Responses of pea plants to heat stress and spermine treatment

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Abstract

The effects of high temperature and the polyamine spermine on the physiological and biochemical status of garden pea plants were investigated. The plants were preliminary treated with 1 mM spermine and 24 h later were subjected for 48 h to conditions with daily temperature up to 38°C. High temperature stress caused more than 20% decrease of leaf pigments content and significant suppression of net photosynthesis rate. An enhanced level of lipid peroxidation was observed in leaves suggesting that oxidative stress occurred. A decrease in the content of free proline, total phenolics, and hydrogen peroxide accompanied by an increase of the activity of catalase, superoxide dismutase, and guaiacol peroxidase was established in plants subjected to high temperature. The harmful physiological effects of high temperature were alleviated by spraying the plants with spermine. The preliminary application of spermine retarded leaf pigment loss and maintained photosynthetic rate and antioxidant enzyme activities, as well as content of non-enzymatic antioxidants.

Key words: antioxidant enzymes, high temperature stress, photosynthesis, *Pisum sativum*, polyamine, stress markers.

Introduction

The elevated air temperature is one of the major abiotic stress factors related to the global climate changes (Wahid et al., 2007). Heat stress influences principal physiological and biochemical processes and thus affects the overall plant growth and productivity. Under heat stress conditions, an accumulation of reactive oxygen species (ROS) occurs and free radicals damage plant biomembranes (Wahid et al., 2007). To cope with the unfavourable environmental conditions plants have developed a complex of antioxidant defense systems, which include non-enzymatic and enzymatic antioxidants. The plants are capable of coping with the oxidative stress by activation of some or all of these systems (Gill, Tuteja, 2010 a). The endogenous plant defence system can be strengthened by application of plant growth regulating substances. Polyamines are organic compounds with straight C_3-C_{15} aliphatic structure bearing at least two primary amino groups and one or more internal imino groups. Polyamines participate in a number of important developmental processes in plants. Because of their polycationic nature, polyamines possess free radical scavenging features and antioxidant activity. They stabilize the negatively charged macromolecules and may confer plant tolerance to different biotic and abiotic stresses

(Groppa, Benavides, 2008; Gill, Tuteja 2010 b). The application of polyamines decreased electrolyte leakage and malondialdehyde from different tissue sections of soybean seedlings subjected to high temperature (HT) stress, suggesting protection of membrane integrity (Amooaghaie, Moghym, 2011). Exogenous spermidine noticeably enhanced the activities of antioxidant enzymes superoxide dismutase, ascorbate peroxidase, and catalase in HT-treated cucumber seedlings (Tian et al., 2012). The authors concluded that foliar spray with spermidine effectively improved the total antioxidant capacity of cucumber seedlings, which enhanced plant tolerance. Pre-treatment of soybean seedlings with putrescine, spermine and spermidine at 28°C for 2 h prior to heat-shock (45°C for 2 h), enhanced the recovery growth of roots and hypocotyls (Amooaghaie, Moghym, 2011). It is believed that spermine (Spm) possesses the highest protective role toward unfavourable conditions because of its highest positive charge due to its four amine groups (Bouchereau et al., 1999).

Pea is a traditionally cultivated spring crop in Bulgaria. High yield, shorter vegetation period and early ripening characterize garden pea variety 'Ran-1'. Elevated temperatures (>30°C) during pea flowering

period can cause blossom shedding. Rapid temperature changes have become be a frequent phenomenon in the region recently, and the search for quick solutions to environmental stress problems is important. This study is an attempt to evaluate the protective role of the polyamine spermine against high temperature stress.

Materials and methods

Plant material and treatments. Garden pea (*Pisum sativum* L., var. 'Ran-1') plants were grown as a water culture under controlled growth conditions (12/12 h photoperiod, 150 µmol m^{-2} s^{-1} photon flux density, 24/22°C day/night temperatures, air humidity about 80%) during April 2013 and May 2014. Twenty-one-days old plants (3–4 leaf stage) were divided into two groups:

1) control – non-treated and 2) spermine (Spm)-treated (1 mM). Tween 80 (0.01%) was used as a surfactant. Twenty-four hours after Spm treatment, half of the control and Spm-treated plants were subjected to high temperature (HT) for 2 days. During each day of the HT-stress program the temperature was progressively elevated from 22°C to 38°C (9:00 a.m. to 12:00 a.m.), and was kept till 17:00 p.m., after that it was decreased to 22°C. The air humidity was up to 50%. After the end of the stress program (day 24), the plants were transferred to initial growth conditions for 4 days to recover. The physiological responses of plants were determined 24 h (day 23) and 48 h (day 24) of the HT-stress program, and 24 h (day 25) and 96 h (day 28) of the recovery period (Fig. 1).
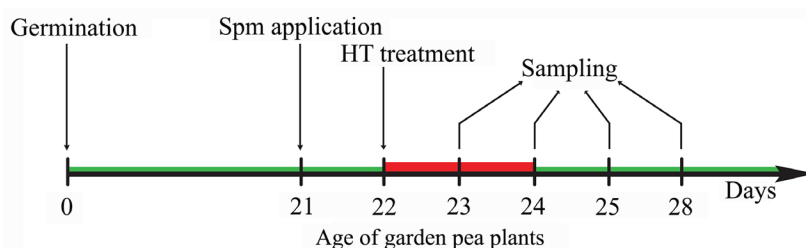
Note. High temperature (HT) treatment was held during days 22–24, and during days 24–28 the plants were transferred to initial growth conditions for recovery.

Figure 1. Scheme of treatment

Biochemical analyses. Selected parameters such as photosynthesis rate, contents of leaf photosynthetic pigments, free proline, free thiol-containing compounds, malondialdehyde, total phenols, and hydrogen peroxide as well as the activities of catalase, guaiacol peroxidase, and superoxide dismutase were measured in the 3rd leaf pair. Plant material was collected from 3–5 plants and composite samples were used for biochemical analyses. Photosynthetic pigments were extracted with 80% acetone from two leaf discs (8 mm diameter) and determined according to the method of Arnon (1949). Net photosynthesis rate was determined by a portable photosynthesis measuring system LCPro+ (ADC, UK). Fresh leaf material (approximately 300 mg) was homogenized with 0.1% (w/v) trichloroacetic acid for determination of free proline, soluble phenols, free thiol-containing compounds, hydrogen peroxide (H$_2$O$_2$) and malondialdehyde (MDA). Free proline was measured as described by Bates et al. (1973) by using of nynhidryne reagent and absorbance was read at 520 nm. Total phenolics content was determined according to the method of Swain and Goldstein (1964) by using of Folin-Ciocalteu reagent supplemented with sodium carbonate and absorbance was read at 725 nm. Gallic acid was used as a referent standard. Content of free thiol-containing compounds was determined with Ellman's reagent and absorbance was read at 412 nm (Edreva, Hadjiiska, 1984). Malondialdehyde content was estimated as a parameter reflecting biomembrane integrity deterioration. It was determined as thiobarbituric acid-reagent product according to Kramer et al. (1991) by using extinction

coefficient 155 mM^{-1} cm^{-1}. Hydrogen peroxide content was estimated spectrophotometrically by the method of Alexieva et al. (2001). The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H$_2$O$_2$.

For the assay of antioxidant enzymes, fresh plant material (approximately 200 mg) was homogenized in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (w/v). The homogenates were centrifuged at 12 000 × g for 15 min. Catalase (CAT, EC 1.11.1.6) activity was measured by following the decomposition of hydrogen peroxide and was determined by monitoring the absorbance decrease at 240 nm (ε = 36.8 mM^{-1} cm^{-1}) for 60 s (Aebi, 1984). Guaiacol peroxidase (POX, EC 1.11.1.7) activity was measured by using of guaiacol as electron donor and the absorbance increase due to the guaiacol oxidation at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) was recorded for 60 s (Dias, Costa, 1983). Total superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitrobluetrazolium (NBT). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm (Beauchamp, Fridovich, 1971).

All spectrophotometrical measurements were made on Multiskan Spectrum (Thermo Electron Corporation, Finland). The kinetic measurements of catalase and guaiacol peroxidase activities were made on spectrophotometer Shumadzu UV-1601 UV-Visible (Shimadzu, Japan).

Statistical analysis. All experiments were repeated three times with three replicates each. The data reported are mean values ± SE. The significance of differences was statistically analyzed using Duncan's multiple range test at a level of significance of 0.05.

Results

The leaf pigments were decreased significantly only in the HT-treated plants as compared to the respective control. Chlorophyll *a* was decreased by 13%, and remained lower than the control by 20% till the end (day 28) of the experimental period (Fig. 2 A). Similar tendency was observed for chlorophyll *b* content (Fig. 2 B). Carotenoides (Fig. 2 C) were also lessened in HT-treated plants and reached 70% of the control levels at the end of the recovery period. Spermine increased the leaf pigments content and the values in both Spm-treated

variants (alone applied and combined with HT) remained higher then these measured in the control plants. Net photosynthesis rate (Fig. 2 D) was considerably reduced by 39% after 24 h of HT treatment, then it maintained relatively stable level but it was significantly lower as compared to control value at the 28th day (4 days of recovery period). However, the Spm application considerably increased net photosynthesis rate starting from day 23 (first day of stress programme) – by 13, 20, 41 and 42 %, respectively as compared to control values. When Spm was applied before HT treatment, the net photosynthesis rate initially was decreased by 12% (day 23 – first day of stress programme), then an increase by 23% was detected on day 25 (first day of recovery period), which did not change significantly till the end of the experiment (day 28 – fourth day of recovery period).
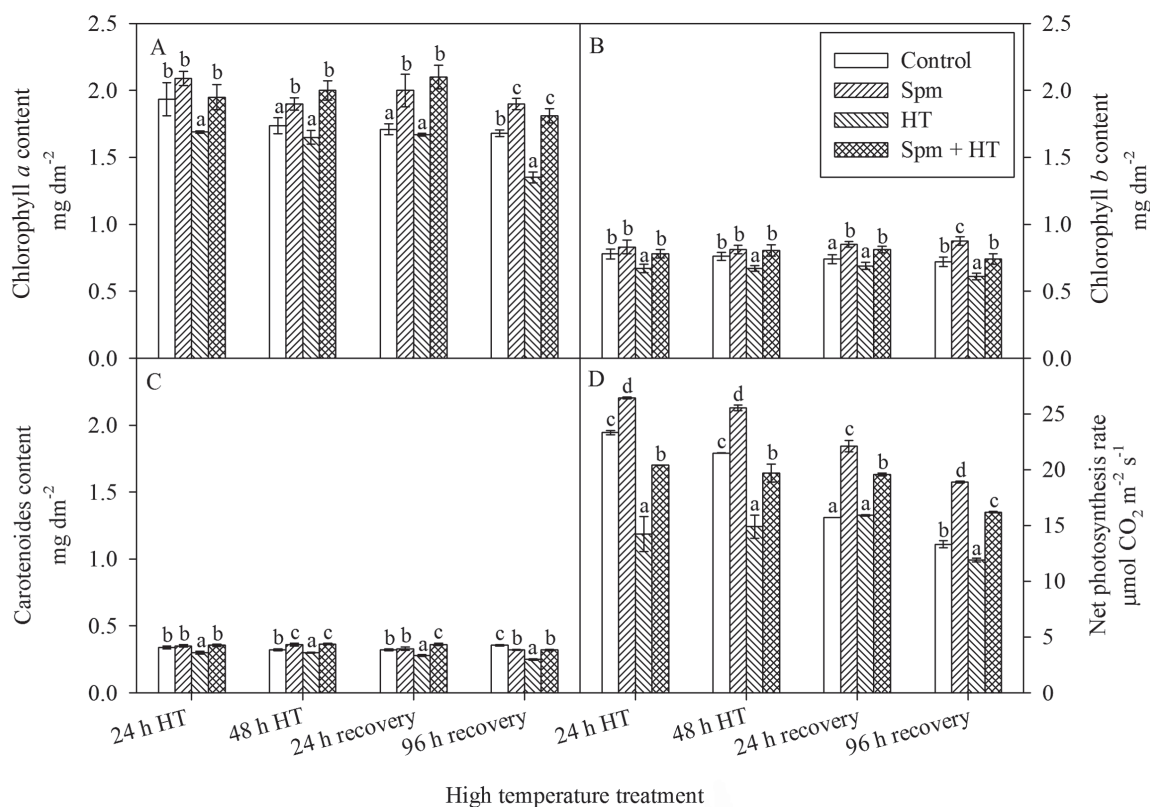
Note. Different letters designate statistically significant difference at $p < 0.05$.

Figure 2. Chlorophyll *a* (A), chlorophyll *b* (B) and carotenoides (C) content, and net photosynthesis rate (D) of garden pea plants sprayed with 1 mM spermine (Spm) and subjected to high temperature (HT) treatment for 48 h

MDA content of HT-treated plants was increased by 24% at day 23 (after first day of stress programme), and remained higher by 20% than the control (Fig. 3A) at day 28 (fourth day of recovery period). The application of Spm alone did not alter significantly MDA level and it was near the control values during all measurement points. Immediately after HT treatment MDA accumulation was 10% in Spm + HT-treated plants and remained constant till the end of the recovery period (day 28).

An increase of free proline by 18% was established only after HT treatment at the first

measurement point (day 23), then a significant decrease – by 30% (day 24), 21% (day 25) and 17% (day 28) was detected and proline levels remained below the control value (Fig. 3 B). Spermine application caused an enhancement in free thiols content (Fig. 3 C) by 25% after first day of stress programme (day 23), 34% after second day of stress programme (day 24) and 51% at first day of recovery period (day 25), respectively in alone Spm-treated plants and by 13% (day 23), 30 % (day 24) and 31% (day 25), respectively in Spm + HT-treated plants as compared to the control values. At the fourth day of

recovery period (day 28), the levels in Spm-treated and control plants were nearly equal. The content of free thiols in HT-treated plants was 18% below the control level at fourth day of recovery period (28th day).

Initially at day 23 an increase (by 13%) of phenolics amount was established in HT-treated plants, then the level was decreased by 17% after second day of stress programme (day 24), 13% at first day of recovery period (day 25) and 9% at fourth day of recovery period (day 28) as compared to the respective controls (Fig. 3D). At first, total phenolics amount was raised in plants treated with Spm by 20% after first day of stress programme (day 23), 10% after second day of stress programme (day 24) and 30% at first day of recovery period (day 25), respectively. When combined with HT, Spm also enhanced noticeably total phenolics content as compared to HT-treated plants with exception of data obtained on day 23 (after first day of stress programme). At the end of the experiment (fourth day of recovery period – day 28) no differences were established between control, Spm-treated and combine-treated plants.
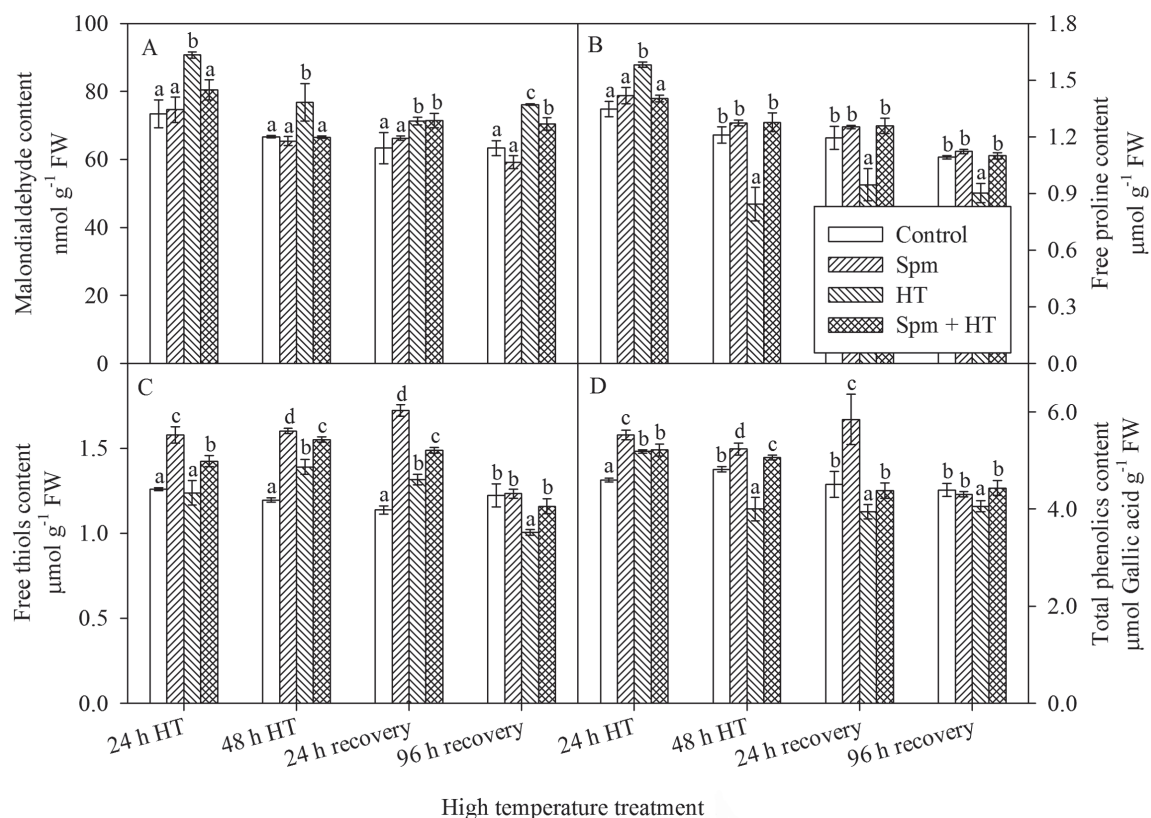
Note. Different letters designate statistically significant difference at $p < 0.05$; FW – fresh weight.

Figure 3. Malondialdehyde (A), free proline (B), free thiol-containing compounds (C) and total phenolics (D) content of garden pea plants sprayed with 1 mM spermine (Spm) and subjected to high temperature (HT) treatment for 48 h

Spermine application caused significant increase in H_2O_2 level in plants – by 46% after first day of stress programme (day 23), 51% after second day of stress programme (day 24), 40% at first day of recovery period (day 25) and 39% at fourth day of recovery period (day 28), respectively to the control levels (Fig. 4 A). When combined with HT, Spm treatment resulted in rise of H_2O_2 up to 20% only at the last measurement point (day 28). HT stress led to a decrease in H_2O_2 content, especially at the 28th day (by 40%) as compared to the respective controls.

SOD activity was enhanced by 30% after first day of stress programme (day 23), 39% after second day of stress programme (day 24), 24% at first day of recovery period (day 25) and 15% at fourth day of recovery period (day 28), respectively by the alone application of Spm (Fig. 4 B). Initially after first day of stress programme

(day 23) the HT treatment led to a decrease in SOD activity by 15%, and then a significant increase was established, which was maintained during the recovery period. The combined treatment did not alter considerably SOD activity. The CAT activity (Fig. 4 C) was enhanced significantly only by the HT treatment and at the end of the recovery period (day 28) it reached 51% above the control.

The application of Spm led to some decrease in POX activity (Fig. 4 D) by 5% after first (day 23) and second (day 24) days of stress programme, and by 7% at first day of recovery period (day 25) and 9% at fourth day of recovery period (day 28). High temperature treatment decreased POX activity by 17% after first day of stress programme (day 23), and then (at day 24) an increase by 24% was detected. The enzymatic activity of POX in HT-treated only plants remained higher than the respective
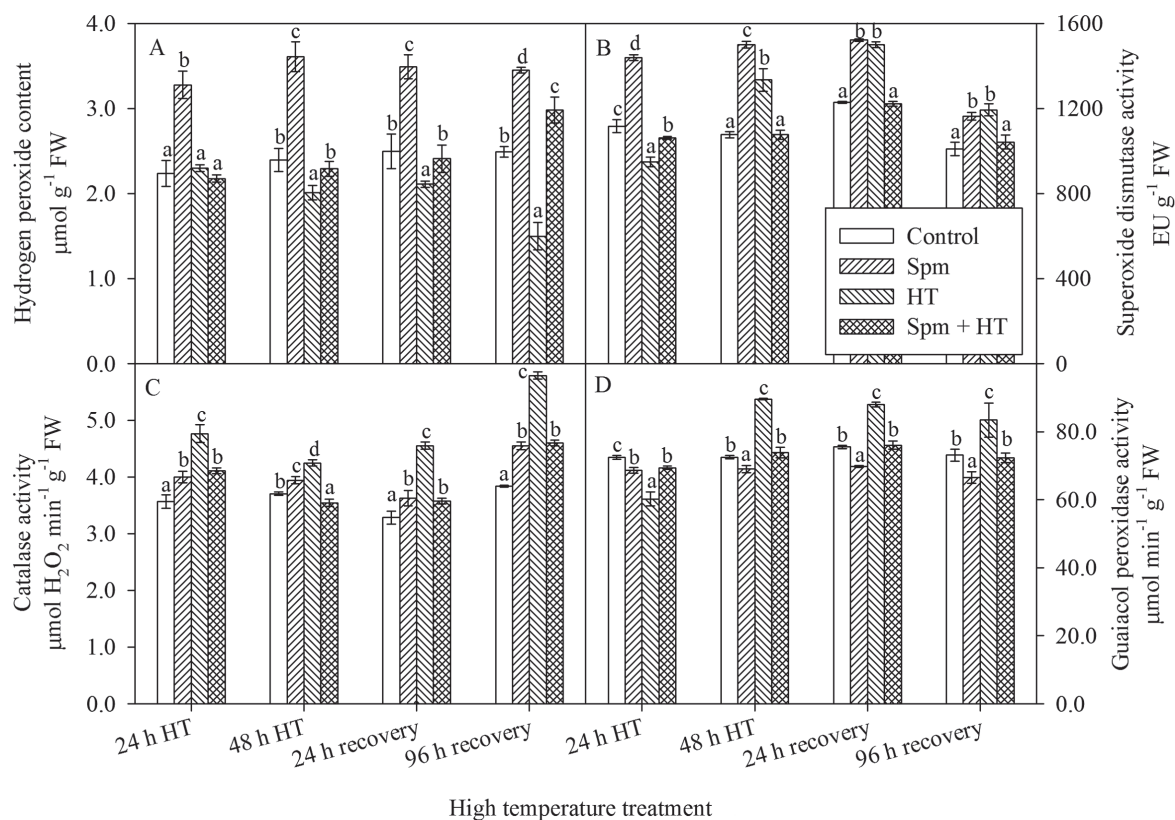
Note. Different letters designate statistically significant difference at $p < 0.05$; FW – fresh weight.

Figure 4. Hydrogen peroxide content (A), activity of superoxide dismutase (B), catalase (C) and guaiacol peroxidase (D) of garden pea plants sprayed with 1 mM spermine (Spm) and subjected to high temperature (HT) treatment for 48 h

controls during the recovery period (17% at first day of recovery – 25 day and 14% at fourth day of recovery – day 28). The POX activity in plants treated with Spm + HT was near to control values after second day of stress programme (day 24) and this trend remained constant during recovery period.

Discussion

Leaf pigments were slightly increased in Spm-treated plants as compared to the control but Spm treatment considerably enhanced net photosynthesis rate similarly to those results reported by other authors and reviewed by Shu et al. (2012). The leaf pigments declined considerably under HT conditions, suggesting structural damage to the chloroplasts as reported previously (Wahid et al., 2007; Kumar et al., 2012). As a result, the net photosynthesis rate was inhibited (Shu et al., 2012). In Spm + HT-treated plants leaf pigment amount was near the control values during the whole experimental period, which led us to suppose that Spm lessened leaf pigment losses induced by HT. So, we support the assumption proposed by other authors (Stoynova et al., 1999; Hamdani et al., 2011) that under various stresses Spm application protected the photosynthetic apparatus. Further, this reflected on the net photosynthesis rate, which was higher in Spm + HT-treated peas than that measured in HT-treated plants. Our results are in accordance with those reported in other studies that

Spm had a beneficial effect on the net photosynthesis rate in cucumber seedlings grown on excess of NO_3^- (Wang et al., 2010) and rice seedlings subjected to drought stress (Farooq et al., 2009). We suppose that preliminary application of Spm allowed garden pea plants to adapt easier to HT stress by maintaining net photosynthesis rate to valuable levels.

The temperature stress causes generation of ROS (Gill, Tuteja, 2010 a) which damage cellular biomembranes and as a consequence an accumulation of MDA occurs (Wahid et al., 2007). The increase of MDA concentration found after HT exposure indicates that oxidative damage is taking place in membranes of garden pea plants. However, less MDA enhancement was detected in combined treatment (Spm + HT). Exogenous Spm decreased the lipid peroxidation under HT stress. This result is in accordance with previous reports where the application of polyamines decreased malondialdehyde in various plant seedlings, suggesting protection of membrane integrity (Amooaghaie, Moghym, 2011; Tian et al., 2012). The fact that MDA amount was lower in plants subjected to combined treatment than in HT-treated plants showed that Spm might assist plants to overcome the negative effect of the temperature stress. In view of the fact that Spm plays an important role in stabilizing the function of cell biomembranes (Groppa, Benavides, 2008; Gill, Tuteja, 2010 b) we also propose that possibly

Spm was conjugated to membrane phospholipids and stabilized cell constituents. Thus, Spm could diminish the damage of membrane integrity and protect pea against HT stress.

The generation of H_2O_2 from the electron transport chains in mitochondria and chloroplasts is a normal physiological process (Gill, Tuteja, 2010 a). Under optimum temperature conditions, the endogenous concentration of H_2O_2 depends on the balance between the rate of its production and utilization (mainly by CAT and different peroxidases). SOD catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide. Catalase decomposes H_2O_2 into water, whereas peroxidases utilize H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Gill, Tuteja, 2010 a). However, under stress conditions, the overproduction of H_2O_2 prevails and disturbs a number of metabolic processes. Unexpectedly, we found that H_2O_2 content was increased considerably only by Spm when applied alone. The increase of SOD activity by Spm suggested an enhanced production of H_2O_2. An evidence exists that the application of polyamines can cause an increase in the activities of antioxidant enzymes which scavenge H_2O_2 (Singh, Malik, 2011; Tian et al., 2012; Li et al., 2014). This effectively improved the total antioxidant ability of plants, and an excess of H_2O_2 was not observed. However, in our study CAT activity was not increased considerably, and POX activity was even slightly inhibited by Spm application. Another source of H_2O_2 is the oxidation of polyamines by di- and polyamine oxidases (Gill, Tuteja, 2010 b) and the observed Spm-increased H_2O_2 could be due to substrate-activated polyamine oxidases.

In HT-treated plants, H_2O_2 was constantly decreasing during the experimental period. It has to be noted that although SOD activity was enhanced, the activities of the enzymes detoxifying H_2O_2 were also steadily increased. An increase in CAT and POX activity is supposed to be an adaptive reaction to prevent the damage by reducing the toxic levels of H_2O_2 produced during the cell metabolism and renders protection against oxidative stress provoked by NaCl (Sudhakar et al., 2001). An increased POX (Liu, Huang, 2000) and CAT (Sairam et al., 2000) activity was reported in a number of plant species under HT stress. Further, an increase in CAT and POX activity is also associated with accelerated scavenging of H_2O_2 in HT-treated plants (Liu, Huang, 2000; Sairam et al., 2000; Tian et al., 2012). Although the activities of antioxidant enzymes were mainly increased by HT stress, the exogenous Spm had an attenuating effect on the enzymatic activities measured in Spm + HT-treated plants. Spm pretreatment normalized the level of H_2O_2 and the activities of antioxidant enzymes in Spm + HT-treated plants as compared to the HT-treated only plants. This provides evidence that preliminary application of Spm facilitates the adaptation reactions of garden pea plants to HT stress explicated by better physiological and biochemical status.

Glutathione is the most abundant low- molecular thiol-containing compound in living cells, which plays defence roles in ROS detoxification (Gill, Tuteja, 2010 a). The insufficient increase and further drop of thiol compounds below the control in HT-treated plants may be an indicator for impaired redox state of the cells. Spm application significantly augmented thiol-containing compounds either alone or in combination with HT. The initial increase of free thiol levels by Spm diminished with time and finally reached the corresponding control values. Usually polyamine application leads to increase in antioxidant capacity of plants. For example, Verma and Mishra (2005) reported that exogenous putrescine increased glutathione concentrations, which improved salinity tolerance and adaptation of seedlings of Indian mustard. The higher content of low-molecular thiols in Spm + HT-treated plants as compared to its quantity in HT-treated, led us to assume that preliminary spraying with Spm provoked an adaptation response.

Plant phenolics and proline are components of the non-enzymatic defense (Gill, Tuteja, 2010 a). Accumulation of plant phenolics under high temperature conditions could be considered as an acclimation mechanism against thermal stress as previously proposed by Rivero et al. (2001). The exact role of proline accumulation in plants is still under discussion but it is thought to play an adaptive and signal role in plant stress tolerance (Szabados, Savouré, 2010). The pretreatment with Spm increased the content of phenolic compounds and proline in Spm + HT-treated plants as compared to the HT-treated only. Thus, we suggest that spraying with Spm has a favourable role for maintaining phenolic compounds and proline in HT-treated plants near the control levels.

Conclusions

1. Treatment of plants with spermine (Spm) prior to high temperature (HT) stress provoked less decrease in leaf pigment content, net photosynthesis rate, total phenolics content, free proline and thiol-containing compounds as compared to those in plants treated with high temperature only.

2. The activities of antioxidant enzymes in Spm + HT-treated plants were not as much altered as those measured in HT-treated garden pea plants.

3. Exogenous application of spermine could alleviate the harmful physiological effects on garden pea plants provoked by rapid increase of the temperature.

Acknowledgments

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Karščio ir apdorojimo sperminu įtaka žirniams

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Santrauka

Tiriant aukštos temperatūros ir poliamino spermino poveikį žirnių fiziologinei bei biocheminei būklei, augalai pirmiausia buvo paveikti 1 mM spermino ir po 24 val. perkelti 48 valandoms į 38 °C paros temperatūros sąlygas. Dėl aukštos temperatūros streso poveikio lapų pigmentų kiekis sumažėjo daugiau nei 20 %, o fotosintezės greitis gerokai sulėtėjo. Paveikus aukšta temperatūra buvo nustatytas padidėjęs augalų lapų lipidų peroksidacijos lygis, rodantis, kad įvyko oksidacinis stresas. Laisvojo prolino, fenolinių junginių ir vandenilio peroksido kiekio sumažėjimas sietinas su katalazės, superoksido dismutazės ir gvajakolio peroksidazės aktyvumo padidėjimu paveikus aukšta temperatūra. Žalingas aukštos temperatūros poveikis augalų fiziologinei būklei buvo sušvelnintas augalus purškiant sperminu. Augalus paveikus sperminu vėl padidėjo prarastas pigmentų kiekis ir fotosintezės greitis, taip pat antioksidacinių fermentų veikla ir nefermentinių antioksidantų kiekis.

Reikšminiai žodžiai: antioksidantiniai fermentai, aukštos temperatūros stresas, fotosintezė, *Pisum sativum*, poliaminai, streso žymekliai.