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Exogenous Application of the Polyamine Spermine Delays Natural Leaf Senescence in Wheat Through Protecting Chlorophyll from Degradation and Preventing Oxidative Stress

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Abstract

Senescence is a genetically regulated process that involves decomposition of cellular structures and the mobilization of the products to other plant parts. The progression of developmental senescence in wheat plant under the effect of foliar application with 100 µM of the polyamine spermine (Spm) was studied. Biochemical changes were recorded in the fourth leaf starting from the onset of senescence at 30 day after sowing (DAS) until the end of leaf senescence at 50 DAS. Leaf senescence was delayed in Spm-treated plants through maintaining water content, preventing chlorophyll degradation and enhancement of total soluble sugars, starch accumulation and total protein compared to controls. In addition, reactive oxygen species and lipid peroxidation were lower in Spm-treated plants than control values. Catalase (CAT) activity was obviously lowered due to Spm-treatment; however, guaiacol peroxidase activity was slightly decreased below the control.

Keywords: Wheat; Leaf senescence; Spermine; Chlorophyll; Sugars; Protein, Reactive oxygen species; Antioxidant enzymes.

Introduction

Leaf senescence is an important agronomic trait that affects crop yield and quality. It is involving a series of cytological and biochemical changes, which cause degradation of macromolecules and remobilization of nutrients from senescing tissues to reproductive, young or storage tissues. The morphological, physiological and molecular changes that occur during senescence process include chlorophyll loss, reduction in photosynthesis, membrane degradation, lipid peroxidation and disintegration of chloroplasts (Noodén et al., 1997).

Chlorophyll breakdown is a key step in the senescence process as it is the visible symptom of senescence leading to loss of green color of leaves. The decrease in chlorophyll biosynthesis or its degradation can lead to reactive oxygen species production and cell death so that the genes involved in chlorophyll degradation are under transcriptional control (Ho¨rtensteiner, 2009).

Sugars play an important role in energy supply during plant metabolism and development in addition to the regulation of senescence program initiation through induction of hexokinase-mediated sugar signaling pathway (Dai et al., 1999). However, in contrast to the decrease in amino acids and photosynthetic pigment contents in senescing leaves of many plant species

(Jongebloed et al. 2004), the sugar content may increase (Noodén et al., 1997). This may be due to the degradation of starch into hexoses, the increased availability of carbon skeleton as a result of retarded amino acid synthesis, the inhibition of sugar export because of sieve tube occlusion (Jongebloed et al., 2004) and/or probable synthesis of sugar from fatty acids by gluconeogenesis.

Protein hydrolysis is an essential catabolic process related with leaf senescence and plays an important role in nutrient mobilization out of the leaf. Protein degradation is caused by de-novo synthesis of proteolytic enzymes, activation of pre-existing proteases, or decompartmentalization of proteases and their substrates (Prochazkova and Wilhelmova, 2007).

Reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen, superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radical are generated under steady-state conditions as a by-product of major metabolic processes such as the C_2 cycle or photorespiration. Although ROS may play a beneficial role as signaling molecules in sub toxic levels (Choudhury et al., 2013) their production contributes to the progression of leaf senescence, as the antioxidant capacity of the leaf declines with age. Therefore, the production of ROS takes place in synchrony with their scavenging to maintain redox homeostasis in plants by developing protection mechanisms through enzymatic and non-enzymatic detoxification of excess ROS (Mittler, 2002). Catalase (CAT) and peroxidase (POD) are among the enzymatic antioxidants. CAT scavenges H_2O_2 to form H_2O and O_2^- and it is the most efficient scavenger of H_2O_2 (Asada and Takahashi, 1987). POD reduces H_2O_2 using several reductants, such as ascorbate, guaiacol and phenolic compounds (Apel and Hirt, 2004).

Polyamines (PAs) are small, positively-charged organic molecules that present in all living organisms. The main forms of PAs are putrescine (Put), spermine (Spm) and spermidine (Spd). PAs play an important role in cell division, differentiation, reproduction, dormancy-break and senescence delay. It was well-known that PAs are effective anti-senescence agents, exerting their effect via delay of chlorophyll loss, membrane damage and RNase and protease activities (Evans and Malmberg, 1989). It was found that internal PAs and PA synthetase activity were low in senescent tissues. Numerous studies have linked PAs to the regulation of plant cell senescence. However, there are contradictions about whether PA levels increase or decrease during senescence. Leaves of monocots and dicots undergo rapid senescence and accumulate large amounts of putrescine in accordance with chlorophyll degradation under the influence of osmotic stress (Cohen, 1998). The levels of PAs are not constant during plant senescence as they increase in the beginning of the process then decrease in the later stages and this pattern depends on if the senescence is natural or induced by external factors. Spm promotes leaf senescence and programmed cell death in leaves through production of H_2O_2 (Cohen, 1998).

Studying the basis of senescence onset is important for future agronomic improvements. Delaying onset or decreasing the progression of senescence can lead to the increase of plants biomass and crop yield. So; this work aims to examine the role of exogenous application of the polyamine Spm in delay of natural leaf senescence in wheat through monitoring some morphological and biochemical changes such as chlorophyll degradation, reactive oxygen species accumulation and antioxidant enzyme activities.

Plant material and growth conditions

Seeds of wheat cultivar; Misr 2 were surface-sterilized in a 1% sodium hypochlorite for 10 min, followed by extensive washing with sterile distilled water. Seeds were soaked in tap water for 12 h then spread on a filter paper overnight for air-drying, and were sown in plastic pots (13 cm in diameter). Eight seeds were sown in each pot filled with clay and sand mixture at a ratio of 2:1 respectively and seedlings were thinned to four per pot. The plants were adequately irrigated with tap water until developing the fourth leaf (30 DAS). Seedlings were supplied with half strength Long Ashton nutrient solution twice (one week and two weeks after sowing). Pots were then divided into two groups; the first group was irrigated with tap water and sprayed three times, each with 30 ml of 0.01% the Tween-20 surfactant to serve as control and the second was sprayed with 100 µM Spm in 0.01% Tween-20. The concentration of Spm was selected based upon a preliminary experiment and spraying was applied at 30, 34 and 38 DAS. All pots were adequately irrigated with tap water until the end of experiment (50 DAS).

Plants were grown in the growth house under normal growth conditions of wheat until the end

of the experiment.
Plants were harvested randomly from each treatment and used for growth parameters measurements. The fourth youngest leaf was also collected, frozen immediately in liquid nitrogen and stored at -80 °C for physiological, biochemical and gene expression analyses.

Determination of growth parameters

Three replicates of seedling leaves from different treatment were harvested for determination of fresh weight (FW). The leaves were then oven-dried at 80 ºC for 2 days for recording the dry weight (DW).

Determination of photosynthetic pigments

Fresh leaf tissue was ground in liquid N_2 and extracted with 85 % acetone then centrifuged at 1000 × *g* for 10 min. The supernatant was adjusted to a final volume of 10 ml and the absorbance was recorded at 470, 645, 663 nm for estimation of Chl. a, Chl. b and carotenoids according to the formulae of Lichtenthaler and Wellburn (1983).

Determination of total soluble protein

Soluble leaf proteins were extracted by grinding in a chilled mortar and pestle. A known fresh weight of leaves was grinded in extraction buffer. The slurry was centrifuged for 10 min at 10000 ×*g*. Then 0.1 ml of the supernatant was mixed with 5 ml of the Coomassie Brilliant Blue dye reagent (CBB) and absorbance was measured at 595 nm by spectrophotometer using slandered curve of bovine serum albumin (Bradford, 1976).

Determination of total sugars

A known dry mass of wheat leaves was extracted by 80% ethanol and centrifuged at 12000 x*g* for 10 min: the extract was used to estimate sucrose and total soluble sugars (TSS) while the debris was used to estimate starch.
For sucrose determination, the alcoholic extract was digested in 5.4 N KOH in a boiling water bath for 10 minutes, anthrone reagent was added to the cooled reaction product; and the mixture was then incubated in a boiling water bath for 8 minutes. After cooling, the absorbance was recorded at 625 nm (Laurentin and Edwards, 2003).
For total soluble sugar determination, anthrone reagent was reacted with the ethanolic extract in a boiling water bath for 10 minutes then the absorbance of the cooled reaction mixture was measured at 625 nm (Schluter and Crawford, 2001).
For starch determination, the pellet left after complete extraction of TSS in ethanol was completely air-dried. To extract starch, 52% perchloric acid were mixed with the air- dry residue and incubated for 20 minutes at 0 °C. The mixture was centrifuged at 4,000 ×*g* for 5 minutes and the pellet was extracted once more then the supernatants were combined to be raised to a known volume with distilled water. To determine starch, the extract was reacted with anthrone reagent and heated in a boiling water bath for 8 minutes. After cooling, the absorbance was measured at 625 nm (Sadasivam and Manickam, 1996).

Determination of hydrogen peroxide (H_2O_2) content

The content of H_2O_2 in the leaves was measured according to Alexieva et al. (2001). Fresh leaf tissues were homogenized in liquid nitrogen and suspended in 5 ml of chilled 0.1% (w/v) trichloro acetic acid (TCA). The homogenate was centrifuged at 12,000 ×*g* for 15 min, and 0.5 ml of the supernatant was mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide (KI). The reaction was developed for 1 h in darkness and absorbance was measured at 390 nm.

Lipid peroxidation measured as malondialdehyde (MDA)

MDA content of the leaves was assayed according to Heath and Paker (1968). About 300 mg of the frozen material was ground in 2 ml of 0.1% (w/v) trichloroacetic acid and centrifuged. An aliquot of 0.5 ml of the supernatant was reacted with 2 ml of 20% (w/v) trichloroacetic acid containing 0.5% thiobarbituric acid at 95 °C for 30 min and cooled in an ice bath. The resulting mixture was centrifuged at 12,000 ×*g* for 10 min and absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated by using an extinction coefficient (ε) of 155 mM^{-1} cm^{-1}.

Assay of Catalase (CAT) activity

The activity of CAT was assayed by determining the rate of change in the absorbance at 240 nm of a reaction mixture (3 ml) that consisted of 50 mM potassium phosphate, pH 6.9, 11.6 mM H_2O_2 and 10 mM dithiotretol at 25 °C, and the decreased absorbance of H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) at 240 nm was recorded 1 min later. One unit of CAT equals 1.0 mmol of H_2O_2 reduced into water min^{-1} mg^{-1} protein (Aebi, 1984).

Guaiacol peroxidase (GPOX) activity

The activity of GPOX was assayed in a reaction mixture that consisted of 50 mM potassium phosphate, PH 6.4, 0.3 mM guaiacol, 0.14mM H_2O_2 at 470 nm by its ability to convert guaiacol to tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}) according to the method of Chance and Maehly (1955).

Statistical analysis

Analyses of variance (ANOVA) were carried out for physiological traits using three replicates in the Microsoft Excel software. Least significant difference (LSD) was calculated at 0.05.

Results and discussion

Changes in growth parameters

Fresh weight was gradually increased from 30 to 44-day-old in control and Spm-treated plants, and then suddenly decreased at 50 DAS. Fresh weight was always higher in Spm-treated plants than controls (Fig 1A). Dry weight was higher in Spm-treated plants than untreated plants, and then decreased on the day 50. (Fig.1B). In accordance with our results, many studies have intensively investigated the role of exogenous PAs in plant development and their mechanism of action and have shown that PAs are closely associated with plant growth promotion (Sequeramutiozabal et al., 2016). Polyamines are considered as a class of plant growth regulators. Foliar spray with Spm significantly improved growth of valerian (*Valeriana officinalis* L.) (Xu et al., 2014). In agreement with our results, Hawkins and Lewis (1993) found that, Spm and Put increased leaf area and fresh and dry weights of roots and shoots of strawberry. Exogenous PAs, especially Spm and Spd, resulted in improvement of reactive oxygen metabolism and photosynthesis, which improved plant growth and relieved the impact of plant stress (Baniasadi et al., 2018).

Water content % was higher in Spm-treated plants above control plants as time progressed beyond 44 DAS otherwise, there was no significant change in the WC % in both treated and untreated plants leaves and remained constant. (Fig. 1C). Because foliar spray brings PAs in direct contact with leaf surface which can improve the water status of the epidermal and underlying cells, and this effect appears to be persistent throughout the late experimental period (Farooq et al., 2009 a ;b). Exogenous application of PAs induces stomatal closure in *Arabidopsis* (Yamaguchi et al., 2007) which prevents water loss. Another finding concerning Spm-pretreated seedlings suggested reducing or slowing water transpiration under drought stress conditions (Fu et al., 2014). Stomatal closure and decreasing water to RWC, which is a direct indicator of plantwater status under drought conditions (Lugojan and Ciulca, 2011).

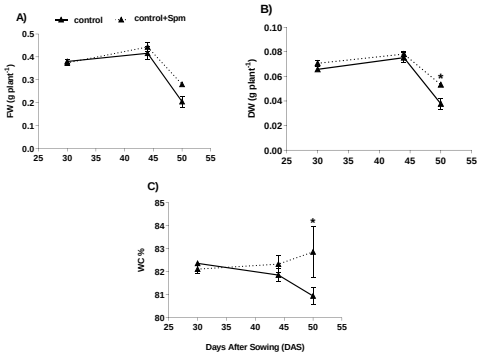

Fig. 1. Changes in growth parameters during developmental senescence in the fourth youngest leaf of Misr2 wheat cultivar under normal watered conditions with or without 100 µM spermine (Spm) foliar treatment. (**A**): Fresh weight, (**B**): dry weight, (**C**): water content. Data are means ± SE of three measurements. Asterisk shows significant difference.

Changes in photosynthetic pigments

In general, Chl a, Chl b and Carot contents were gradually decreased with the advance of leaf senescence (Fig. 2A, B &C). Spraying plants with Spm caused higher Chl a, Chl b and carotenoids contents compared with non-treated plants especially at 45 DAS. During leaf senescence, chlorophyll and photosynthetic proteins are degraded (Humbeck et al., 1996).There are several factors that can accelerate or delay this breakdown of the photosynthetic apparatus. Many changes during leaf senescence can be inhibited by the

application of exogenous PAs (Cai, 2009). Accordingly, the maintenance of photosynthetic pigments in Spm-treated plants with enhanced growth and production is in accordance with Mustafavi et al., (2016). Chloroplasts are the first organ to undergo disorganization during leaf senescence, and chlorophyll catabolites are transported and accumulated in the vacuole (Ling and Jarvis, 2016). During leaf development, carotenoid levels increase to protect chlorophyll from photo-oxidation and maintain photosynthetic activity but during leaf senescence, instead, they act as scavengers of ROS to ensure protection against oxidative stress and consequently, their level declines (Biswal, 1995).

Senescence of detached *Marchantia* thallus discs was monitored by measuring the decrease in chlorophyll. Polyamine treatment prevented Chl loss and preserved thylakoid membrane structure. Serafni-Fracassini et al., (2010) found that chlorophyll rapidly degraded and Put accumulated during senescence, while the exogenous addition of Spd or Spm inhibited protein degradation and reduced chlorophyll losses.

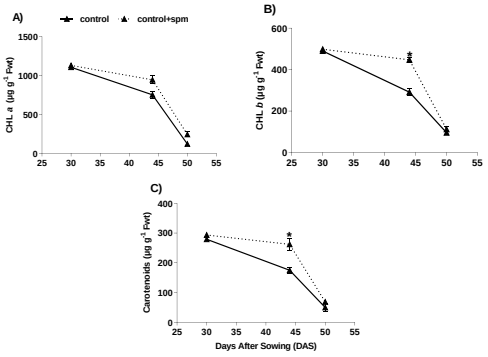

Fig. 2. Changes in Chlorophyll a (**A**), chlorophyll b (**B**) and carotenoids (**C**) during developmental senescence in the fourth youngest leaf of Misr2 wheat cultivar under normal watered conditions with or without 100 µM spermine (Spm) foliar treatment. Data are means ± SE of three measurements. Asterisk shows significant difference according to analysis of variance (two-way ANOVA) statistical analysis at $P \leq 0.05$ from the untreated control.

Changes in sugar levels

Sucrose accumulated similarly in the fourth leaf of both control and Spm- treated plants with the progress of time up to the 44th DAS; thereafter, it leveled off in the untreated plants but continued to increase in Spm- treated plants leading to higher levels of sucrose in Spm-treated leaves above the control at by the 50th DAS (Fig. 3A). The magnitude of total soluble sugars accumulation was significantly increased with the progress of time from 30 to 44 DAS then, a dramatic decrease was recorded. The concentration of total soluble sugars was higher at last interval in Spm- treated compared to non-treated plants (Fig. 3B). Starch was progressively accumulated along over the experimental period in treated and non- treated leaves. Foliar spermine application enhanced starch content over control value at day 50 concomitantly with the decreased total soluble sugars (Fig. 3C).

Soluble sugars are osmotically active organic substances, which could balance the vacuole water potential with that of the cytoplasm (Arzani, 2008). Our results showed a decrease in sucrose and total soluble sugars during natural senescence; but application of PA caused further increase of soluble and insoluble sugar of senescent leaves. This result is consistent with that of Chen et al. (2011). Sugars play an important role in energy supply during plant metabolism and development in addition to the regulation of senescence program initiation through induction of hexokinase-mediated sugar signaling pathway (Dai et al., 1999). Noh and Amasino (1999) have reported that low sugar contents induce leaf senescence. However, our experiment indicated that Spm treatment can delay plant senescence through high sucrose and soluble sugar levels. Moreover, our evidence supports the role of Spm in senescence retardation through increasing starch accumulation, meaning its role in improving the photosynthate product.

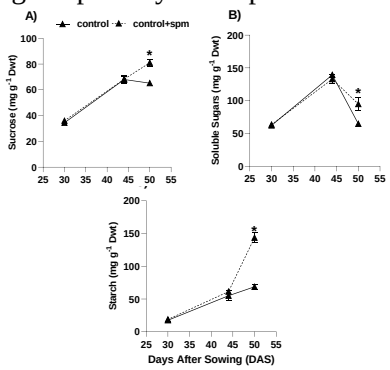

Fig. 3. Changes in sugar levels during developmental senescence in the fourth leaf of Misr 2 wheat cultivar under normal watered conditions with or without 100 µM spermine (Spm) foliar treatment. (**A**): Sucrose, (**B**): soluble sugars, (**C**): starch. Data are means ± SE of three measurements. Asterisk shows significant difference according to analysis of variance (two-way ANOVA) statistical analysis at $P \leq 0.05$ from the untreated control.

Changes in hydrogen peroxide (H_2O_2) and lipid peroxidation index (MDA content)

Figure 4A&B shows that, H_2O_2 and MDA contents increased gradually with increasing plant age in both control and Spm-treated plants. However, foliar spray with Spm clearly decreased the content of H_2O_2 and MDA compared to the control plants at all intervals. During leaf senescence, the pattern of ROS accumulation in cells is characterized by an initial increment followed by a decline (Khan and Khan 2017). The disorganization of the cell membrane through lipid peroxidation generates high levels of ROS; as a direct consequence, the detoxification systems can be impaired or being less efficient**.** Delayed leaf senescence was found to be associated with a higher Spm level, reduced reactive oxygen species (ROS) production (Sobieszczuk-Nowicka, 2017). Our results are in accordance with many studies (Amooaghaie and Moghym, 2011; Minocha et al., 2014; Mustafavi et al., 2016) where exogenous PAs alleviate growth inhibition possibly due to the protection of membranes and minimization of oxidative damage.

It has been shown that polyamines are effective scavengers of superoxide free radicals generated in a number of chemical systems in the cell, and that these free radicals cause organelle deterioration (Kaur-Sawhney et al. 1986). The increased production of superoxide molecules associated with advancing senescence induces alterations in the molecular organization of the lipid bilayer. Drolet et al. (1986) showed that exogenous application of polyamines protecte the membranes from the superoxide molecules due to their ability to scavenge free radicals.

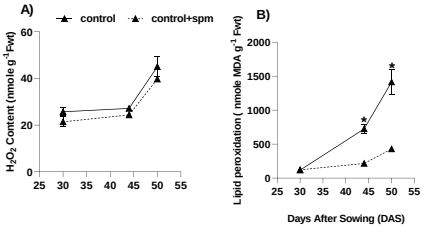

Fig. 4. Changes in hydrogen peroxide and lipid peroxidation during developmental senescence in the fourth leaf of Misr 2 wheat cultivar under normal watered conditions with or without 100 µM spermine (Spm) foliar treatments. (**A**): Hydrogen peroxide (**B**): lipid peroxidation. Data are means ± SE of three measurements. Asterisk shows significant difference according to analysis of variance (two-way ANOVA) statistical analysis at $P \leq 0.05$ from the untreated control.

Changes in total soluble protein

In general, there was a gradual decrease in the total soluble protein content in all samples with increasing leaf age. Total soluble protein content was slightly higher in Spm- treated plants compared to control plants (Fig. 5A). Protein breakdown is one of the most important catabolic processes associated with leaf senescence with an essential role in nutrient recycling, especially nitrogen. Increases in proteinase activity have been found during leaf senescence (Peterson and Huffaker 1975). In agreement with our results, Cohen et al. (1979) found that Spermidine and spermine enhanced protein retention in barley leaf discs during senescence. Also, Serafini-Fracassini et al. (2010) found that exogenous application of Spd or Spm inhibited protein degradation in *Lactuca sativa*

Changes in antioxidant enzymes

Catalase (CAT) activity was increased gradually along the experimental period both in Spm-treated and non-treated plants. CAT activity was obviously inhibited in plants sprayed with Spm compared with control plants (Fig. 5B).

In general, guaiacol peroxidase (GPOD) activity increased gradually with the advance of plant senescence up to 44 days then it decreased at day 50 in all plants. Foliar spray with Spm had no significant effect on GPOD enzyme activity (Fig. 5C). In accordance with our results, Serafini-Fracassini et al. (2010) has been shown that in *Arabidopsis*, CAT activity decreased at a the early stage of bolting

To avoid potential damage caused by ROS to cellular components, as well as to maintain growth, metabolism, development, and overall productivity, the balance between production and elimination of ROS at the intracellular level must be tightly regulated and/or efficiently metabolized. This equilibrium between the production and detoxification of ROS is sustained by enzymatic and nonenzymatic antioxidants (Mittler et al., 2004). The enzymatic components comprise several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and peroxiredoxins (Prxs). In wheat, several studies have reported changes in the activity of many enzymes of the antioxidant defense system in plants to control oxidative stress induced by environmental stresses causing alterations in the activity of SOD, APX,

CAT, glutathione reductase GR and GPX and in the ROS concentration (Huseynova et al., 2014; Wu et al. 2018). The increase in H_2O_2 levels is enforced by a decrease in ascorbate peroxidase 1 (APX1) activity (Zimmermann et al., 2006). In rice, increased expression levels of antioxidant enzymes and genes have been related to the response to stress factors (Caverzan et al., 2014). The present results showed an increasing H_2O_2 levels during natural senescence, CAT activity decreased, and GPX being slightly decreased in Spm- treated leaves.

Delayed leaf senescence was found to be associated with a higher Spm level, reduced reactive oxygen species (ROS) production, and increased nitric oxide levels (Sobieszczuk-Nowicka, 2017).

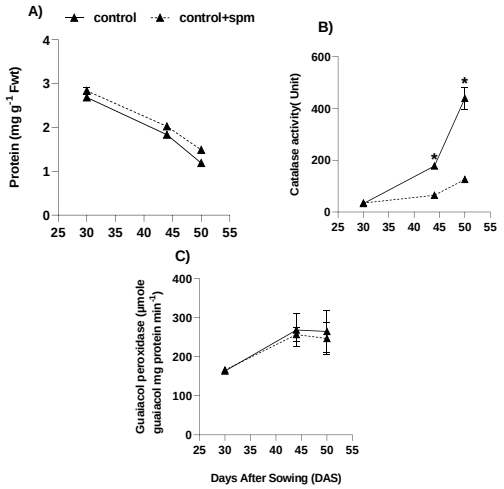

Fig. 5. Changes in protein content and the activity of antioxidant enzymes during developmental senescence in the fourth leaf of Misr2 wheat cultivar under normal watered conditions with or without 100 µM spermine (Spm) foliar treatment. (**A**): Protein content, (**B**): catalase, (**C**): guaiacol peroxidase. Data are means ± SE of three measurements. Asterisk shows significant difference according to analysis of variance (two-way ANOVA) statistical analysis at $P \leq 0.05$ from the untreated control.

Conclusion

Natural senescence in wheat can be delayed using foliar spray with 100 µM spermine at the vegetative stage through some biochemical changes including maintaining water content, preventing chlorophyll degradation and enhancement of soluble sugars, starch and total protein at late stages of senescence. In addition, reactive oxygen species and lipid peroxidation contents were lowered in Spm-treated plants than control values concomitantly with decreased catalase and guaiacol peroxidase activity due to Spm-treatment.

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الملخص العربي

عنوان البحث: المعالجة الخارجية بالإسبرمين تؤدى إلى تأخير الشيخوخة الطبيعية في أوراق نبات القمح من خلال حماية أصباغ البناء الضوئي من التكسير ومنع الإجهاد التأكسدي

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الشيخوخة هي عملية منظمة جينيا وتتضمن تكسير المكونات الخلوية ثم نقل النواتج لأجزاء النبات الأخرى. لكي تتم دراسة مراحل تقدم الشيخوخة الطبيعية في نبات القمح ومعرفة تأثير المعالجة الخارجية بالرش الورقي للبولي امين؛ الإسبرمين تركيز (١٠٠µM)، تم قياس التغيرات الكيميائية في الورقة الرابعة لنبات القمح عند مراحل زمنية مختلفة من شيخوخة الأوراق من اليوم ٣٠ إلى اليوم ٥٠ بعد البذر وقد وجد تأخير الشيخوخة الطبيعية للأوراق في النباتات المعالجة بالاسبرمين عن الأوراق التي لم تعالج عن طريق الحفاظ على المحتوى المائي للورقة ومنع تكسير أصباغ البناء الضوئي وتحسين تراكم السكريات الذائبة والنشا وكذلك البروتين الكلي حتى مراحل متأخرة من الشيخوخة. هذا بالإضافة إلى أن كمية الشقوق الحرة النشطة والمالون داي ألدهيد كانت أقل في الأوراق المعالجة بالاسبرمين مقارنة بالأوراق التي لم ترش. كذلك أدت المعالجة بالاسبرمين إلى تغير مضادات الأكسدة الإنزيمية مثل الكاتاليز والبيروكسيديز.