Table 2). The active grain filling period of the two varieties was as follows: HT + Spd ≥ HT + Spm > HT > CK (Table 2, Year, 2018), but the difference was not significant, showing that high temperature stress of two varieties at the early stage of grain-filling may have the effect of heat training and prolong the grain-filling period, and high temperature stress reduced grain weight mainly by reducing grain filling rate. The maximum grain filling rate, final grain weight, average grain filling rate and active grain filling period of the two varieties under HT + Spm and HT + Spd were higher than HT, indicating that exogenous spraying Spm and Spd could alleviate the heat injury of grain filling, the wheat yield under high temperature stress can be increased.

Polyamines in grains. With the grain filling process, the content of Put in the grains of the two varieties basically show a decreasing trend, while the content of Spm and Spd basically show a trend of increasing first

Year	Varieties	Treatment	Final grain mass (g)		Maximum grain filling rate (g·d ⁻¹)		Mean grain filling rate (g·d ⁻¹)		Grain filling period (d)
			Superior	Inferior	Superior	Inferior	Superior	Inferior	
2018	XC 6	CK	0.054 ^a	0.042 ^a	0.0051 ^a	0.0040 ^a	0.00169 ^a	0.00131 ^a	32 ^a
		HT	0.048 ^d	0.035 ^d	0.0032 ^d	0.0022 ^c	0.00145 ^c	0.00106 ^c	33 ^a
		HT + Spm	0.052 ^{bc}	0.040 ^{bc}	0.0035 ^{bc}	0.0025 ^b	0.00158 ^b	0.00121 ^b	33 ^a
		HT + Spd	0.050 ^c	0.038 ^c	0.0037 ^b	0.0027 ^b	0.00147 ^c	0.00112 ^c	34 ^a
	XC 31	CK	0.056 ^a	0.042 ^a	0.0037 ^a	0.0031 ^a	0.00170 ^a	0.00127 ^a	33 ^a
		HT	0.047 ^d	0.035 ^d	0.0032 ^b	0.0019 ^c	0.00138 ^c	0.00103 ^c	34 ^a
		HT + Spm	0.053 ^{bc}	0.040 ^b	0.0031 ^b	0.0027 ^b	0.00151 ^b	0.00114 ^b	35 ^a
		HT + Spd	0.051 ^c	0.038 ^c	0.0037 ^a	0.0029 ^{ab}	0.00146 ^c	0.00109 ^c	35 ^a
2019	XC 6	CK	0.0553 ^a	0.0437 ^b	0.0050 ^a	0.0037 ^b	0.00179 ^a	0.00141 ^b	31 ^a
		HT	0.0507 ^c	0.0377 ^d	0.0033 ^d	0.0023 ^c	0.00169 ^d	0.00126 ^d	30 ^a
		CK + Spm	0.0557 ^a	0.0463 ^a	0.0052 ^a	0.0043 ^a	0.00174 ^b	0.00145 ^a	32 ^a
		CK + Spd	0.0547 ^a	0.0453 ^a	0.0053 ^a	0.0041 ^a	0.00171 ^{bc}	0.00142 ^{ab}	32 ^a
		HT + Spm	0.0524 ^b	0.0403 ^c	0.0039 ^c	0.0029 ^d	0.00174 ^{bc}	0.00134 ^c	30 ^a
		HT + Spd	0.0513 ^{bc}	0.0393 ^c	0.0043 ^b	0.0034 ^c	0.00171 ^{bc}	0.00131 ^c	30 ^a
	XC 31	CK	0.0477 ^b	0.0387 ^c	0.0035 ^b	0.0031 ^a	0.00144 ^b	0.00117 ^c	33 ^a
		HT	0.0423 ^e	0.0327 ^f	0.0025 ^d	0.0020 ^c	0.00125 ^c	0.00096 ^f	34 ^a
		CK + Spm	0.0497 ^a	0.0417 ^a	0.0036 ^{ab}	0.0033 ^a	0.00151 ^a	0.00126 ^a	33 ^a
		CK + Spd	0.0483 ^b	0.0403 ^b	0.0038 ^a	0.0027 ^{ab}	0.00146 ^b	0.00122 ^b	33 ^a
		HT + Spm	0.0453 ^c	0.0367 ^d	0.0027 ^d	0.0021 ^c	0.00133 ^c	0.00108 ^d	34 ^a
		HT + Spd	0.0437 ^d	0.035 ^e	0.0030 ^c	0.0023 ^{bc}	0.00128 ^d	0.00103 ^e	34 ^a

Table 2. Grain-filling characteristics of two varieties (XC 6 and XC 31) at normal temperature (CK), normal temperature plus spermine (CK + Spm), normal temperature plus Spermidine (CK + Spd), high temperature (HT), high temperature plus spermine (HT + Spm), and high temperature plus Spermidine (HT + Spd) with the days after flowering in 2018 and 2019. Values (means, $n = 3$) followed by different letters among different treatments are significantly different according to the Duncan's multiple range tests ($P < 0.05$).

and then decreasing (Fig. 2). After HT treatment (13 DAF), the Put content in the grains of XC 6 decreased slightly compared with CK, the content of Spd was not significantly increased, while that of Spm was significantly increased; the content of Put, Spm, Spd in the grains of XC 31 were significantly decreased compared with CK, which indicated that there were differences in response to high temperature stress between different varieties. Compared with HT (13 DAF), HT + Spd significantly increased the content of Put in the grains of two varieties, both Spm and Spd content in the grains of XC 6 were significantly decreased, while in the grains of XC 31, Spd content was significantly increased and the decrease of Spm content was not significant. Compared with HT, HT + Spm made no significant difference in the Put and Spd content in the grains of XC 6, while the content of Spm was significantly decreased. However, the contents of Put, Spm and Spd in XC 31 grains increased significantly. At 25 DAF, there was no significant difference in the content of Put in the grains of the two varieties among HT, HT + Spm and HT + Spd treatments, HT + Spm and HT + Spd made the content of Spd in the grains of XC 6 significantly higher than HT, while of XC 31 significantly lower than HT. At 31 DAF, HT + Spd made the Spd content in the grains of XC 6 significantly higher than that in the other three treatments (CK, HT and HT + Spm), and for XC 31 the Spm content in the grains was the highest among the four treatments (CK, HT, HT + Spm and HT + Spd).

Antioxidant enzymatic activities in grain. With the grain filling process, the activities of CAT and SOD in the grains of the two varieties showed a decreasing trend (Fig. 3A–C), POD activity and MDA content showed a first increasing and then decreasing trend (Fig. 3B–D). After HT treatment (13 DAF), the activity of SOD and CAT and MDA content in the grains of the two varieties were significantly higher than CK, while POD activity of CK was significantly higher than that of HT, indicating that high temperature stress at the early stage of grain filling led to destruction and lipid peroxidation of cell membrane, and XC 6 and XC 31 can quickly activate the antioxidant system to adapt to high temperature stress, mainly through increasing the activity of SOD and CAT. Compared with HT (13 DAF), HT + Spm and HT + Spd could increase the activity of CAT and POD and decrease SOD activity and MDA content of the two varieties, and the effect of Spd was better than that of Spm. At 25 DAF, the activity of SOD, POD and CAT in the grains of the two varieties under HT was significantly lower than those under CK; compared with HT, HT + Spm and HT + Spd significantly increased the activity of SOD, POD and CAT, while MDA content of grains was decreased significantly in XC 6 not significantly in XC 31, and the increasing or decreasing effect of Spm was better than that of Spd, indicating that the damage of heat stress in the early stage of grain filling is a continuous process, exogenous spraying polyamine to alleviate the damage on grain filling caused by high temperature stress is a dynamic process.

Soluble sugar and proline content in grain. During the grain filling, the soluble sugar (SS) content in the grains of the two varieties under CK showed a change trend of first decreasing and then increasing, while

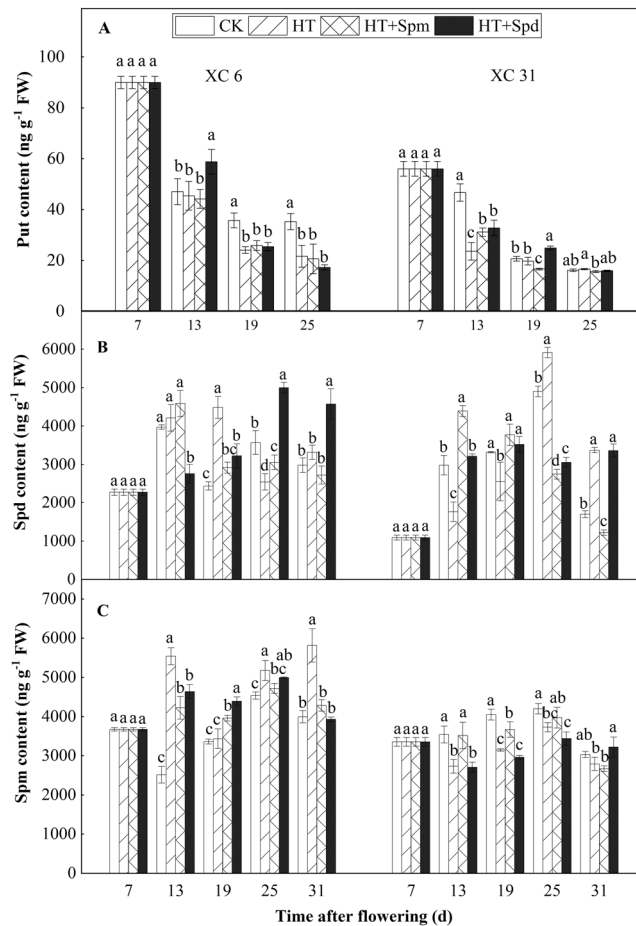


Figure 2. Changes in content of putrescine (A) PUT, spermidine (B) Spd and spermine (C) Spm of two varieties (XC 6 and XC 31) at normal temperature (CK), high temperature (HT), high temperature plus spermine (HT + Spm), and high temperature plus Spermidine (HT + Spd) with the days after flowering in 2018. Bars indicate SD (n = 3). The same letters within each panel imply no statistically significant differences.

under HT, it showed a wave change trend of first decreasing, then increasing and then decreasing (Fig. 4A). After HT (13 DAF), the contents of SS and proline (Pro) in the grains of XC 6 were not significantly different from those in CK, while for XC 31, they were significantly different, that is, compared with CK, SS increased by 13% and Pro decreased by 48%. Compared with CK, HT treatment made SS content in the grains of XC 6 significantly higher at 19 and 31 DAF and of XC 31 significantly higher at 13 and 19 DAF, indicating there were differences in the response of SS to high temperature stress between XC 6 and XC 31. After HT (13 DAF), Pro content in grains of the two varieties was significantly lower than CK at 19 and 25 DAF, while HT + Spm and HT + Spd could significantly increase the Pro content in the grains of two varieties at 25 DAF and significantly decrease the SS content at 13 and 19 DAF, indicating that exogenous spraying Spm and Spd under high temperature stress had significant effects on the contents of SS and Pro in grains.

Correlation analysis of investigated parameters in the grains of wheat plants. The correlation analysis showed that grains weight (GW) was negatively correlated with the contents of PUT, CAT, MDA, Pro and activity of SOD in the grains, positively correlated with the contents of Spd and activity of POD, but not significantly correlated with the contents of Spm and SS (Table 3); The content of Put was negatively correlated with the content of Spd, and positively correlated with the contents of MDA, Pro and activity of SOD, CAT; The content of Spm was positively correlated with that of Spd; The content of Spd was negatively correlated with MDA and Pro content and activity of SOD; SOD activity was positively correlated with CAT activity, MDA and Pro content, and negatively correlated with POD activity; POD activity was negatively correlated with CAT activity, MDA and SS content; CAT activity was significantly positively correlated with MDA and Pro content; MDA content was significantly positively correlated with SS and Pro content; SS content was positively correlated with Pro content.

Discussion

Effects of PAs on the grain filling of wheat under heat stress. In our two-year experiment, high temperature stress treatment for 5 consecutive days at 7 days after flowering significantly decreased the yield of XC 6 and XC 31, which is caused by the decrease of GNP and grain weight (GWP and TGW). The decrease of

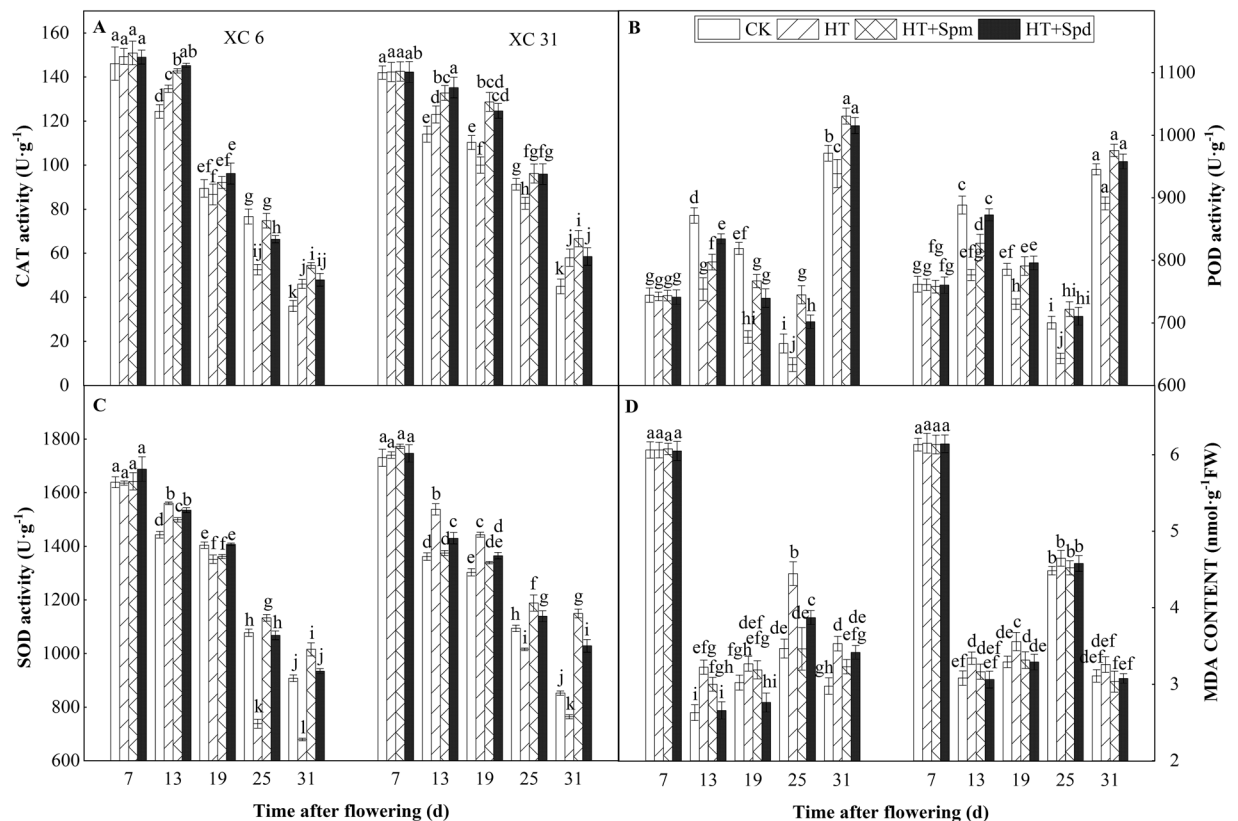


Figure 3. Changes in activity of catalase (A) CAT, peroxidase (B) POD, superoxide dismutase (C) SOD, and content of malonaldehyde (D) MDA of two varieties (XC 6 and XC 31) at normal temperature (CK), high temperature (HT), high temperature plus spermine (HT + Spm), and high temperature plus Spermidine (HT + Spd) with the days after flowering in 2018. Bars indicate SD (n = 3). The same letters within each panel imply no statistically significant differences.

grain weight is mainly related to the significant decrease of grain filling rate. This is consistent with the previous studies that wheat suffered high temperature stress during the grain filling period, resulting in the decrease of grain weight and filling rate^{25,26}. It was also found that the inhibitory effect of high temperature stress on inferior grains was greater than that on superior grains, which was consistent with previous studies^{11,27,28}. Previous studies have shown that rice (*Oryza sativa* L.) can cope with high temperature stress by accumulating polyamines under drought stress^{29–31}. Exogenous application of polyamines increased plant tolerance to drought or osmotic stress^{32,33}, the contents of Spd and Spm were positively correlated with the grain weight of rice, and negatively correlated with the Put content²⁴, while some studies also suggested that there was no significant correlation between the grain filling rate and Put content³⁴. In this study, it was found that exogenous spraying Spm and Spd could significantly increase the final grain weight of superior and inferior grains in two varieties under high temperature stress. Correlation analysis indicated that the endogenous Spd contents of the grains were positively and very significantly correlated with grain weight, while endogenous put content was negatively and very significantly correlated with grain weight. Endogenous Spm content was positively correlated with grain weight, but not significantly, and significantly positively correlated with Spd content in grains. These results showed that exogenous Spd and Spm could effectively alleviate the injury of high temperature stress on the grain filling of XC 6 and XC 31, and this alleviating process is related to the change of endogenous polyamine content regulated by exogenous polyamine. In this study, it was not found that HT, HT + Spd and Spm had a significant effect on the grain filling time. This may be due to in shihezi, xinjiang, China, at the late stage of wheat grain filling (about June 30), daytime temperatures can reach as high as 37°C, grain filling between treatments is forced to stop, it is difficult to observe the difference in grain filling period between treatments in this case. So, we plan to conduct experiments in the greenhouse next, control the temperature at the later stage of grain filling and make a further observation.

Relationship among antioxidant enzymatic activities, osmotic adjustable substance and grain filling of wheat. High temperature is a serious abiotic stress factor, which affects various physiological and biochemical changes in plant growth and development^{35,36}. Plants have developed many mechanisms to alleviate the damage effects of heat stress, ROS can induce the increase in the expression of protective enzymes, such as SOD, POD, CAT and other antioxidant enzymes, MDA is the final product of plasma membrane peroxidation, and its molality concentration can reflect the degree of plant damage^{37–40}. In this study, it was found that in the early stage of high temperature stress, SOD activity, CAT activity and MDA content in the two varieties were significantly increased compared with CK, this is consistent with the research result that high temperature

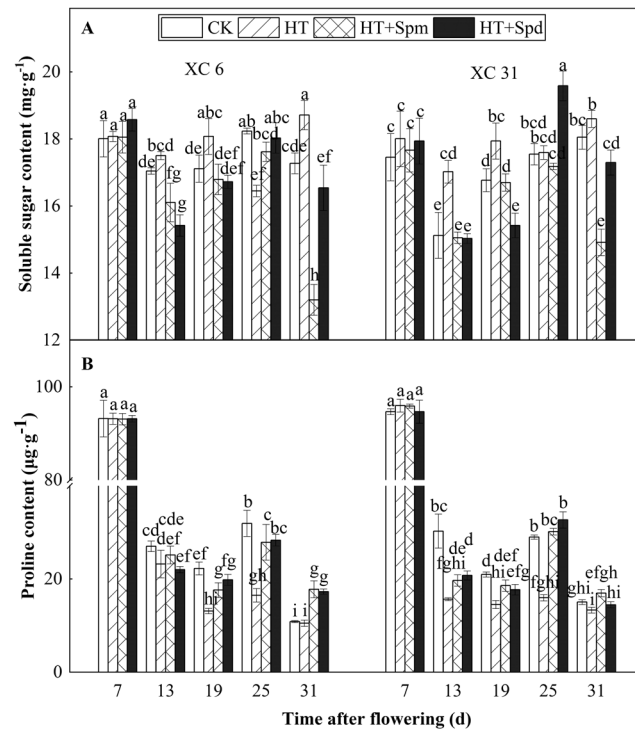


Figure 4. Changes in content of soluble sugar (A) SS and proline (B) Pro of two varieties (XC 6 and XC 31) at normal temperature (CK), high temperature (HT), high temperature plus spermine (HT + Spm), and high temperature plus Spermidine (HT + Spd) with the days after flowering in 2018. Bars indicate SD (n = 3). The same letters within each panel imply no statistically significant differences.

	G W	Put	Spm	Spd	SOD	POD	CAT	MDA	SS	Pro
G W	1									
Put	-0.815**	1								
Spm	0.2	-0.053	1							
Spd	0.406**	-0.396*	0.321*	1						
SOD	-0.881**	0.710**	-0.295	-0.401*	1					
POD	0.320*	-0.187	-0.205	-0.12	-0.328*	1				
CAT	-0.885**	0.713**	-0.233	-0.24648	0.922**	-0.381*	1			
MDA	-0.639**	0.644**	-0.059	-0.450**	0.483**	-0.442**	0.471**	1		
S S	-0.116	0.18	0.08	-0.054	0.008	-0.450**	-0.013	0.461**	1	
Proline	-0.780**	0.828**	-0.142	-0.555**	0.695**	-0.304	0.645**	0.915**	0.343*	1

Table 3. Correlation analysis of the investigated parameters in the grains of two varieties (XC 6 and XC 31) at normal temperature (CK), high temperature (HT), high temperature plus spermine (HT + Spm), and high temperature plus Spermidine (HT + Spd) with the days after flowering in 2018. Significant correlations at 0.05 levels were highlighted in bold. Investigated parameters: G W: grain weight; contents of Put: putrescine; Spd: spermidine and Spm: spermine; MDA: malonaldehyde; S S: soluble sugar; Pro: proline; enzyme activities of SOD: superoxide dismutase; POD: peroxidase; CAT: catalase. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

treatment in early stage could enhance SOD and CAT activities in wheat leaves⁴¹, POD activity was inhibited by high temperature, it is consistent with the research of Zhang *et al.* (2015) that POD activity was decreased by high temperature⁴². This study also found that SOD, POD and CAT activities in the grains of the two varieties were lower than CK in the later stage of grainfilling under HT, indicating that the damage of cell membrane caused by high temperature stress in the early stage of grain filling resulted in lipid peroxidation of cell membrane, Through increasing SOD and CAT activities, wheat could cope with high temperature stress, however, the activities of SOD, CAT and POD decreased in the later grainfilling period, which led to the intensification of membrane lipid peroxidation. Previous studies have shown that PA, a necessary compound for many cell functions (including the response to environmental stress), is not only a direct protective substance, but also a signal to trigger some adaptive mechanisms^{43,44}, and can scavenge active oxygen radicals, stabilize the structure of biological membrane, and interact with biological macromolecules^{45,46}. In this study, it was found that under high temperature stress

(13 DAF), exogenous Spm and Spd could significantly increase the activity of CAT and POD, and decrease the activity of SOD and MDA content in the grains of the two varieties; At 25 DAF, exogenous Spm and Spd could significantly increase the activity of SOD, POD and CAT and decrease the content of MDA in the grains of the two varieties under high temperature stress, indicating that alleviating effect of exogenous spraying PAs on the damage of wheat grain filling caused by high temperature stress was a dynamic process. Further study is needed on the molecular mechanism of polyamines increasing the activity of antioxidant enzymes.

Many plants can sustain growth in abiotic conditions, one of the key adaptive mechanisms is accumulation of amino acids, sugars, sucrose, and amines (such as polyamines) works as osmotic adjuster under abiotic stresses^{47–49}. This study found that after HT (13 DAF), SS content in the grains of two varieties increased, which was consistent with the results of Gao *et al.* study⁵⁰, however, the decrease of Pro content in grains was different from previous studies which showed that Pro content in leaves increased under high temperature stress⁵¹, this difference in the results may be due to the different responses of different tissues of wheat to high temperature stress; Compared with HT (13 DAF), SS content of XC 6 was not significantly increased, while that of XC 31 was significantly increased, indicating that the effect of heat stress on XC 31 was greater than XC 6; Exogenous Spm and Spd could significantly increase the content of Pro (19 and 25 DAF), and significantly decrease the content of SS (13 and 19 DAF) in grains of two varieties under HT, indicating that exogenous spraying Spm and Spd had significant effect on the content of SS and Pro in the grains under high temperature stress.

Conclusions

Our results showed that high temperature stress significantly inhibited grain filling of XC 6 and XC 31, and XC 6 and XC 31 have different responses to high temperature stress in two years. Exogenously spraying Spd and Spm could alleviate the inhibition of grain filling under high temperature stress. Alleviating process is closely related to endogenous polyamine content (Put, Spd and Spm), antioxidant enzyme activity (SOD, POD, CAT) and osmotic adjustment substances content (SS and Pro) in grains. Whether exogenous polyamines can alleviate high temperature stress through modulating gene expression needs further study.

Materials and methods

Study site description. This study was conducted from 2018–2019 at a research station of Shihezi University, Xinjiang, in northwestern China (45°19'N, 74°56'E). The annual mean precipitation of the experimental station is 550 mm, the average annual maximum and minimum temperatures during the crops growing season were 36.9°C and 9.4°C, respectively, and the annual mean temperature is 12.9°C. The total yearly sunshine duration is 2196 h, and the period is 220 days. The soil at the experiment site is moderate fertility, the readily available N, P and K quantities were 0.058 g kg⁻¹, 0.025 g kg⁻¹, and 0.149 g kg⁻¹ respectively, The organic matter concentration of the 0–20 cm topsoil was 12.34 g kg⁻¹, and the pH was 7.35 (2018)⁵².

Experimental design and treatments

The first experiment. The experiment was performed in the field. Two wheat varieties⁵³, XC 6 (a heat-resistant variety) and XC 31 (a heat-sensitive variety), were grown. The seeds were sown on 24 March in 2018. The sowing density was 150 kg hm⁻², with a row spacing of 0.20 m. The diammonium phosphate (N content was 16.5%, containing P₂O₅ 47.5%) 155 kg hm⁻² were used as base fertilizer; 70, 150, 80 and 80 kg hm⁻² urea were applied respectively in the 3-leaf stage, jointing stage, booting stage and filling stage (drip fertilization).

The experiment was a 2 × 2 × 2 (two levels of temperature and two polyamines and two varieties) factorial design, with 8 treatment combinations. Each of the treatments contained three plots as replicates in a complete randomized block design. Before the high temperature treatment, the plants with the same growth (Same flowering time) were selected and marked. At 7 day after flowering (DAF), the shed was kept in the field for 5 days, and the plastic film was put down at 10:00–18:00 h each day (20 cm off the ground for ventilation) to increase the temperature. The temperature inside and outside the shed during the treatment was recorded with an automatic thermometer (the thermometer was suspended 15 cm above the wheat canopy). The temperature changes during the treatment period in 2018 and 2019 are shown in Table 4. It can be seen from the average temperature inside and outside the shed that the effect of high temperature stress has been achieved (Table 4).

Treatment and labeling methods are: control (normal growth conditions outside the greenhouse), recorded as CK; artificially simulated high temperature, recorded as HT; artificially simulated high temperature plus exogenous application of spermine (1 mM L⁻¹), recorded as HT + Spm; artificially simulated high temperature plus spermidine (1 mM L⁻¹) was recorded as HT + Spd. Spm and Spd were purchased from Sigma Company (USA) with purity Spd were purchased freely. Exogenous spraying PAs started from the day before high temperature treatment, lasting for 5 days, at 20:00 h of everyday, PAs were sprayed on the flag leaves and panicles, each for 20 ml (CK and HT spraying with water).

The second experiment. The experiment was also performed in the field. The same two varieties, XC 6 and XC 31, were used in exogenous PAs application treatments. Each variety received six treatments at 7 day after flowering (DAF), as follows: (1) CK: normal growth conditions outside the greenhouse; (2) CK + Spm: normal growth conditions outside the greenhouse plus exogenous application of spermine (1 mM L⁻¹); (3) CK + Spd: normal growth conditions outside the greenhouse plus spermidine (1 mM L⁻¹); (4) HT: artificially simulated high temperature; (5) HT + Spm: artificially simulated high temperature plus exogenous application of spermine (1 mM L⁻¹); (6) HT + Spd: artificially simulated high temperature plus spermidine (1 mM L⁻¹).

At 7 DAF, 1 mM L⁻¹ Spm and 1 mM L⁻¹ Spd were sprayed on the flag leaves and panicles with a sprayer. Exogenous spraying PAs started from the day before high temperature treatment, lasting for 5 days, at 20:00 h of everyday. All of the solutions contained 0.1% (V/V) ethanol and 0.01% (V/V) Tween –20. The same volume of deionized water containing the same concentrations of ethanol and Tween –20 was applied to CK and HT. Each

Years	Date (m-d)	Temperature(°C)					
		Outside the shed			Inside the shed		
		Lowest	Highest	Average	Lowest	Highest	Average
2018	06-07	27.8	34.4	31.1	26.7	42.4	34.88
	06-08	28.1	35.1	30.45	28.2	37.9	32.29
	06-09	27	36.5	32.33	29.5	38.9	34.62
	06-10	27.7	35.8	32.08	27.7	41.7	37.75
	06-11	26.4	36.1	30.93	28	41.7	34.24
2019	06-05	23.7	34.8	29.69	23.9	41.6	34.24
	06-06	29.4	35.4	31.94	29.5	41.6	37.09
	06-07	18.2	30.9	27.02	18.3	40.4	32.78
	06-08	29.7	33.3	31.8	28.9	37.9	34.47
	06-10	24.4	30.5	26.77	29.5	39.4	32.14

Table 4. Changes of temperature inside and outside the shed during the treatment periods in 2018 and 2019. The temperature was not recorded on June 9, 2019 because of rain, so it was postponed for one day.

treatment had three replicates with a completely randomized block design. The Spm and Spd were purchased from Sigma Company (USA).

Measurement. Two hundred panicles that flowered on the same day were chosen and tagged in each plot. Tagged spikes from each plot were sampled at 5-d intervals from 7 DAF to maturity. All grains from each spike were removed. Grains on a spike were divided into superior grains and inferior grains. The most basal grains in the middle panicles (4 to 12 spikelets) from the bottom of a spike were considered superior grains, and the most distal grains in the middle panicles (4 to 12 spikelets) from the bottom of a spike were considered inferior grains⁵⁴. Half of the sampled grains were used for measurements of PAs, antioxidant enzymatic activities (SOD, POD and CAT) and content of osmotic adjustment substances (SS and Pro) and MDA. The other half of the grains were dried at 70 °C and weighed until a constant weight was observed.

Yield and yield components. Randomly selected 15 plants of wheat, to determine the grain number per panicle (GNP), grain weight per panicle (GWP); randomly selected 1000 grains of each treatment to test the thousand grain weight (TGW), repeating for 3 times to measure TGW; Theoretical yield (TY) = number of panicle per hectare (300 × 10000) × grain number per panicle (GNP) × thousand grain weight (TKW) × 10⁻⁶ × 85%.

Grainfilling process. Samples were taken at 0, 5, 15, 20 and 25 days of high temperature treatment, and wheat grains were divided into superior and inferior grains according to the classification method. After fixing for 30 minutes at 105 °C, they were dried and weighed at 70 °C. The grain-filling period = The date of death of wheat plants (more than 50%) – The date of flowering of wheat plants (more than 50%).

Detection of free and soluble-conjugated PAs. Samples were taken at 0, 5, 15, 20 and 25 days of high temperature treatment. Spd, Spm, and Put were extracted and measured according to Cheng *et al.*⁵⁵. Concentrations of free and conjugated PAs were determined using a modified high performance liquid chromatography (HPLC) method. Briefly, approximately 0.5 g fresh weight (FW) of samples was homogenized in a pre-chilled mortar and pestle in 5 mL 10% perchloric acid, incubated on ice for 1.5 h, and then centrifuged at 18514 g for 20 min at 4 °C. Seven micro liter benzoyl chloride and 1 mL 2 M NaOH were then added to 500 μL of the supernatant. The reactions were allowed to proceed at 37 °C for 30 min and then 2 mL ether and 2 mL saturated NaCl were added to the reactions. The reactions were shaken for 5 min and then 1 mL of the ether phase was removed and dried under vacuum. The dried reactions were re-dissolved in 100 μL methanol before HPLC analysis. The HPLC was performed on an Agilent 1200 system (Agilent, USA) with an Agilent XDB-C18 (4.6 mm × 150 mm) column. The HPLC conditions were as follows: liquid phase with a methanol: water ratio of 60:40 (v/v), 1 mL/min of flow rate, 10 μL of sample per injection, detection at 30 °C with a wave length of 254 nm, and 30 min of retention time. Peak areas and retention times were measured by comparison with standard Put, Spd, and Spm. The concentrations of PAs (ng of PAs g⁻¹ fresh callus weight) were determined using a standard curve prepared with known amounts of standard Put, Spd, and Spm. The assays were technically repeated three times.

Determination of antioxidant enzymes activity. Samples were taken at 0, 5, 15, 20 and 25 days of high temperature treatment. Generally, 0.1 g grain sample was ground by adding 0.9 mL extracting solution of SOD, POD and CAT, respectively, at 0 °C using test kit (Jiancheng, Nanjing, China)⁸. The samples were centrifuged at 3500 r/min for 10 min. For the SOD activity, the 0.02 mL supernatant was added with 0.02 mL reagent I and 0.2 mL reagent II and then kept for 20 min at 37 °C. For the POD activity, 2.4 mL reagent I, 0.3 mL reagent II and 0.2 mL reagent III were mixed with 0.1 mL sample. The mixture was kept for 30 min at 37 °C and then 1.0 mL reagent IV was added. The solution was centrifuged at 3500 r/min lasting 10 min. The activity of SOD and POD were determined at 450 nm and 420 nm using a UV-2450 spectrophotometer (Shimadzu, Japan), respectively. For the CAT activity, the 0.02 mL sample was mixed with 3 mL substrate reaction solution. The absorbance at 240 nm was measured immediately and 60 s later using the UV spectrophotometer.

Detection of MDA, soluble sugar, and proline content and proline content. Samples were taken at 0, 5, 15, 20 and 25 days of high temperature treatment. The 0.2 g grain sample was ground by adding 1.8 mL extracting solution using MDA, SS and Pro testkit (Jiancheng, Nanjing, China)⁸. The samples were centrifuged at 3,500 r/min lasting 10 min. Afterwards, 0.05 mL supernatant was mixed with 0.1 mL reaction solution, well-blended and water-bathed for 20 min at 95 °C to determine MDA content. Meanwhile, 0.5 mL sample was mixed with 1 mL reagent I and 1 mL reagent II and the mixture was water-bathed for 30 min at 95 °C to detect proline content. Then, the MDA, SS, and Pro content were measured at 532 nm, 620 nm and 520 nm, respectively, using a UV-2450 spectrophotometer (Shimadzu, Japan).

Statistical analysis. Three independent repetitions were performed for each experiment, and representative data are presented. The results were the means of at least 3 replicates for measurements of the grain weight, yield trait, spectrophotometric and HPLC determinations. The data were statistically evaluated using the standard deviation and Duncan's multiple range tests methods. The SPSS 22.0 statistical program (Statistical Package for the Social Sciences) was used to examine correlations between the parameters. The PAs, antioxidant enzyme activity (SOD, POD and CAT) and the content of osmotic adjustable substances (SS, and Pro) and MDA, were presented as the date in 2018–2019.

Data availability

All data generated or analysed during this study are included in this published article.

Received: 24 October 2019; Accepted: 10 April 2020;

Published online: 04 May 2020

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Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (31260357) and the Research Fund for the Doctoral Program of the Xinjiang Production and Construction Corps (2016AC027).

Author contributions

Jianguo Jing and Suyan Guo were responsible for supervision, soluble sugar, proline and polyamine determination, and measurements of the antioxidant enzymes activity, statistical analyses, writing and visualization. Youfang Li, was responsible for yield traits measurement, Weihua Li is responsible for reviewing and editing. All authors have reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-020-64468-5>.

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