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非生物逆境下亞托敏及硒處理對小麥幼苗生理之影響

Physiological Effects of Azoxytrobin and Selenium on Wheat Seedlings under abiotic Stresses

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中文摘要

本研究的目的主要在評估亞托敏（azoxystrobin, AZ）和硒（Selenium, Se）處理對小麥品種台中選 2 號（*Triticum aestivum* L. variety Taichung No. 2, TCS2）在高溫和鹽逆境下的作用機制。在亞托敏實驗中，探討殺真菌劑亞托敏前處理對小麥幼苗於突然來襲的熱逆境下（Heat stress, HT, 46℃）的生理機制影響。本試驗分別以 0.4, 4, 40, 80 和 120 mg L^{-1} 的 AZ 預處理 4 天，再針對有經過 AZ 預處理和未處理 AZ 的幼苗施以 46 ºC 1 小時的高溫逆境，緊接著進行 1000 μmol m^{-2} s^{-1} 之高光處理 20 分鐘。 高溫誘導的氧化逆境導致未處理亞托敏幼苗葉片中的還原力降低及丙二醛含量增加，抗壞血酸過氧化物酶（ascorbate peroxidase, APX）和過氧化氫酶（catalase, CAT）酶活性增強。然而在高溫逆境下的 AZ 預處理卻導致葉綠素螢光，APX 和 CAT 活性以及清除 DPPH 能力的降低，並且隨著 AZ 處理濃度的增加，熱逆境所引起的生理損傷亦加劇。AZ 預處理雖可增加光合色素的含量，然而綜合上述生理指標的結果表明 AZ 無法為小麥台中選 2 號在熱逆境下提供保護作用。在硒與鹽害實驗中，主要探討 22 μM 之硒處理在不同濃度（0, 100, 200, 300 和 400 mM NaCl）的鹽逆境下對台中選 2 號幼苗的保護作用。研究結果顯示，經過硒處理之小麥在葉綠素螢光參數，抗氧化酵素 (過氧化氫酶 CAT)，抗氧化物質(總酚，類黃酮及黃青素含量)，抗氧化能力 (清除 DPPH 自由基能力、還原力分析)，光合作用色素 (總葉綠素及類胡蘿蔔素)，株高及根長皆有較佳之表現，顯示 22 μM 的硒處理確實對台中選 2 號幼苗在鹽脅迫下具有保

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護效果。在硒及鹽害下之滲透逆境及離子逆境實驗之研究中，旨在區分鹽逆境引起的滲透和離子脅迫對台中選 2 號的影響，並評估 22 μM Se 處理對此二種脅迫之調節效果。我們假設耐鹽性較強的台中選 2 號較易受到滲透脅迫的影響，而 22 μM 的 Se 處理對鹽害逆境所導致之滲透脅迫及離子脅迫皆發揮保護作用。為了驗證此一假設，本實驗將聚乙二醇（PEG）和鹽 (NaCl) 調成 3 種等滲濃度，最終滲透勢分別為 -1.05 MPa（24%（w / v）PEG 和 200 mM NaCl ）， - 1.33 MPa（26.5%（w / v）PEG 和 250 mM NaCl）和-1.57 MPa（29%（w / v）PEG 和 300 mM NaCl）。研究結果顯示，相較於 PEG 所造成之滲透逆境，鹽處理所造成的之離子逆境可使葉綠素螢光（ChlF）參數有較佳之表現，光合色素（總葉綠素和類胡蘿蔔素） 之降解速度較慢，抗壞血酸過氧化物酶（APX）活性較好，且丙二醛 （MDA）累積也較少。顯示在鹽害逆境下的滲透脅迫比離子脅迫對台中選二號造成更大之生理影響。同時，我們觀察到 22 μM Se 處理對鹽害逆境下之滲透脅迫及離子脅迫均無法提供保護效果，顯示硒的有效處理濃度還需要更進一步的實驗加以闡明。整體而言，本研究結果分別為亞托敏及硒處理下對台中選 2 號之小麥品種在熱逆境和鹽脅迫下之生理機制提供了更好的理解。

關鍵詞：小麥、台中選二號、亞托敏、硒、高溫逆境、鹽逆境、滲透逆境、離子逆境、聚乙二醇

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Abstract

The objective of this study was to evaluate the effect of azoxystrobin (AZ) and Selenium (Se) on wheat (*Triticum aestivum* L.) variety Taichung No. 2 'Taichung SEL.2' (TCS2) in high temperature and salt stress. In the AZ exp., the physiological mechanism of the fungicide AZ in protecting against heat (HT, 46 ˚C) stress in wheat TCS2 seedlings was investigated. Seedlings were pretreated with 0.4, 4, 40, 80, and 120 mg L^{-1} of AZ for 4 d. Next, AZ-pretreated and untreated seedlings were subjected to HT for 1 h followed by 1000 μmol m^{-2} s^{-1} lighting for 20 min. HT induced oxidant stress which resulted in a decrease in the reducing power, an increase in malondialdehyde, and enhanced enzyme activities of ascorbate peroxidase (APX) and catalase (CAT) in leaves of untreated seedlings. However, AZ-pretreated seedlings under HT displayed reductions in chlorophyll fluorescence, APX and CAT activities, and the 1,1-diphenyl-2-picryl-hydrazyl scavenging capacity. Physiological damage caused by HT was aggravated by an increase in the AZ concentration. In addition, increased photosynthetic pigments were also observed in leaves of AZ-pretreated and HT-exposed seedlings. The results suggest that AZ does not provide a protective effect against HT stress. In the Se & salt exp., the mitigative effects of 22 μM Se on TCS2 were investigated under different salt stress levels (0, 100, 200, 300, and 400 mM NaCl). Results of the antioxidative capacity showed that

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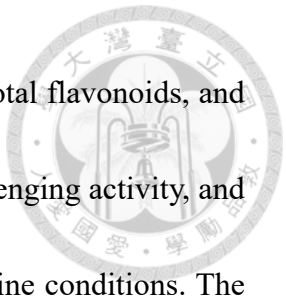

catalase (CAT) activity, non-enzymatic antioxidants (total phenols, total flavonoids, and anthocyanins), 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH) radical-scavenging activity, and the reducing power of Se-treated seedlings were enhanced under saline conditions. The more-stabilized chlorophyll fluorescence parameters (maximal quantum yield of photosystem II (F_v/F_m), minimal chlorophyll fluorescence (F_0), effective quantum yield of photosystem II (Φ_{PSII}), quantum yield of regulated energy dissipation of photosystem II (Y(NPQ)), and quantum yield of non-regulated energy dissipation of photosystem II (Y(NO)) and the less-extensive degradation of photosynthetic pigments (total chlorophyll and carotenoids) in Se-treated seedlings were also observed under salt stress. The elongation of shoots and roots of Se-treated seedling was also preserved under salt stress. Protection of these physiological traits in Se-treated seedlings might have contributed to stable growth observed under salt stress. The present study showed the protective effect of Se on the growth and physiological traits of wheat seedlings under salt stress. In the Se and salt/PEG exp., the aim was to distinguish the effects of osmotic and ionic stress induced by salt stress on TCS2 and evaluate the effect of 22 μM Se treatment on these two stresses. We hypothesized that TCS2 is more susceptible to osmotic stress, and 22 μM Se treatment protects against osmotic stress and ionic stress caused by salt stress. In order to verify this hypothesis, this experiment adjusted polyethylene glycol (PEG) and salt (NaCl) to three isotonic concentrations, and the final osmotic potential was -1.05 MPa

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(24% (w / v) PEG and 200 mM NaCl), - 1.33 MPa (26.5% (w / v) PEG and 250 mM NaCl) and -1.57 MPa (29% (w / v) PEG and 300 mM NaCl). According to the results of chlorophyll fluorescence parameters, APX activity, photosynthetic pigments (total chlorophyll and carotenoids), malondialdehyde (MDA), and shoot height. The osmotic stress caused by PEG was more harmful than the ionic stress caused by salt stress to TCS2. Also, we observed that 22μM Se treatment could not protect against osmotic stress and ionic stress under salt stress. The effective treatment concentration of Se required furthering experimentation. Overall, the results of this study provide a better understanding of the physiological mechanisms of TCS2 under thermal and salt stress with the treatment of AZ and Se.

Key words: wheat; Taichung SEL.2; azoxystrobin; selenium; heat stress; salt stress; osmotic stress; ionic stress; polyethylene glycol

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Lan CY, Lin KH, Chen CL, Huang WD, Chen CC (2020). Comparisons of chlorophyll fluorescence and physiological characteristics of wheat seedlings influenced by iso-osmotic stresses from polyethylene glycol and sodium chloride. Agronomy, 10(3): 325

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Introduction

Wheat (*Triticum aestivum* L.), the world's third most important primary cereal with more than 600 million tons of global production, preceded only by corn and rice (Asseng *et al.*, 2011), provides the main source of carbohydrates for 35% ~ 40% of the world's population (Chen *et al.*, 2017). However, under the influence of global warming, extreme environmental stresses such as heat, drought (Fábián *et al.*, 2019), and salinity (Yadav *et al.*, 2019) have impacted wheat (*Triticum aestivum* L.) production around the world with increasing frequency and intensity. Therefore, it is crucial to understand the mechanisms by which plants encounter abiotic stresses to promote crop performance under adverse conditions. Many protective chemicals have been used to mitigate the adverse effects caused by abiotic stresses, such as salicylic acid (Borsani *et al.*, 2001; Lu *et al.*, 2009), caffeic acid (Bhardwaj and Ramandeep, 2017), NO (Song *et al.*, 2013), H_2O_2 (Uchida *et al.*, 2002), spermidine (Mostofa *et al.*, 2014), and magnetized water (Hasan *et al.*, 2018). In addition, azoxystrobin (AZ) (Grossmann and Retzlaff, 1997) and selenium (Se) (Djanaguiraman *et al.*, 2005) are certified as agents for promoting yields and delaying senescence in some crops.

Azoxystrobin (AZ) is one of the widely used strobilurins used as systemic fungicides (Giuliani *et al.*, 2011). It is also certified as a chemical agent for promoting

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yields and delaying senescence in some crops, such as rice and wheat (Grossmann and Retzlaff, 1997; Wu and Tiedemann, 2001). Therefore, it is also classified as a plant bioregulator (Rademacher, 2004). Previous studies report that strobilurins stimulate nitrogen assimilation (Debona *et al.*, 2016), mediate plant hormones, cause leaf aging delays, increase chlorophyll concentrations (Grossmann and Retzlaff, 1997), mitigate oxidative stress (Wu and von Tiedemann, 2001; Zhang *et al.*, 2010), and improve plant water use efficiency and stabilize yields during droughts (Giuliani *et al*., 2011; Cantore *et al.*, 2016). Furthermore, strobilurins also enhance the activity of nitrate reductase, a key enzyme involved in plant nitrogen assimilation, while increasing nitrogen assimilation (Debona *et al.*, 2016). Conversely, Nason *et al*., (2007), Swoboda and Pedersen (2009), and Amaro *et al*., (2018) found that strobilurins might not be able to provide protective effects under stress conditions. The effects of strobilurins are complex and require further clarification.

Selenium (Se) is considered a beneficial element to plants (Pilon-Smits *et al*., 2009). Previous studies indicated that Se can delay senescence (Djanaguiraman *et al*., 2005; Xue *et. al*., 2001) and promote the vegetative and reproductive growth of plants (Hajiboland *et al*., 2012). Previous research reveals that the possible protective mechanisms of Se in plants against stresses include the enhancement of antioxidant enzyme activity (peroxidase (POD) and catalase (CAT), etc.) and increase the levels of

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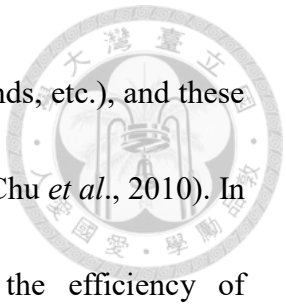

antioxidant compounds (anthocyanins, flavonoids, phenolic compounds, etc.), and these antioxidant features thus reduce stress-induced oxidative situations (Chu *et al*., 2010). In addition, Se can improve plant photosynthesis by increasing the efficiency of photosystem II (PSII), enhancing chlorophyll fluorescence, and reducing the degradation of chlorophyll concentration (Chu *et al*., 2010). Moreover, Se contributes to water status regulation in plants by promoting water uptake efficiency in roots and reducing water loss from tissues (Kuznetsov *et al*., 2003). In addition, Se stimulates plant growth by promoting the integrity of the membrane system, which results in root and shoot elongation and biomass accumulation (Djanaguiraman *et al*., 2005; Xiaoqin *et al*., 2009; Hawrylak-Nowak *et al*., 2009; Hu *et al*., 2013; Sun *et al*., 2010). Research by Hawrylak-Nowak (2009) shows that Se particularly supports root system development. However, Se exerts a dual effect in plants (Djanaguiraman *et al*., 2005), in that while it can stimulate plant growth and provide beneficial effects as a micronutrient at low concentrations, it is harmful to plants at higher concentrations. The positive effects of Se depend on its form, dose, and plant genotype (Sieprawska *et al*., 2015). Two forms of inorganic Se can be found, depending on the pH and redox potential of the soil, one being selenite and the other selenate, each of which exhibits different availabilities to and effects on plants. In our study, wheat was treated with sodium selenite (Na_2SeO_3) in an acidic nutrient solution, primarily as $HSeO_3^-$ (Guerrero *et al*., 2014). The recommended Se doses for hydroponic

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conditions are usually < 1 mg L^{-1} (29 μM) (Pilon-Smits *et al*., 2009). Conversely, toxic effects can occur when the Se dosage is > 0.35 mg L^{-1} (10 μM). The effects and beneficial dosages of Se on wheat (*Triticum aestivum* L.) cultivar 'Taichung SEL.2' (TCS2) need to be explored.

In the present study, the functions and effects of AZ and Se on wheat (*Triticum aestivum* L.) cultivar 'Taichung SEL.2' (TCS2) were investigated. In AZ experiments, the effects of AZ applications on the ChlF and antioxidant activity of wheat seedlings grown under high heat (HT) were evaluated. In Se and salt experiments, the growth and physiological effects of Se on wheat subjected to salt stress were conducted. In Se and salt/PEG experiments, the effects of applying Se to wheat under three iso-osmotic concentrations of salt and PEG were evaluated.

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Chapter 2. Physiological Effects of the Fungicide Azoxystrobin on Wheat Seedlings under Extreme Heat

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Abstract

Azoxystrobin (AZ) is not only a fungicide used for disease control, but also a protective chemical for crops against specific stresses. The physiological mechanism of the fungicide AZ in protecting against heat (HT, 46 °C) stress in wheat (*Triticum aestivum* L.) seedlings was investigated. 'Taichung SEL.2' variety seedlings were pretreated with 0.4, 4, 40, 80, and 120 mg L^{-1} of AZ for 4 d. Next, AZ-pretreated and untreated seedlings were subjected to HT for 1 h followed by 1000 μmol m^{-2} s^{-1} lighting for 20 min. HT induced oxidant stress which resulted in a decrease in the reducing power, an increase in malondialdehyde, and enhanced enzyme activities of ascorbate peroxidase (APX) and catalase (CAT) in leaves of untreated seedlings. However, AZ-pretreated seedlings under HT displayed reductions in chlorophyll fluorescence, APX and CAT activities, and the 1,1-diphenyl-2-picryl-hydrazyl scavenging capacity. Physiological damage caused by HT was aggravated by an increase in the AZ concentration. In addition, increased photosynthetic pigments were also observed in leaves of AZ-pretreated and HT-exposed

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seedlings. The results suggest that AZ does not provide a protective effect against HT stress.

Keywords: antioxidant activity; azoxystrobin; chlorophyll fluorescence; heat stress; wheat

Introduction

Under the influence of global warming, heat waves have impacted wheat production around the world with increasing frequency and intensity (Teixeira *et al.*, 2013; Asseng *et al.*, 2014). Heat is one of the main environmental factors influencing wheat yields and physiological senescence, such as inhibiting antioxidant enzyme activities and enhancing lipid peroxidation (Zhao *et al*., 2007). Maintaining wheat's physiological functions under sudden heat waves is an urgent issue, as well as preserving wheat yields and quality from acute and unexpected heat waves. In order to promote crop performances under adverse conditions, applying protective chemicals is one preventive strategy to protect against stresses (Gondim *et al.*, 2012).

Introduced in the 1990s, strobilurins are a group of chemicals used as systemic fungicides, and one of the most widely used strobilurin is azoxystrobin (AZ) (Giuliani *et*

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al., 2011). Strobilurins are also certified as chemical agents for promoting yields and delaying senescence in some crops, such as rice and wheat (Grossmann and Retzlaff, 1997; Wu and Tiedemann, 2001). Therefore, strobilurins are also classified as plant bioregulators (Rademacher, 2004). Previous studies reported that strobilurins stimulate nitrogen assimilation (Debona *et al.*, 2016), mediate plant hormones, cause leaf aging delays, increase chlorophyll concentrations (Grossmann and Retzlaff, 1997), mitigate oxidative stress (Wu and Tiedemann, 2001; Zhang *et al.*, 2010), and improve plant water use efficiency and stabilize yields during droughts (Giuliani *et al*., 2011; Cantore *et al.*, 2016). Furthermore, strobilurins also enhance the activity of nitrate reductase, a key enzyme involved in plant nitrogen assimilation, while raising nitrogen assimilation (Debona *et al.*, 2016). Grossmann and Retzlaff (1997) demonstrated that kresoxim-methyl, one type of strobilurin with auxin-like activity, induced plant morphogenesis and differentiation, reduced the activity of aminocyclopropane-1-carboxylic acid synthase which is involved in the ethylene synthesis pathway, and increased chlorophyll (Chl) concentrations in leaves, suggesting that strobilurins might provide protective effects against senescence. Moreover, application of AZ increased the antioxidant enzyme activity with a reduction in free radicals in plants, and reduced protein concentrations and electrolyte leakage from leaves (Wu and von Tiedemann, 2001; Zhang *et al*., 2010). Cantore *et al*. (2016) and Giuliani *et al*. (2011) indicated that AZ and other strobilurin

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fungicides reduced stomatal conductance and water evaporation, and promoted the water use efficiency (WUE) in tomatoes cultivated in an arid environment. The effects of AZ on the WUE of plants are related to an increase of endogenous abscisic acid and mediation of stomatal closure (Venancio *et al.*, 2003). However, Nason *et al*. (2007) observed that the beneficial effects which strobilurins provided of alleviating the WUE and evapotranspiration of crops grown in water deficient environments were extremely limited, and this resulted in a reduction in the maximum quantum yield (Fv/Fm). Debona *et al*. (2016) revealed that AZ mediated stomatal movements and inhibited the photosynthesis capacity, but did not influence Chl fluorescence (ChlF) or levels of photosynthetic pigments. The effects of strobilurins on stomata are complex and require further clarification. In addition, Swoboda and Pedersen (2009) illustrated that pyraclostrobin, a kind of strobilurin, did not promote the growth or yields of soybeans. Amaro *et al*. (2018) observed that AZ blocked electron transfer of the cytochrome-*bc1* complex in mitochondria and inhibited the respiration of plants, followed by a decrease in adenosine triphosphate and a reduction in the osmotic potential of guard cells, which was associated with the degree of stomatal opening. The main mechanism of AZ in reducing pathogen-induced oxidative stress from pathogen infections was to limit the expansion of the pathogen rather than increasing the antioxidant activity (Debona and Rodrigues, 2016). Strobilurin-induced delay of senescence in plants is well described.

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Unfortunately, strobilurin might not be able to provide protective effects when a plant is under a water deficit or is infected with a pathogen. However, Pedersen (2016) found that AZ promoted endogenous cytokinins and phenolic components of creeping bentgrass for maintaining physiological functions under heat stress. Nevertheless, there is no study on whether strobilurins can also provide crops with protective effects during heat stress. In this study, the effects of AZ applications on the ChlF and antioxidant activities of wheat seedlings grown under high heat (HT) were evaluated.

Materials and Methods

Plant and growth conditions

Wheat (*Triticum aestivum* L.) cultivar 'Taichung SEL.2' (TCS2), one of the most widely grown wheat cultivars in Taiwan, was used in this study. The seeds were sterilized with 1% hydrogen peroxide for 5 min, washed with distilled water, and germinated in Petri dishes on wetted filter paper at 25 °C in the dark. After 24 h of incubation, uniformly germinated seeds were selected and cultivated in 150 ml beakers containing one-fifth-strength Hoagland nutrient solution (Hoagland and Arnon, 1950), and the solution was replaced every 2 d. Hydroponically cultivated wheat seedlings were raised in growth chambers with fluorescent lamp lighting at 30 and 25 °C at day and night, respectively,

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under a 12-h photoperiod. The photosynthetic photon flux density (PPFD) was uniformly set to 300 μmol m^{-2} s^{-1}.

Experimental treatments

Hydroponically grown seedlings that had reached stage Z1.0 (Zadoks *et al*., 1974) on day 4 were treated with the AZ fungicide (250 g AI L^{-1}, AmistarR, Syngenta Limited, Waterford, Ireland) at concentrations of 0.4, 4, 40, 80, and 120 mg L^{-1} for 4 d. AZ was added to the nutrient solution according to the concentration of each AZ pretreatment. After AZ treatment, these seedlings were placed in a high temperature of 46 °C for 1 h in the dark as the HT condition. There were also a group of seedlings grown under HT without AZ pretreatment. Untreated seedlings were used as a control (CK). The experiment was independently performed three times for a randomized design of growth conditions.

Measurements of chlorophyll fluorescence (ChlF)

The fluorescence parameters in seedling leaves were determined after 1 h of HT in the dark. ChlF was measured in the middle portion of the first leaf of each seedling taken at ambient temperature with Chl fluorimeter imaging-PAM (Walz, Effeltrich, Germany). Actinic light and saturating light intensities were set to 500 and 7200 μmol m^{-2} s^{-1} of

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photosynthetically active radiation (PAR), respectively, and then the effective quantum yield of photosystem (PS)II under illumination (Φ_{PSII}) in leaves was determined after 1000 μmol m^{-2} s^{-1} (300 μmol m^{-2} s^{-1} for CK) lighting for 20 min. The minimal (F_0) and maximal (F_m) ChlF, maximum quantum yield of PSII (F_v/F_m), Φ_{PSII}, non-photochemical quenching (NPQ), the quantum yield of regulated energy dissipation of PSII (Y(NPQ)), the quantum yield of non-regulated energy dissipation of PSII (Y(NO)), and the relative electron transfer rate (ETR) were measured and calculated according to previously described methods (Vankooten and Snel, 1990; Kramer *et al*., 2004).

Measurement of ascorbate peroxidase (APX) and catalase (CAT) activity

APX activity was determined using the method of Nakano and Asada (1981). Briefly, 0.06 g of the latest newly expanded leaf was placed in 2 mL sodium phosphate buffer (50 mM, pH 6.8) in an ice bath for extraction and centrifuged at 4 °C and 12,000 g for 20 min. The supernatant (0.1 mL) was collected, followed by the sequential addition of 2.7 mL of potassium phosphate buffer (150 mM, pH 7.0), 0.4 mL of ethylenediaminetetraacetic acid (EDTA, 0.75 mM), 0.5 ml of H_2O_2 (6 mM), 0.5 mL of H_2O, and 0.5 mL of ascorbate (1.5 mM) and then mixed well. The absorbance at 290 nm (A290) of the sample solution was determined every 15 s for 1 min using a spectrophotometer (Hitachi U3010, Tokyo, Japan). The blank containing the same mixture with no enzyme extract was also measured.

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CAT activity was measured based on the method of Kato and Shimizu (1987). Briefly, 0.03 g of the latest newly expanded leaf was placed in 2 mL of sodium phosphate buffer (50 mM, pH 6.8) in an ice bath for extraction and centrifuged at 4 °C and 12,000 g for 20 min. The supernatant (0.2 mL) was collected, followed by adding 2.7 mL of sodium phosphate buffer (100 mM, pH 7.0), 0.05 mL of H_2O, and 0.05 ml of H_2O_2 (1 M), and then mixing well. The absorbance of the sample solution at 240 nm (A240) was determined every 15 s for 1 min. The blank containing the same mixture with no enzyme extract was also measured.

Measurement of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging capacity and the reducing power

The DPPH scavenging capacity was determined using the method of Shimada *et al.* (1992). Briefly, 160 μL of a methanol extract of the sample combined with methanol or standard solution of butylated hydroxytoluene (BHT) was added to 40 μL of a freshly prepared DPPH solution (1mM) to initiate the antioxidant-radical reaction at room temperature. The control was 160 μL of sample extract, methanol, or BHT solution diluted to 200 μL. The absorbance of the reaction mixture was determined at 517 nm during a 30-min reaction time. The DPPH scavenging capacity was calculated using a curve of BHT standards. Results are expressed as μg BHT equivalent g^{-1} dry weight (DW).

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The reducing power was determined using the method of Oyaizu (1986). Briefly, 0.3 mL of the methanol extract from a leaf was placed in 0.3 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.3 mL of 1% $K_3Fe(CN)_6$ in a water bath at 50 °C for 20 min, immediately placed in 0.3 mL of 10% TCA in an ice bath, and then centrifuged at 9000 rpm for 10 min. The supernatant (0.5 mL) was mixed well with 0.5 mL distilled water and 0.1 mL $FeCl_3 \cdot H_2O$ (0.1%). The absorbance of the reaction mixture was determined at 700 nm during the 10-min reaction. The reducing power was calculated using a curve of BHT standards. Results are expressed as mg BHT equivalent g^{-1} DW.

Determination of the malondialdehyde (MDA) concentration

MDA was determined using a previously described method (Heath and Packer, 1968). Briefly, lyophilized sample powder (0.03 g) was mixed with 1 mL of 5% TCA, and then centrifuged at 10,000 rpm and 20 °C for 5 min. The supernatant (250 μL) was added to 1 mL of 0.5% thiobarbituric acid (TBA) which was made up with 20% TCA. The mixture was placed in a water bath at 95 °C for 30 min, and then immediately cooled in an ice bath. The reaction mixture was centrifuged at 3000 rpm and 20 °C for 10 min, and the absorbance was determined at 532 and 600 nm. The blank was the same reaction mixture with no sample extract.

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Determination of the photosynthetic pigment concentrations

The photosynthetic pigment concentrations were determined using the method of Yang *et al*. (1998). Briefly, 0.01 g of lyophilized sample powder was extracted with 10 ml of an 80% acetone solution, and then centrifuged at 4500 rpm for 5 min. The supernatant of the sample extract was tested to determine the absorbance of Chl *a*, Chl *b*, and carotenoids (Cars) in acetone at 663.6, 646.6, and 440.5 nm, respectively.

Statistical analyses

All measurements were evaluated for significance using an analysis of variance (ANOVA) followed by a least significant difference (LSD) test at the $p < 0.05$ level. All statistical analyses were conducted using R i386 3.5.1 software (https://cran.r project.org/bin/windows).

Results and Discussion

Chlorophyll fluorescence (ChlF)

The response of ChlF can be used to evaluate the physiological condition of photosynthetic tissues in plants. Our preliminary data (data not shown) indicated that the light saturation point in leaves of seedlings was at 500 μmol m^{-2} s^{-1}. F_v/F_m in leaves was

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determined after dark adaption with HT (CK at 30 °C) for 1 h. With the exception of Φ_{PSII}, other ChlF parameters were determined at 500 μmol m^{-2} s^{-1}. Φ_{PSII} was determined after 1000 μmol m^{-2} s^{-1} treatment (CK under 300 μmol m^{-2} s^{-1}) for 20 min. F_v/F_m and Φ_{PSII} are widely used to estimate the status under heat stress (Sun *et al*., 2006), and F_v/F_m in CK seedling leaves was 0.80. The difference in F_v/F_m of leaves of untreated seedlings grown under HT and CK was insignificant. However, F_v/F_m values of AZ-pretreated seedlings grown under HT were significant lower than that of CK, and continued to decrease from 0.48 to 0.22 with an increase in the pretreated AZ concentration from 0.4 to 120 mg L^{-1} (Fig. 2-1). A similar trend was observed in Φ_{PSII} in leaves of heated seedlings after exposure to light at 1000 μmol m^{-2} s^{-1}, and CK seedlings exposed to light at 300 μmol m^{-2} s^{-1} for 20 min (Fig. 2-2). Meanwhile, an increase in F_0 and a decrease in F_m were observed in leaves of AZ-pretreated seedlings. Furthermore, a rise in the AZ concentration significantly influenced F_0, but not F_m. F_0 is a fluorescent signal when the PSII reaction center is fully open (Sun *et al*., 2006), and an increase in F_0 usually indicates that a plant is under stress (Song *et al*., 2013). NPQ indicates the ability of plants to dissipate excess light energy, and is one of protective mechanisms against high light stress, while Y(NPQ) and Y(NO) are important indicators of photo-protection and photo-damage, respectively (Kramer *et al*., 2004). Lower NPQ levels were detected in leaves of seedlings pretreated with higher AZ concentrations and exposed to HT (Fig. 2-3), and the dynamics of Y (NPQ)

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also showed a similar pattern. On the other hand, Y(NO) in seedling leaves was enhanced with an increase in the AZ concentration, indicating that AZ pretreatment provided no protective effect for photosynthesis against HT. ChlF is an ideal tool for evaluating damage to a plant's photosynthetic tissues. In this study, decreases in F_v/F_m and Φ_{PSII} in seedling leaves with an increase in the AZ concentration suggest that the damage level of the D1-protein was more severe at higher pretreatment AZ concentrations. On the other hand, the increase in F_0 also suggests that the light-harvesting complex had suffered irreversible damage, and/or the ability to transmit light energy from the antenna system to the PSII reaction center had degraded (Song *et al.*, 2013). Meanwhile, the fall in NPQ and Y (NPQ) and increase in Y (NO) in seedling leaves also indicate loss of photo-protective ability and expansion of photo-damage (Kramer *et al.*, 2004). These responses of ChlF parameters are consistent with results of previous studies (Nason *et al*., 2007; Debona *et al*., 2016) and suggest that an increase in the pretreated AZ concentration caused greater physiological damage to the photosystem in leaves. These disadvantageous effects of strobilurin on ChlF might have resulted from blockage of the transmission of electrons between PSII and PSI because of the combination of strobilurin and Qi in the cytochrome *bf* complex in chloroplasts (Nason *et al*., 2007). However, other studies reported that the foliar application of AZ might inhibit stomatal movement rather than ChlF, and would result in inefficient gas exchange (Debona *et al*., 2016; Amaro *et*

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al., 2018). In this study, AZ was added to the hydroponic solution rather than being applied to the foliage, and the impact of strobilurin on ChlF should be due to blockage of the transmission of electrons between PSII and PSI.

Activities of APX and CAT, DPPH scavenging capacity, and reducing power

APX activity in leaves of untreated seedlings grown under HT improved 86% more than that of CK leaves (Fig. 2-4), indicating that HT induced APX activity. However, a continuous reduction in APX activity of AZ-pretreated and heated seedlings from 1.36 to 0.55 μmol ascorbate min^{-1} mg^{-1} protein was observed with an increase in the AZ concentration up to 40 mg L^{-1}. APX activities in leaves of seedlings subjected to pretreatment with AZ at 40 mg L^{-1} or higher were significantly lower than that of CK leaves. The dynamics of CAT activity in seedlings showed a different pattern from the results of APX.

CAT activity of HT seedlings improved 24% more than that of CK seedlings (0.43 μmol H_2O_2 min^{-1} mg^{-1} protein), but did not increase as sharply as did APX. Furthermore, the sudden reduction in CAT activity from 0.50 to 0.35 μmol H_2O_2 min^{-1} mg^{-1} protein was determined in leaves of seedlings pretreated with AZ 40 mg L^{-1} and grown under HT, implying that both APX and CAT activities in leaves of AZ pretreated and heated seedlings were inhibited, and the critical concentration of AZ pretreatment was 40 mg L^{-}

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[1]. The difference in the DPPH scavenging capacity between CK (24.6 μg BHT equivalent g^{-1} DW) and HT leaves of seedlings (24.4 μg BHT equivalent g^{-1} DW) was insignificant, but a significant reduction in DPPH scavenging capacity from 24.4 to 14.6 μg BHT equivalent g^{-1} DW was observed in AZ-pretreated and heated seedlings with an increase in the AZ pretreatment concentration (Table 2-1). Nevertheless, the reducing power in leaves of HT seedlings (19.4 mg BHT equivalent g^{-1} DW) was significant lower than that of CK seedlings (21.6 mg BHT equivalent g^{-1} DW), but the difference in the reducing power in leaves of seedlings among the AZ-pretreated and heated groups was insignificant. APX and CAT are involved in the antioxidant system to protect against stress-induced reactive oxygen species (ROS). Wu and Tiedemann (2001) showed that AZ induced a delay in senescence which resulted from an increase in superoxide dismutase activity. Zhang *et al*. (2010) also suggested that AZ enhanced the activity of antioxidant enzymes, including CAT, and induced a delay in senescence. In our study, the activities of APX and CAT in seedling leaves were enhanced after being exposed to HT for 1 h, but suppression of enzyme activities was observed in AZ-pretreated seedlings. Furthermore, AZ also reduced the DPPH radical scavenging capacity in leaves of seedlings after HT exposure, suggesting that AZ was unable to provide a protective effect against HT in seedlings, but in fact damaged the antioxidant system in seedling leaves. These results are consistent with a previous study by Debona and Rodrigues (2016) who

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observed that AZ suppressed stress induced activities of APX and CAT in rice leaves. Moreover, Amaro *et al*. (2018) also reported that strobilurins, with the exception of AZ, improved the activity of the antioxidative system.

Malondialdehyde (MDA) concentration

In plants, the concentration of MDA, a product of lipid peroxidation, reflects the status of heat-induced damage (Lu *et al.*, 2009; Bhardwaj and Ramandeep, 2017). Fig. 2-5 shows that the level of MDA in leaves of HT seedlings (111 nmol g^{-1} DW) was higher than that of CK seedlings (100 nmol g^{-1} DW). Meanwhile, a stable level (109~114 nmol g^{-1} DW) of MDA in leaves of heated seedlings was observed among all AZ pretreatment concentrations. Zhang *et al*. (2010) observed that a reduction in MDA was accompanied by enhanced antioxidant enzyme activity. In our study, HT resulted in higher MDA levels in seedling leaves, but AZ neither enhanced nor suppressed the level of MDA in leaves of seedlings after exposure to HT. In addition, a similar trend was observed in the reducing power in leaves of seedlings after HT treatment. Debona and Rodrigues (2016) reported that glutathione, a chemical involved in the non-enzymatic system, inhibited oxidative stress. There might be another antioxidant mechanism, which was undermined in this study, in the AZ-treated seedlings against oxidant stress.

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Photosynthetic pigments

Both Chl and Cars are involved in the light reaction of photosynthesis. Levels of total Chl (7.88 mg g^{-1} DW), which is the sum of Chl *a* and Chl *b* concentrations, and Cars (1.07 mg g^{-1} DW) in leaves of seedlings exposed to HT were consistent with those of CK seedlings. In addition, total Chl and Car concentrations in leaves of AZ-pretreated and heated seedlings exhibited a significant upward trend from 7.88 to 8.78 mg g^{-1} DW and from 1.07 to 1.23 mg g^{-1} DW, respectively, with an increase in the AZ concentration applied (Table 2-2). However, differences in Chl *a*/*b* ratios in leaves were insignificant among all experimental treatments. Biotic and/or abiotic stresses usually lead to reductions in the concentration of photosynthetic pigments (Ashraf and Harris, 2013; Chen *et al*., 2016). The lower Chl concentration in leaves might result from an imbalance between the biosynthetic and degradative pathways of Chl (Chen *et al.*, 2015), but levels of photosynthetic pigments in leaves of HT seedlings were consistent with those of CK seedlings. Short-term exposure to HT probably did not effectively induce sharp reductions in pigment levels in leaves. On the other hand, AZ increased the accumulation of photosynthetic pigments in seedling leaves. A similar result was also presented in previous studies (Grossmann and Retzlaff, 1997; Wu and Tiedemann, 2001), and the phenomena, such as a reduction in Chl degradation or a delay in leaf yellowing, is called a ‘greening effect’. Our results showed that even though AZ induced a greening effect, it

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was obviously unable to protect against oxidant stress which was also caused by AZ. Song *et al*. (2013) reported that an enhanced Chl/Car ratio could mitigate heat-induced oxidative stress. In our study, a stable ratio of Chl/Car was observed in AZ-treated seedling leaves, and this might be another reason that AZ was unable to provide a protective effect against heat stress in wheat seedlings. Strobilurin is one of the most important fungicides for plant disease control. In addition, strobilurin is also considered a chemical to improve crop physiology. The effect of strobilurins on wheat grown under a well-controlled environment without stress and applied during the later growth stages induced a delay in senescence and promoted grain yields (Wu and Tiedemann, 2001; Zhang *et al*., 2010). Nevertheless, each strobilurin produced a dynamic effect on the plant's physiology and growth (Amaro *et al.*, 2018), and the responses of crops to strobilurin are dramatically diverse at different growth stages (Zhang *et al*., 2010), and some specific physiological effects are only fully presented with sufficient N fertilizer (Ishikawa *et al*., 2012). A previous study reported that strobilurins alleviated paraquat-induced stress (Wu and Tiedemann, 2001), but reduced photosynthesis during a drought (Nason *et al*., 2007). The heat-induced oxidant stress decreased the Chl concentration and CAT activity, increased the MDA concentration, and ultimately reduced wheat yields (Zhao *et al*., 2007). In our study, the effect of AZ on the wheat seedlings exposed to sudden HT was also observed. Further studies on the protective effect of strobilurins in

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crops are needed.

Conclusions

This study explored the physiological mechanism of AZ in wheat seedlings exposed to sudden HT. AZ treatment displayed an ability to increase the concentrations of photosynthetic pigments in seedling leaves of wheat, but impacted ChlF, APX and CAT activities. There were no trends observed in the responses of the reducing power or MDA concentration in leaves to AZ concentrations. Therefore, AZ provided limited support for the physiological functioning of wheat seedlings under HT stress.

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Table 2-1. Effect of azoxystrobin (AZ) on the level of the DPPH radical scavenging capacity (expressed in μg BHT equivalent g^{-1} dry weight (DW)) and reducing power (express in mg BHT equivalent g^{-1} DW) in seedlings leaves collected from the control (CK) or heated (HT) condition

Treatment	DPPH radical scavenging capacity	Reducing power
CK	24.6 ± 0.8 a	21.6 ± 0.2 a
HT	24.4 ± 0.2 a	19.4 ± 0.6 b
AZ 0.4 mg L^{-1} + HT	21.3 ± 0.3 b	19.9 ± 0.2 b
AZ 4 mg L^{-1} + HT	21.3 ± 0.3 b	19.0 ± 0.6 b
AZ 40 mg L^{-1} + HT	14.8 ± 0.4 c	19.5 ± 0.6 b
AZ 80 mg L^{-1} + HT	15.7 ± 0.1 c	19.4 ± 1.1 b
AZ 120 mg L^{-1} + HT	14.6 ± 0.9 c	19.5 ± 0.9 b

Within columns, means followed by the same letter do not significantly differ according to LSD test ($p < 0.05$).

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Table 2-2. Effect of azoxystrobin (AZ) on the level of total chlorophyll (Chl; expressed in mg g-1 DW), the Chl *a*/*b* ratio, carotenoids (Car; expressed in mg g^{-1} DW), and the Chl/Car ratio in seedling leaves collected from the control (CK) or heated (HT) condition.

Treatment	Total Chl	Chl a/b	Car	Chl/Car
CK	7.76 ± 0.19 b	2.14 ± 0.02 a	1.05 ± 0.03 c	7.39 ± 0.33 a
HT	7.88 ± 0.12 b	2.13 ± 0.01 a	1.07 ± 0.03 c	7.35 ± 0.11 a
AZ 0.4 mg L^{-1} + HT	8.35 ± 0.21 ab	2.14 ± 0.02 a	1.11 ± 0.06 bc	7.35 ± 0.31 a
AZ 4 mg L^{-1} + HT	8.83 ± 0.53 a	2.14 ± 0.01 a	1.14 ± 0.05 ab	7.58 ± 0.26 a
AZ 40 mg L^{-1} + HT	8.88 ± 0.64 a	2.14 ± 0.03 a	1.16 ± 0.03 ab	7.65 ± 0.44 a
AZ 80 mg L^{-1} + HT	8.69 ± 0.56 a	2.13 ± 0.01 a	1.17 ± 0.06 ab	7.69 ± 0.59 a
AZ 120 mg L^{-1} + HT	8.78 ± 0.14 a	2.13 ± 0.02 a	1.23 ± 0.01 a	7.15 ± 0.08 a

Within columns, means followed by the same letter do not significantly differ according to the LSD test ($p < 0.05$).

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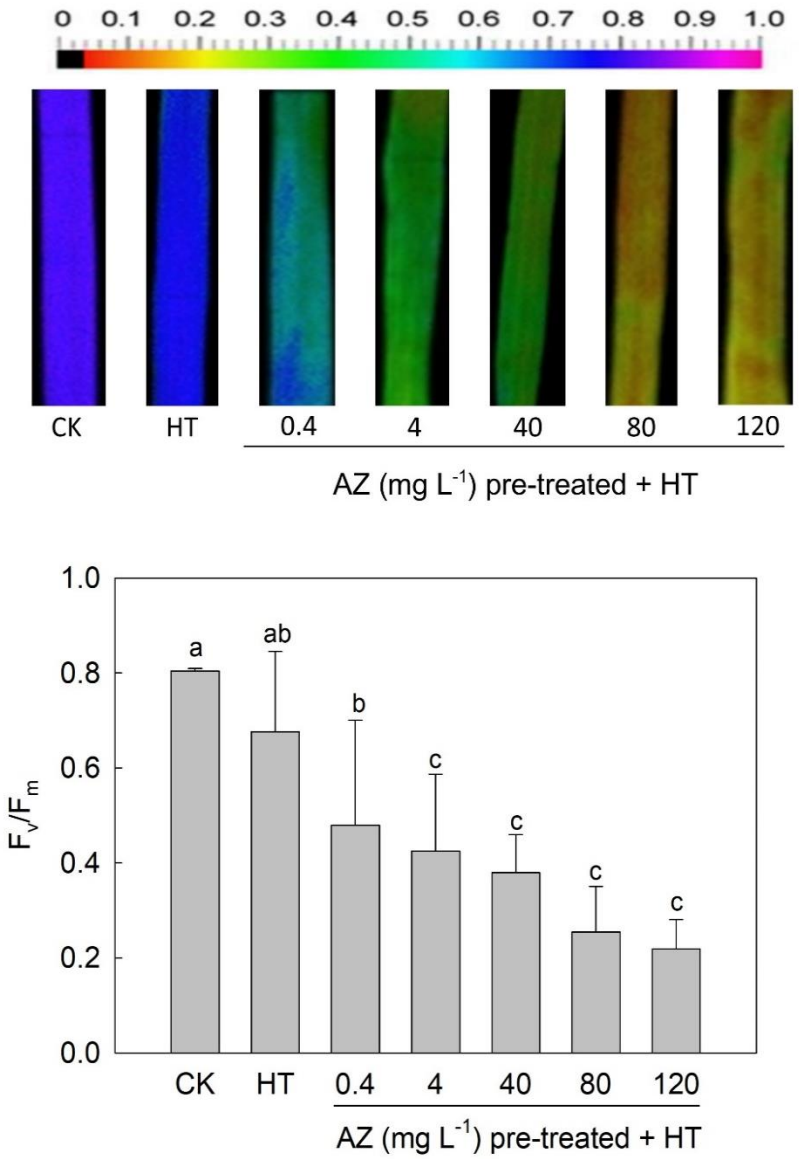

Figure 2-1. Images of chlorophyll fluorescence in the maximum quantum yield of photosystem II (Fv/Fm). The mean ± standard deviation (error bar) of F_v/F_m in leaves was determined in wheat seedlings treated with azoxystrobin (AZ) and grown under control (CK) or heated (HT) conditions. Values followed by different letters statistically significantly differ at $p < 0.05$.

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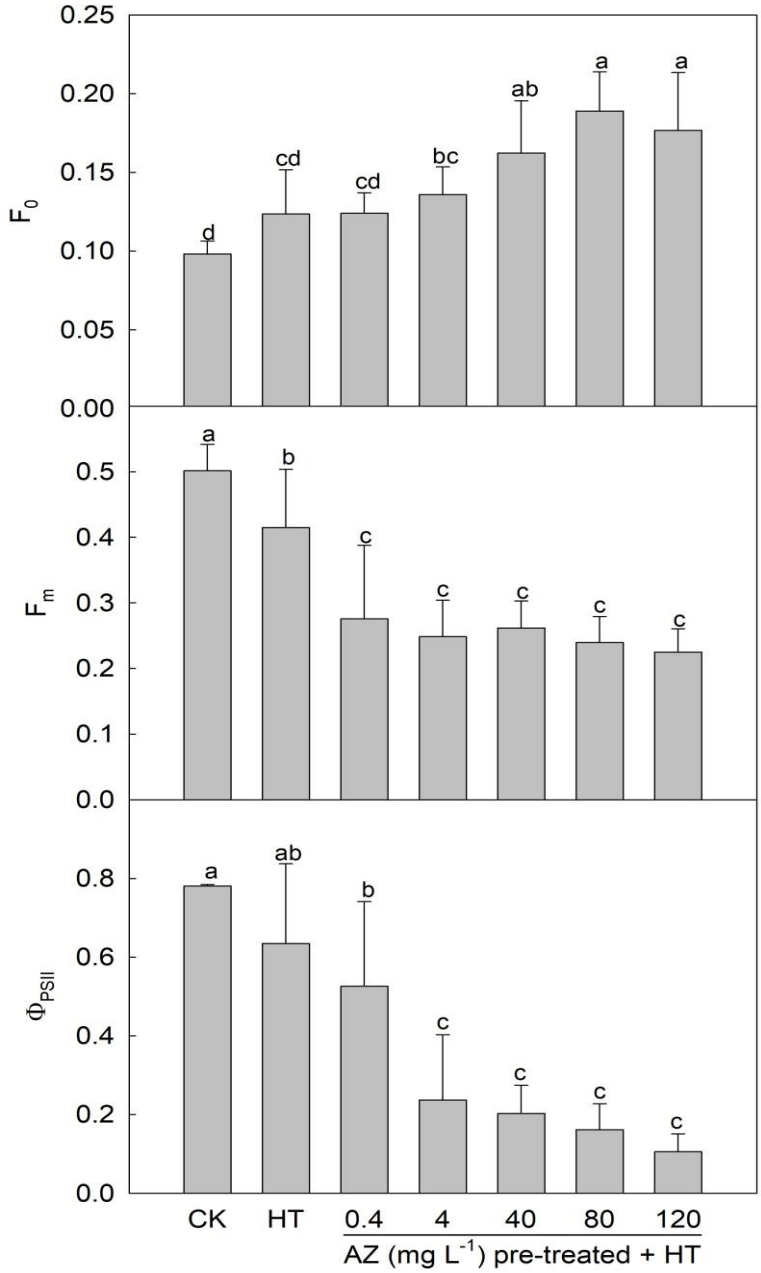

Figure 2-2. The mean ± standard deviation (error bar) of the minimal (F_0) and maximal (F_m) chlorophyll fluorescence, and the effective quantum yield of photosystem II (PSII) under illumination (Φ_{PSII}) of leaves collected from wheat seedlings treated with azoxystrobin (AZ) and grown under the control (CK) or heated (HT) environment. Values followed by different letters statistically significantly differ at $p < 0.05$.

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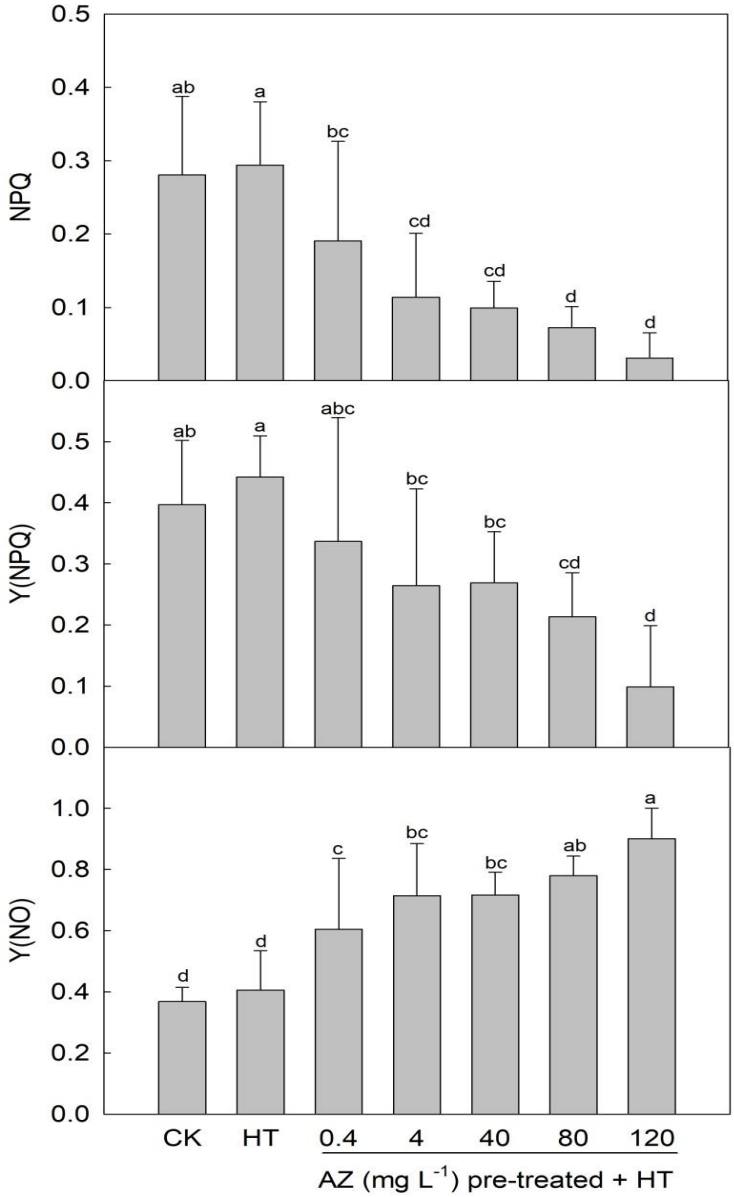

Figure 2-3. The mean ± standard deviation (error bar) of non-photochemical quenching (NPQ), the quantum yield of regulated energy dissipation of photosystem II (PSII) (Y(NPQ)), and the quantum yield of non-regulated energy dissipation of PSII (Y(NO)) of leaves collected from wheat seedlings treated with azoxystrobin (AZ) and grown under the control (CK) or heated (HT) environment. Values followed by different letters statistically significantly differ at $p < 0.05$.

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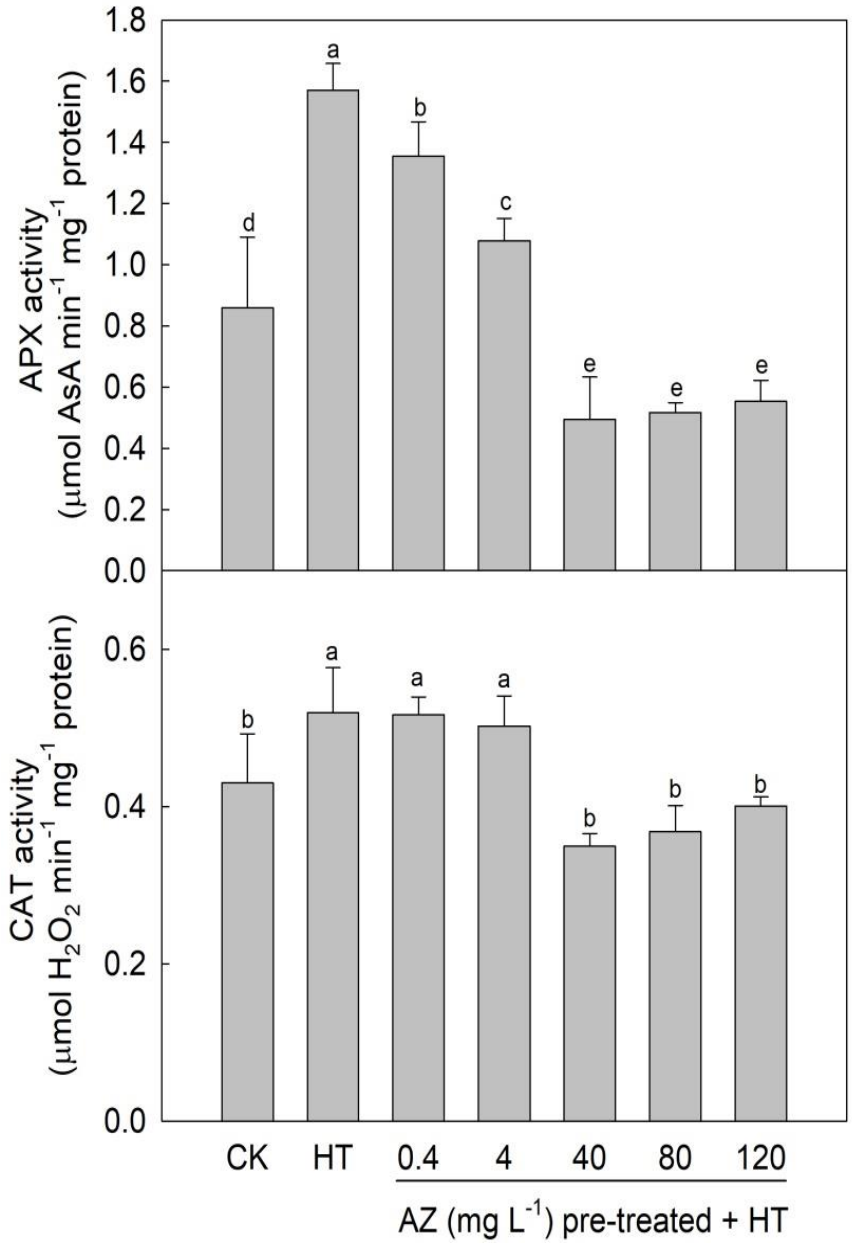

Figure 2- 4. The mean ± standard deviation (error bar) of ascorbate peroxidase (APX) and catalase (CAT) activities of leaves collected from wheat seedlings treated with azoxystrobin (AZ) and grown under the control (CK) or heated (HT) environment. Values followed by different letters statistically significantly differ at $p < 0.05$.

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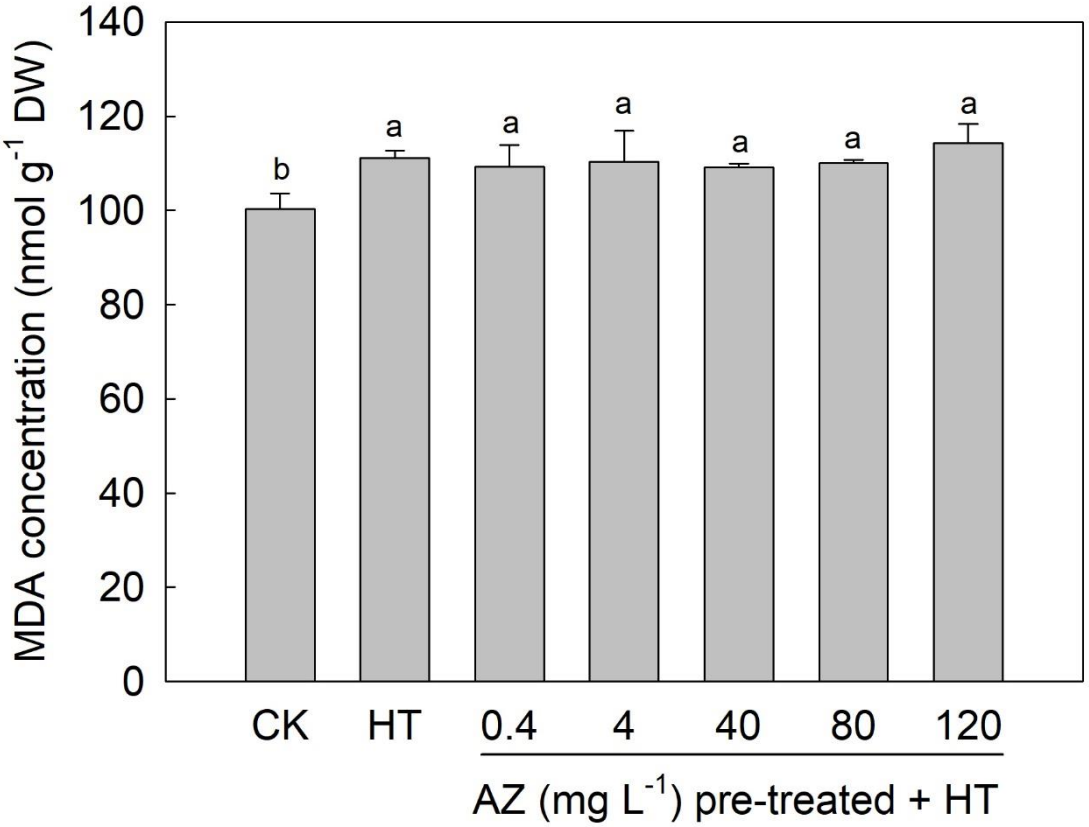

Figure 2-5. The mean ± standard deviation (error bar) of malondialdehyde (MDA) of leaves collected from wheat seedlings treated with azoxystrobin (AZ) and grown under the control (CK) or heated (HT) environment. Values followed by different letters statistically significantly differ at $p < 0.05$.

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Chapter 3. Protective Effects of Selenium on Wheat Seedlings under Salt Stress

Lan CY, Lin KH, Huang WD, Chen CC (2019). Protective Effects of Selenium on Wheat Seedlings under Salt Stress. Agronomy, 9(6): 272.

Abstract

Wheat is a staple food worldwide, but its productivity is reduced by salt stress. In this study, the mitigative effects of 22 μM selenium (Se) on seedlings of the wheat (*Triticum aestivum* L.) cultivar Taichung SEL. 2 were investigated under different salt stress levels (0, 100, 200, 300, and 400 mM NaCl). Results of the antioxidative capacity showed that catalase (CAT) activity, non-enzymatic antioxidants (total phenols, total flavonoids, and anthocyanins), 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH) radical-scavenging activity, and the reducing power of Se-treated seedlings were enhanced under saline conditions. The more-stabilized chlorophyll fluorescence parameters (maximal quantum yield of photosystem II (F_v/F_m), minimal chlorophyll fluorescence (F_0), effective quantum yield of photosystem II (Φ_{PSII}), quantum yield of regulated energy dissipation of photosystem II (Y(NPQ)), and quantum yield of non-regulated energy dissipation of photosystem II (Y(NO)) and the less-extensive degradation of photosynthetic pigments (total chlorophyll and carotenoids) in Se-treated seedlings were also observed under salt stress. The elongation of shoots and roots of Se-treated seedling was also preserved under salt stress.

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Protection of these physiological traits in Se-treated seedlings might have contributed to stable growth observed under salt stress. The present study showed the protective effect of Se on the growth and physiological traits of wheat seedlings under salt stress.

Keywords: wheat; selenium; salt stress; enzymatic and non-enzymatic activities; antioxidant activity

Introduction

Wheat (*Triticum stivum* L.), the third most important primary cereal with more than 600 million tons of global production, preceded only by corn and rice (Asseng *et al*., 2011), provides the main source of carbohydrates for 35%~40% of the world's population (Chen *et al*., 2017). However, wheat yields are markedly reduced in saline soils, due to improper fertilization that causes osmotic and drought stresses (Egamberdieva, 2009). Therefore, it is imperative to research the effects of salt stress on wheat's physiology.

Selenium (Se) is considered a beneficial element to plants (Pilon-Smits *et al*., 2009). Previous studies indicated that Se can delay senescence (Djanaguiraman *et al*., 2005; Xue *et. al*., 2001) and promote the vegetative and reproductive growth of plants (Hajiboland *et al*., 2012). Studies reported that Se mitigated disadvantageous phenomena caused by

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various stressful situations, such as heat (Iqbal *et al*., 2015; Shang *et al*., 2005; Djanaguiraman *et al*., 2010), cold (Chu *et al*., 2010), heavy metals (Khan *et al*., 2015; Mroczek-Zdyrska and Wójcik, 2012), ultraviolet (UV)-B (Breznik *et al*., 2005; Yao *et al*., 2010; Yao *et al*., 2011; Xue and Hartikainen, 2000), drought (Xiaoqin *et al*., 2009; Nawaz *et al*., 2015), and salt stress (Mona *et al*., 2017; Diao *et al*., 2014). According to previous research, possible protective mechanisms of Se in plants against stresses include its enhancement of antioxidant enzyme activities (peroxidase (POD), catalase (CAT), etc.) and increasing antioxidant compounds (anthocyanins, flavonoids, phenolic compounds, etc.), and these antioxidant systems thus reduce stress-induced oxidative situations (Chu *et al*., 2010). In addition, Se can improve plant photosynthesis by increasing the efficiency of photosystem II (PSII), enhancing chlorophyll fluorescence, and reducing the degradation of chlorophyll concentration (Chu *et al*., 2010). Moreover, Se contributes to water status regulation in plants by promoting the water uptake efficiency from roots and reducing water loss from tissues (Kuznetsov *et al*., 2003). In addition, Se stimulates plant growth by promoting the integrity of the membrane system which results in root and shoot elongation and biomass accumulation (Djanaguiraman *et al*., 2005; Xiaoqin *et al*., 2009; Hawrylak-Nowak, 2009; Hu *et al*., 2013 ; Sun *et al*., 2010). Research by Hawrylak-Nowak (2009) reported that Se particularly supported root system development.

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Salinization degrades land and is serious environmental stress that limits wheat production (Egamberdieva, 2009). There are two phases when plants encounter salt stress. Initially within a few minutes, plants are subjected to osmotic changes which reduce the roots' ability to absorb water. Gradually, the toxicity of NaCl inhibits ion transport and results in leaf senescence and reduced photosynthesis (Läuchli and Grattan, 2007). Few studies have evaluated the mitigation of salt stress in wheat by supplying Se. A study by Yigit *et al*. (2012) reported that organic Se can increase germination percentages and enhance antioxidant activities in wheat exposed to salt stress. Mona *et al*. (2017) evaluated the effect of Se on wheat under salt stress, and their results suggested that supplying Se led to increased germination percentages and growth, and also an enhancement of total soluble sugars. A study by Sattar *et al*. (2017) indicated that foliar application of Se improved the growth and physiological status of wheat seedlings under stressed conditions.

All of the above results provide evidence that supplying Se can alleviate the disadvantageous effects of salt stress on wheat, but the physiological mechanisms which Se trigger need to be further evaluated. In this study, Se played a role as a bioregulator to remediate the physiological status in wheat seedlings cultivated under salt stress. The hypothesis was a hydroponic solution with Se application might improve the growth and physiological performance of wheat subjected to salt stress.

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Materials and Methods

Plant and Growth Conditions

Wheat (*Triticum aestivum* L.) cultivar Taichung SEL. 2 (TCS2), one of the most widely cultivated wheat cultivars in Taiwan, was used in this study. Seeds used in the present study were obtained from the Department of Agronomy, National Taiwan University (Taipei, Taiwan). The seeds were sterilized with 1% hydrogen peroxide for 5 min, washed with distilled water, and germinated in Petri dishes on wetted filter paper at 25 °C in the dark. After 24 h of incubation, uniformly germinated seeds were selected and cultivated in 150 mL beakers containing complete Hoagland's nutrient solution (PhytoTech, Lenexa, KS, USA), which was replaced every 3 days. Hydroponically cultivated wheat seedlings were raised in growth chambers with fluorescent lamp lighting at 25 and 20 °C during the day and night, respectively, under a 12-h photoperiod. The photosynthetic photon flux density (PPFD) was uniformly set to 300 μmol m^{-2} s^{-1}.

Experimental Treatments

Se was added in the form of Sodium selenite (Na_2SeO_3, Sigma-Aldrich Chemie GmbH, Taufk irchen, BY, Germany) to the nutrient solution (pH = 4.6) with the treated concentration of 22 μM once the germinated seeds were cultivated in 150 mL beakers.

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No treatment consisted of the nutrient solution without Se supplementation. Hydroponically grown seedlings that had reached stage Z1.0 (Zadoks *et al*., 1974) on day 6 were treated with sodium chloride (NaCl) at concentrations of 0, 100, 200, 300, and 400 mM for 7 days. The experiment was independently performed three times for a randomized design of growth conditions.

Growth Analysis

Shoot height and root length were measured with a ruler before the measurements of chlorophyll (Chl) fluorescence (ChlF) and sample collection.

Measurements of ChlF

Fluorescence parameters in seedling leaves were determined after Se and salt treatments. ChlF was measured in the middle portion of the first leaf of each seedling taken at ambient temperature with Chl fluorometer imaging-PAM (Walz, Effeltrich, Germany). Actinic light and saturating light intensities were set to 185 and 7200 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), respectively. The minimal (F_0) and maximal (F_m) ChlF, the maximum quantum yield of PSII (F_v/F_m), the effective quantum yield of PSII (Φ_{PSII}), the quantum yield of regulated energy dissipation of PSII (Y(NPQ)) and the quantum yield of non-regulated energy dissipation of PSII (Y(NO)) were

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measured and calculated according to previously described methods (Van and Snel, 1990; Kramer *et al*., 2004).

Measurement of Catalase (CAT) and Ascorbate Peroxidase (APX) Activities

CAT and APX activities were measured according to the methods of Kato and Shimizu (1987) and Nakano and Asada (1981), respectively. Briefly, 0.06 g of the latest newly expanded leaf was placed in 2 mL of sodium phosphate buffer (50 mM, pH 6.8) in an ice bath for extraction and centrifuged at 4 °C and 12,000 rpm for 20 min. For CAT activity, the supernatant (0.2 mL) was collected, followed by the addition of 2.7 mL of sodium phosphate buffer (100 mM, pH 7.0), 0.05 mL of H_2O, and 0.05 mL of H_2O_2 (1 M), and then mixed well. The absorbance of the sample solution at 240 nm (A_{240}) was determined every 15 s for 1 min. A blank containing the same mixture with no enzyme extract was also measured. For APX activity, the supernatant (0.1 mL) was collected, followed by the sequential addition of 2.7 mL of potassium phosphate buffer (150 mM, pH 7.0), 0.4 mL of ethylenediaminetetraacetic acid (EDTA, 0.75 mM), 0.5 mL of H_2O_2 (6 mM), 0.5 mL of H_2O, and 0.5 mL of ascorbate (1.5 mM) and then mixed well. The absorbance at 290 nm of the sample solution was determined every 15 s for 1 min using a spectrophotometer (Hitachi U3010, Tokyo, Japan). The blank containing the same mixture with no enzyme extract was also measured.

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Measurement of 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH)-Scavenging Capacity and the Reducing Power

The methanol extract for analyzing the DPPH scavenging capacity and the reducing power was prepared by adding 12 mL of 100% methanol to 0.02 g of lyophilized sample powder. The mixture was oscillated in an ultrasonic oscillator for 1 h and extracted overnight at 4 °C. The mixture was then centrifuged at 3000 rpm for 20 min, and the supernatant was collected for the following measurement.

The DPPH-scavenging capacity was determined using the method of Shimada *et al*. (1992). Briefly, 160 μL of a methanol extract of the sample combined with methanol or a standard solution of butylated hydroxytoluene (BHT) was added to 40 μL of a freshly prepared DPPH solution (1 mM) to initiate the antioxidant-radical reaction at room temperature. The control was 160 μL of sample extract, methanol, or BHT solution diluted to 200 μL. The absorbance of the reaction mixture was determined at 517 nm during the 30 min reaction time. The DPPH-scavenging capacity was calculated by the percentage of the free radical-scavenging activity.

The reducing power was determined using the method of Oyaizu (1986). Briefly, 0.3 mL of a methanol extract from a leaf was placed in 0.3 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.3 mL of 1% $K_3Fe(CN)_6$ in a water bath at 50 °C for 20 min, immediately placed in 0.3 mL of 10% trichloroacetic acid (TCA) in an ice bath, and then

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centrifuged at 9000 rpm for 10 min. The supernatant (0.5 mL) was well mixed with 0.5 mL distilled water and 0.1 mL $FeCl_3 \cdot H_2O$ (0.1%). The absorbance of the reaction mixture was determined at 700 nm during the 10 min reaction. The reducing power was calculated using a curve of BHT standards. Results are expressed as mg BHT equivalents g^{-1} dry weight (DW).

Determination of Total Phenols, Total Flavonoids, and Anthocyanin Concentration

Phenolic compounds were determined using the method of Kujala *et al*. (2000). Briefly, 0.01 g of lyophilized sample powder was extracted with 1 mL of a 0.3% HCl in a 60% methanol solution, and then centrifuged at 4000 rpm for 10 min. The supernatant (200 μL) was added to 2 mL of 1 N Folin-Ciocalteau reagent (Sigma, St. Louis, MO, USA), mixed well, and allowed to sit for 10 min. Na_2CO_3 (sodium carbonate; 10%) was added to the solution and allowed to sit for 2.5 h. The absorbance was determined at 750 nm. The total phenolic concentration was calculated using a curve of gallic acid standards. Results are expressed as mg gallic acid equivalents (GAE) g^{-1} DW.

The flavonoid concentration was determined according to Chen *et al*. (2015). Sample powder of 0.01 g was extracted with 1 mL of a 1% HCl solution in ethanol and centrifuged at 3000 rpm for 10 min at 4 °C, and the absorbance at a wavelength of 540 nm was measured with a spectrophotometer.

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The anthocyanin concentration was determined using the method of Mancinelli *et al*. (1975). Lyophilized sample power (0.01 g) was extracted with 6 mL of 1% (v/v) HCl of a methanol solution and then centrifuged at 2000 rpm for 15 min. The supernatant of the sample extract was tested to determine the absorbance of 530 and 657 nm, respectively.

Determination of the Photosynthetic Pigment Concentrations

The photosynthetic pigment concentrations were determined using the method of Yang *et al*. (1998). Briefly, 0.01 g of lyophilized sample powder was extracted with 12 mL of an 80% acetone solution, and then centrifuged at 4500 rpm for 5 min. The supernatant of the sample extract was tested to determine the absorbance extents of Chl *a*, Chl *b*, and carotenoids in acetone at 663.6, 646.6, and 440.5 nm, respectively.

Statistical Analyses

All measurements were evaluated for significance using an analysis of variance (ANOVA) followed by a least significant difference (LSD) test and *t*-test at $p < 0.05$. All statistical analyses were conducted using R i386 3.5.1 software (https://cran.r-project.org/bin/windows).

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Results

Growth Analysis

Shoot heights of seedlings without Se treatment gradually declined when the NaCl concentration exceeded 100 mM. Seedlings with Se treatment also showed a similar trend, but shoot heights of Se-treated seedlings were significantly ($p < 0.05$) higher than those without Se when the NaCl concentration exceeded 100 mM (Figure 3-1). Root lengths of Se-treated seedlings also declined with an increase in the NaCl concentration. A similar declining trend was observed in Se-treated seedlings. However, root lengths of Se-treated seedlings were significantly longer ($p < 0.05$) than those of seedlings without Se treatment (Figure 3-1). These results showed that Se effectively promoted the growth of seedlings grown under salt stress.

ChlF

The response of ChlF can be applied as an index to evaluate the physiological condition of the photosynthetic tissues of plants. F_v/F_m in leaves was determined after dark adaption. F_v/F_m in leaves of seedlings without Se treatment suddenly declined as the NaCl concentration exceeded 200 mM, but F_v/F_m in leaves of Se-treated seedlings was significantly enhanced ($p < 0.05$) under salt stress (Figure 3-2). F_0 is a fluorescent signal

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when the PSII reaction center is fully open (Sun *et al*., 2006), and an increase in F_0 usually indicates that a plant is under stress (Song *et al*., 2013). F_0 in leaves of seedlings without Se treatment gradually increased with an increase in the NaCl concentration, but F_0 in Se-treated seedlings was stable (Figure 3-2).

Φ_{PSII} reflects the effective quantum yield of PSII under illumination. Y(NPQ) and Y(NO) are important fluorescence parameters of photo-protection and photodamage, respectively (Kramer *et al*., 2004). In this study, Φ_{PSII}, Y(NPQ), and Y(NO) were determined at an illumination of 185 μmol m^{-2} s^{-1}, and results are presented in Figure 3-3. The value of Φ_{PSII} in leaves of seedlings without Se treatment dramatically decreased with an increase in the NaCl concentration. A similar trend was also observed in results of Φ_{PSII} in leaves of Se-treated seedlings, but Φ_{PSII} values of Se-treated seedlings at 300 and 400 mM NaCl significantly improved ($p < 0.05$). Y(NPQ) in leaves of seedlings without Se treatment was significantly ($p < 0.05$) enhanced by NaCl of < 200 mM, but dramatically declined under more-severe salt stress (> 300 mM NaCl). A similar Y(NPQ) dynamic was determined in Se-treated seedlings, but the value at 400 mM NaCl was significantly higher than that without Se ($p < 0.05$). Y(NO) in leaves of seedlings without Se was maintained at a level of around 0.26-0.30 under NaCl of < 200 mM and was significantly enhanced at 300 and 400 mM ($p < 0.05$). The Y(NO) result for Se-treated

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seedlings also presented a similar trend, but Y(NO) values at 300 and 400 mM NaCl were significantly lower than that without Se ($p < 0.05$).

Activities of CAT and APX, DPPH-Scavenging Capacity, and Reducing Power

Results of the antioxidant enzyme activity and capacity in wheat seedlings of this study are presented in Table 3-1. CAT and APX play important roles in quenching H_2O_2. In this study, results for CAT activity in seedlings without Se treatment showed a descending trend with an increasing NaCl concentration, while CAT activities in Se-treated seedlings significantly ($p < 0.05$) remained at a level of around 1.79~1.56 μmol H_2O_2 min^{-1} mg^{-1} protein until NaCl exceeded 300 mM (Table 3-1). On the other hand, results of APX activities in both Se-treated seedlings and untreated seedlings showed descending trends with an increasing NaCl concentration.

The removal of DPPH radicals and reduction in the reducing power are methods for measuring antioxidant activities (Erel, 2004). The method for determining DPPH free radicals is based on the amount of DPPH free radicals removed. In this study, the ability to clear DPPH in untreated seedlings was reduced from 33.5% to 28.0% with an increasing NaCl concentration, while this ability was significantly induced ($p < 0.05$) at around 31.9% ~ 43.2% in Se-treated seedlings until NaCl exceeded 300 mM (Table 3-1). The reducing power is a method to estimate substances that might own the ability to

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remove free radicals in a plant (Jayanthi and Lalitha, 2011). In this study, the reducing power in untreated seedlings was maintained at a level of 20.5 ~ 19.2 BHT equivalent g^{-1} DW until NaCl exceeded 300 mM, but the reducing power in Se-treated seedlings was significantly enhanced ($p < 0.05$) except at 300 mM NaCl (Table 3-1).

Total Phenols, Total Flavonoids, and Anthocyanin Concentrations

Plants contain a variety of non-enzymatic antioxidants which can scavenge free radicals, including phenols, flavonoids, and anthocyanins (Thiruvengadam and Chung, 2015). Results of total phenols, total flavonoids, and anthocyanin concentrations in seedlings undergoing different treatments in this study are presented in Table 3-2. Total phenols contained in untreated wheat seedlings declined from 60.85 to 51.89 mg GAE g^{-1} DW with an increase in the NaCl concentration, while in Se-treated seedlings, they were effectively enhanced. Similar trends were also observed in the dynamics of total flavonoid and anthocyanin concentrations in seedlings in this study.

Photosynthetic Pigments

Chls and Cars are both involved in the light reaction of photosynthesis. Chl *a*, Chl *b,* and their sum, and carotenoid concentrations in leaves of seedlings from all treatments in this study are presented in Figure 3-4. In this study, Chl *a* and Chl *b* concentrations and

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their sum in leaves of Se-treated seedlings and untreated seedlings were slightly enhanced at 100 mM NaCl, but these Chl concentrations significantly ($p < 0.05$) and sharply declined when seedlings were grown under NaCl of more than 200 mM. A similar trend was also observed in Car concentrations in leaves of Se-treated seedlings and untreated seedlings. However, all values of photosynthetic pigments of Se-treated seedlings were higher than those of untreated seedlings. These results revealed that Se treatment served as a protectant for photosynthetic pigments to prevent their salt-stress induced degradation in wheat seedlings.

Discussion

Although a beneficial micronutrient, Se exerts a dual effect in plants (Djanaguiraman *et al*., 2005): It can stimulate plant growth and provide beneficial effects at low concentration, but it is harmful to plants at higher concentrations. Positive effects of Se depend on its form, dose and the chosen plant genotype (Sieprawska *et al*., 2015). According to the pH and redox potential of soil, two kinds of inorganic Se forms can be found: One is selenite, and the other one is selenate. Each of them exhibits different availabilities and effects to plant. In our study, sodium selenite (Na_2SeO_3) was treated in the acidic nutrient solution, which existed primarily as $HSeO_3^-$ (Guerrero *et al*., 2014).

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The recommended Se doses for hydroponic conditions are usually < 1 mg L^{-1} (29 μM) (Pilon-Smits *et al*., 2009). According to results of our preliminary study for wheat (*T. aestivum* L.) cultivar Taichung SEL. 2, the growth rate of wheat seedlings was strongly retarded at 10 mg Se L^{-1} (294 μM), but was not promoted at 0.5 mg Se L^{-1} (14.7 μM). Se at 1 mg L^{-1} (29 μM) might exceed the plant's threshold, resulting in a slightly disadvantageous effect on the plant. Therefore, 0.75 mg Se L^{-1} (22 μM) was an appropriate concentration for Se treatment in this study.

When plants experience salt stress, electron leakage from chloroplasts and mitochondria might react with O_2 during normal aerobic metabolism to produce reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide ($^{\cdot}O_2^-$), and hydroxyl ($^{\cdot}OH$) radicals (Dionisio-Sese and Tobita, 1998). ROS can immediately react with DNA, lipids, and proteins, thereby possibly causing serious cellular damage (Sato *et al*., 2001). Fortunately, the adverse effects of ROS can be diminished by enzymatic and non-enzymatic defense systems in plants (Gondim *et al*., 2010). Among the enzymatic defense system, CAT and APX play key roles in quenching H_2O_2 (Sato *et al*., 2001 ; Gondim *et al*., 2010). In our study, we found that applying Se improved CAT activities in wheat seedlings grown under all salt treatments, especially at 100 and 200 mM NaCl (Table 3-1). Our results of CAT activity were similar to those of a study by Chu *et al*. (2010) who indicated that Se mitigated cold-induced stress in wheat

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seedlings. Results of Djanaguiraman *et al*. (2010) and Nawaz *et al*. (2015) also supported our results. However, Iqbal *et al*. (2015) found that Se treatment did not increase CAT activity in every wheat cultivar. Furthermore, Djanaguiraman *et al*. (2005) observed that CAT did not participate in active H_2O_2 reduction irrespective of the sampling date (on the 80th and 90th days after sowing). These previous studies suggested that CAT activity was cultivar-specific and variable according to the growth period. In this study, CAT absolutely played an important role in the enzymatic defense system when wheat was suffering from salt stress.

Nonetheless, we found that Se treatment did not promote APX activity in wheat seedlings that were suffering from salt stress at 200 mM NaCl ($p < 0.05$) (Table 3-1). Xue and Hartikainen (2000) also reported that APX activities in ryegrass and lettuce were not enhanced by Se treatment. This phenomenon could be explained by APX and CAT sharing the same substrate, and while Se might increase CAT activity, it would effectively reduce H_2O_2, which might also weaken the substrate to induce APX activity (Shang *et. al.*, 2005). Another possibility could be that APX activities in plants might vary in different cultivars and growth stages (Djanaguiraman *et al*., 2005 ; Iqbal *et al*., 2015). Our results showed that the Se-induced antioxidative activity in wheat seedlings grown under salt stress did not include mediation of APX activity. The beneficial effects of Se treatment on ChlF, total Chl and Car concentrations, and growth of wheat seedlings grown

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under salt stress might have been due to the contribution of CAT activity or other enzymes which were not analyzed in this study.

The antioxidant potential was evaluated by the DPPH free radical-scavenging activity and reducing power. Se treatment enhanced the antioxidant potential of wheat seedlings exposed to salt stress (Table 3-1). Se was also reported to induce enhancement of antioxidant activity in broccoli (Ramos *et al*., 2011) and turnip (Thiruvengadam and Chung, 2015). The non-enzymatic antioxidant system diminishes the adverse effects of ROS in plants (Iqbal *et al*., 2015; Chu *et al*., 2010 ; Thiruvengadam and Chung, 2015). In this study, the concentrations of total phenols, total flavonoids, and anthocyanins in wheat seedlings grown under salt stress were evaluated. Anthocyanins act to reduce photoinhibition and photobleaching of Chl in plants under stress (Steyn *et al*., 2002). In addition, flavonoids and anthocyanins also provide functions as osmoprotectants in plants under stress (Iqbal *et al*., 2015). According to results presented in this study, Se treatment increased the concentrations of total phenols, total flavonoids, and anthocyanins in wheat seedlings grown under salt stress (Table 3-2). These results were consistent with a study by Yao *et al*. (2010), who evaluated the effect of an exogenous Se supply on wheat seedlings under enhanced UV-B.

The ChlF response can be used to evaluate the physiological condition of photosynthetic tissues in the plant. F_v/F_m and Φ_{PSII} are widely used to estimate the status

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under stress (Sun *et al*., 2006). Results of ChlF showed that Se treatment enhanced values of F_v/F_m (Figure 3-2), Φ_{PSII}, and Y(NPQ) (Figure 3-3) and decreased F_0 (Figure 3-2) and Y(NO) (Figure 3-3) in salt-stressed wheat seedlings. These results, which illustrated the stimulating effects of Se treatment on the photochemical activity of PSII and photosynthetic activity in wheat seedlings, are in agreement with a previous report by Diao *et al*. (2014). Se treatment increased the enzymatic and non-enzymatic antioxidative capacities of wheat seedlings grown under salt stress, and reduced ROS accumulation at PSII. Therefore, the salt stress-induced damage in the PSII reaction center complex in wheat seedlings was alleviated (Djanaguiraman *et al*., 2005 ; Khan *et al*., 2015). On the other hand, the insignificant difference between F_v/F_m of wheat seedlings grown at 100 and 200 mM NaCl suggests that TCS2, the tested cultivar in this study, is a salt-tolerant cultivar. The ChlF results were similar to the results of CAT activity which were enhanced by Se treatment (Table 3-1). Therefore, we speculated that CAT plays a key role in quenching H_2O_2 in TCS2, and we propose that application of Se in the soil or coating Se onto seeds might be suitable for TCS2 cultivation in saline areas.

Stress conditions also damage photosynthetic pigments with impaired biosynthesis and/or accelerated degradation of pigments (Chen *et al*., 2015; Ashraf and Harris, 2013). Results of the analysis of photosynthetic pigments in this study (Figure 3-4) suggested that Se treatment significantly increased the Chl and Car concentrations in wheat

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seedlings ($p < 0.05$). The increase in total Chl concentrations might have been due to increases in the Car and anthocyanin concentrations, since these antioxidative metabolites can protect Chl from photo-oxidative destruction and photobleaching under stress conditions (Yao *et al*., 2011 ; Steyn *et al*., 2002). Meanwhile, the enzymatic antioxidant system contributed to efficient ROS scavenging (Hawrylak-Nowak, 2009). Furthermore, Se treatment positively promoted the integrity of the membrane system of chloroplasts (Kong *et al*., 2005). Se induced enhancements of Chl and Car concentrations in wheat seedlings under stress was also reported in a previous study by Iqbal *et al*. (2015).

Se treatment stimulates root and shoot elongation and biomass accumulation in plants facing salt stress, and the protective effects of Se have been evaluated in sorrel (Kong *et al*., 2005), melon (Hu *et al*., 2013), and cucumber (Hawrylak-Nowak, 2009). In addition, Se treatment promotes the growth of soybeans facing senescence (Djanaguiraman *et al*., 2005). According to the results of seedling growth in this study (Figure 3-1), shoot and root elongation were absolutely inhibited under salt stress. However, Se treatment counteracted the stress-induced effects on seedling growth. Se alleviated the disadvantageous effect of salt stress on seedling growth, which might have been associated with enhanced CAT activity and non-enzymatic antioxidants, including total phenols, total flavonoids, and anthocyanins. The Se-induced antioxidant system resulted in activation of antioxidant activity, such as DPPH radical-scavenging activity and

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reducing power, increased levels of photosynthetic pigments, and effective photosynthesis potency. Moreover, Se supported the integrity of the membrane system in chloroplasts and/or mitochondria which might have also contributed to plant morphogenesis (Kong *et al*., 2005).

Conclusions

Compared to seedlings without Se treatment, CAT and antioxidative activities of Se-treated seedlings were enhanced, and the accumulation of non-enzymatic antioxidants, including total phenols, total flavonoids, and anthocyanins, increased under salt stress. Meanwhile, the indices of ChlF and degradation of photosynthetic pigments in Se-treated seedlings had stabilized compared to those in untreated seedlings. The elongation of shoots and roots of Se-treated seedlings was preserved under salt stress. The performance of physiological and morphological traits in Se-treated wheat seedlings was better than in untreated seedlings in a saline environment in this study. These results suggest that wheat cultivation with Se treatment could prevent salt stress-induced disadvantageous effects.

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Table 3-1. Catalase (CAT) and ascorbate peroxidase (APX) activities, DPPH radical-scavenging activity (%), and reducing the power of wheat seedlings.

NaCl (mM)	Se treated	CAT activity (μmol H_2O_2 min^{-1} mg^{-1} protein)	APX activity (μmol AsA min^{-1} mg^{-1} protein)	DPPH radical scavenging activity (%)	Reducing power (BHT equivalent g^{-1} DW)
0	No added	1.53 ± 0.05 ns A	0.22 ± 0.04 a A	33.5 ± 1.1 b B	19.2 ± 0.4 b A
	22 μM	1.56 ± 0.12 ns A	0.18 ± 0.01 b A	43.2 ± 2.4 a A	23.6 ± 0.4 a A
100	No added	1.39 ± 0.09 b A	0.18 ± 0.02 ns AB	36.0 ± 1.5 b A	20.5 ± 0.9 b A
	22 μM	1.79 ± 0.14 a A	0.16 ± 0.03 ns A	40.2 ± 0.2 a B	23.1 ± 0.1 a A
200	No added	1.07 ± 0.19 b B	0.17 ± 0.01 a AB	28.8 ± 2.2 b C	19.7 ± 0.5 b A
	22 μM	1.62 ± 0.26 a A	0.13 ± 0.01 b AB	40.2 ± 1.0 a B	21.7 ± 0.2 a B
300	No added	0.92 ± 0.00 ns BC	0.13 ± 0.03 ns BC	25.4 ± 0.2 b D	19.2 ± 0.5 ns A
	22 μM	0.98 ± 0.19 ns B	0.10 ± 0.04 ns B	31.9 ± 1.2 a C	19.2 ± 0.7 ns C
400	No added	0.76 ± 0.02 ns C	0.10 ± 0.02 ns C	28.0 ± 0.2 ns CD	15.2 ± 0.3 b B
	22 μM	0.89 ± 0.14 ns B	0.11 ± 0.04 ns B	28.9 ± 1.7 ns D	18.3 ± 0.4 a D

Values are the mean ± SD (n = 3). Means with different lowercase letters significantly differ by a t-test between untreated (No added) and Se-treated (22 μM) plants at the same salt concentration. Means with different capital letters significantly differ by an LSD test among NaCl concentrations of 0, 100, 200, 300, and 400 mM in untreated (No added) and Se-treated (22 μM) plants ($p < 0.05$). ns, not significant at the $p < 0.05$ level. AsA, ascorbate; BHT, butylated hydroxytouene.

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Table 3-2. Total phenols, total flavonoids, and anthocyanin concentrations of wheat seedlings.

NaCl (mM)	Se treated	Total phenols concentration (Gallic acid equivalent g^{-1} DW)	Total flavonoids concentration (A_{540} g^{-1} DW)	Anthocyanin concentration (μmol g^{-1} DW)
0	No added	60.9 ± 2.4 b A	33.9 ± 3.0 b AB	117 ± 2 b A
	22 μM	65.1 ± 2.8 a A	41.5 ± 1.0 a A	126 ± 7 a A
100	No added	56.0 ± 0.9 b B	36.0 ± 1.1 b A	117 ± 5 b A
	22 μM	63.4 ± 0.3 a AB	42.9 ± 0.6 a A	121 ± 1 a A
200	No added	56.5 ± 0.5 b B	31.1 ± 2.4 b B	99 ± 0 b B
	22 μM	65.8 ± 2.5 a A	37.9 ± 1.8 a B	111 ± 2 a B
300	No added	58.1 ± 0.1 ns AB	22.6 ± 1.3 b C	76 ± 2 b C
	22 μM	59.7 ± 1.7 ns AB	27.1 ± 0.9 a C	83 ± 0 a C
400	No added	51.9 ± 2.2 b C	17.7 ± 0.7 b C	58 ± 1 b D
	22 μM	59.5 ± 3.2 a B	21.8 ± 0.7 a D	69 ± 5 a D

Values are the mean ± SD (n = 3). Means with different lowercase letters significantly differ by a t-test between untreated (No added) and Se-treated (22 μM) plants at the same NaCl concentration. Means with different capital letters significantly differ by an LSD test among NaCl concentration of 0, 100, 200, 300, and 400 mM in untreated (No added) and Se-treated (22 μM) plants ($p < 0.05$). ns, not significant at the $p < 0.05$ level. DW, dry weight.

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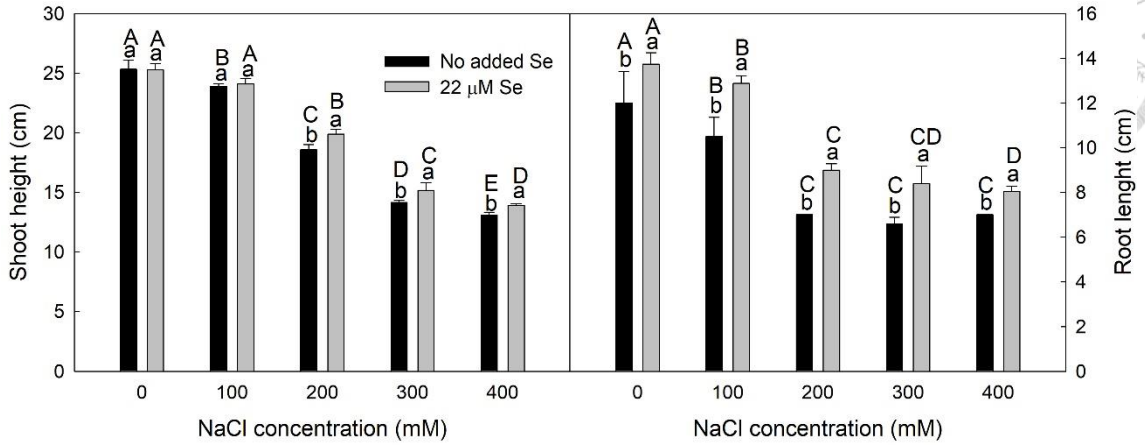

Figure 3-1. Means ± SD (n = 5) of the shoot height and root length of untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under different NaCl concentrations. Values followed by different letters statistically significantly differ at $p < 0.05$. Means with different lowercase letters significantly differ by a t-test between Se treatments (No added Se and 22 μM Se) at the same NaCl concentration. Means with different capital letters significantly differ by an LSD test under different NaCl concentrations in separate untreated and Se-treated groups ($p < 0.05$).

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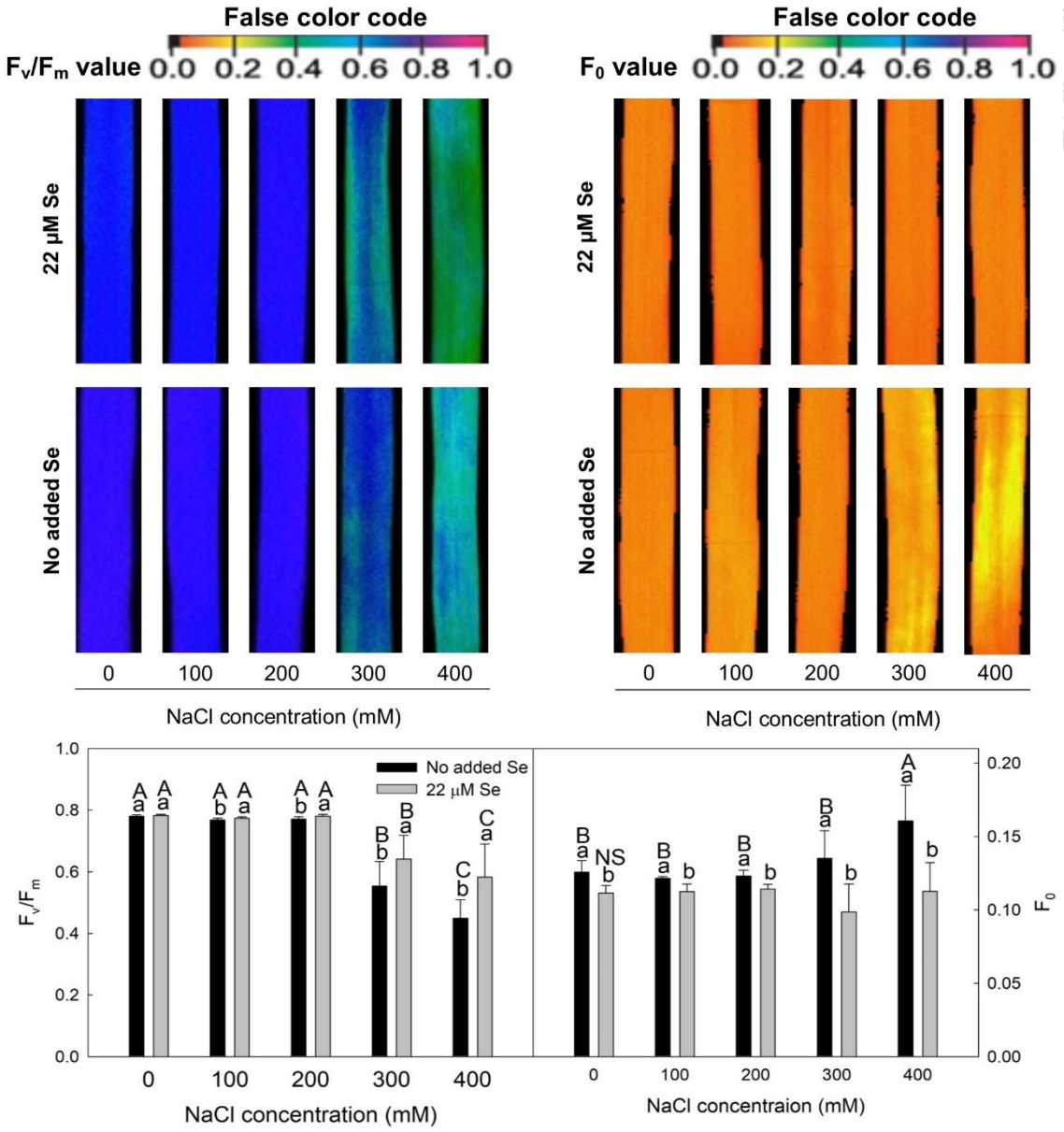

Figure 3-2. Images and the mean ± SD (n = 4) of the maximum quantum yield of photosynthetic system II (Fv/Fm) and the minimal fluorescence of photosynthetic system II (F_0) of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under different NaCl concentrations. The false color code depicted on the top of the images ranges from 0.0 (black) to 1.0 (purple). Values followed by different letters statistically significantly differ at $p < 0.05$. Means with different lowercase letters significantly differ by a *t*-test between Se treatments (No added Se and 22 μM Se) at the same NaCl concentration. Means with different capital letters significantly differ by an LSD test under different NaCl concentrations in separate untreated and Se-treated groups ($p < 0.05$).

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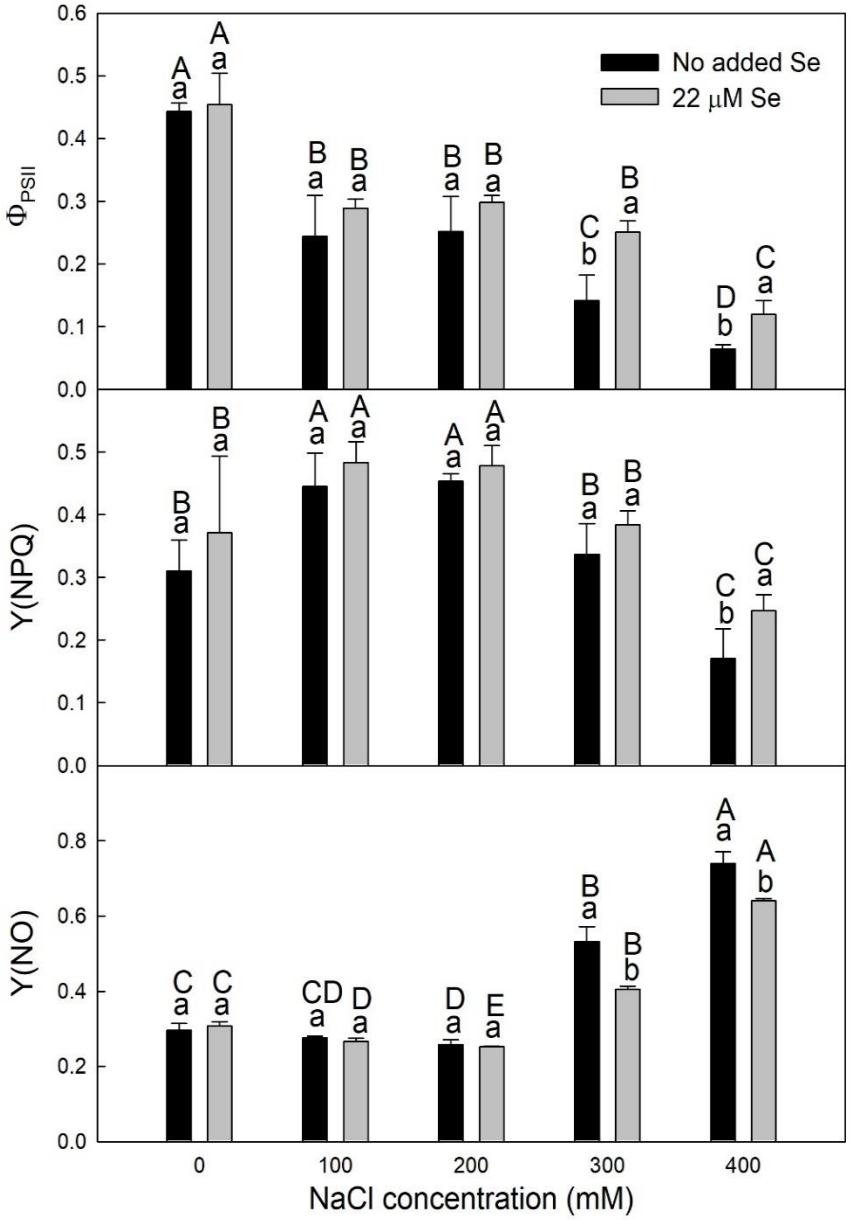

Figure 3-3. Means ± SD (n = 4) of the effective quantum yield of photosystem II under illumination (ΦPSII), quantum yield of regulated energy dissipation of photosystem II (Y(NPQ)), and the quantum yield of non-regulated energy dissipation of photosystem II (Y(NO)) of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under different NaCl concentrations. Values followed by different letters statistically significantly differ at $p < 0.05$. Means with different lowercase letters significantly differ by a *t*-test between Se treatments (No added Se and 22 μM Se) at the same NaCl concentration. Means with different capital letters significantly differ by an LSD test under the different NaCl concentrations in separate untreated and Se-treated groups ($p < 0.05$).

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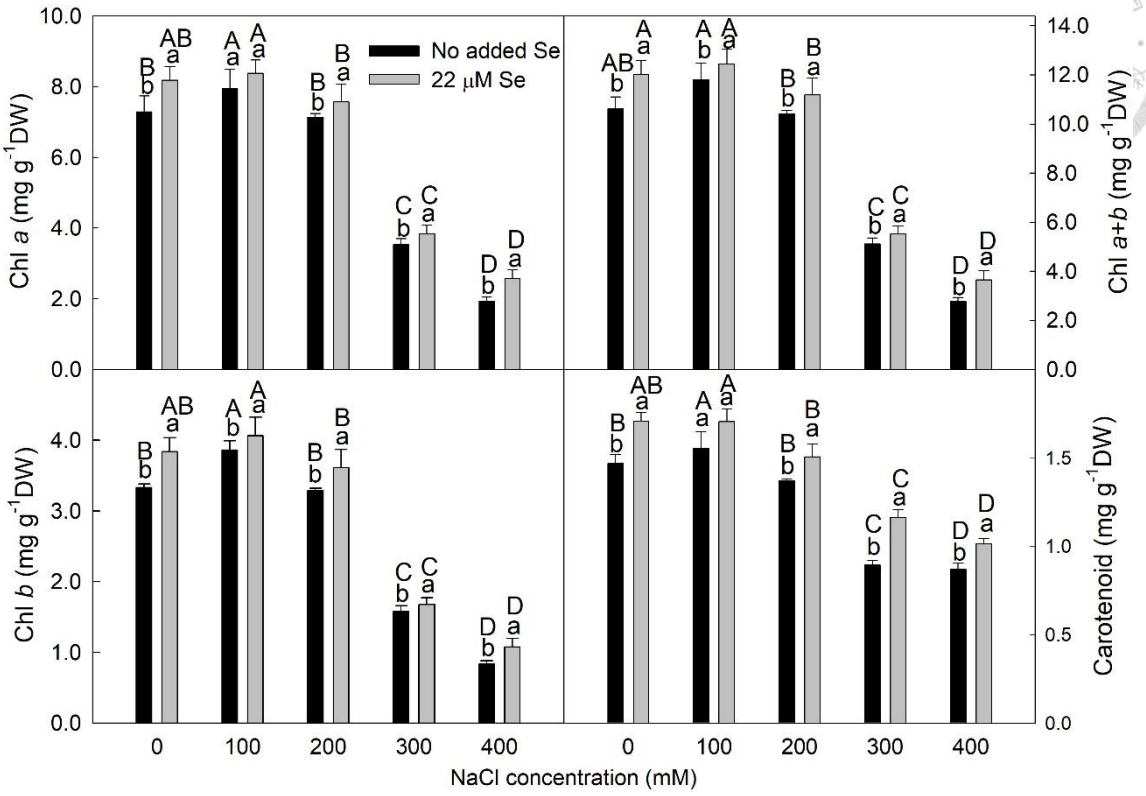

Figure 3-4. Means ± SD (n = 3) of chlorophyll (Chl) *a*, Chl *b* , Chl *a*+*b*, and carotenoids of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under different NaCl concentrations. Values followed by different letters statistically significantly differ at $p < 0.05$. Means with different lowercase letters significantly differ by a *t*-test between Se treatments (No added Se and 22 μM Se) at the same NaCl concentration. Means with different capital letters significantly differ by an LSD test under different NaCl concentrations in the untreated and Se-treated groups ($p < 0.05$). The concentration of Se treatment was 22 μM.

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Chapter 4. Comparison of the chlorophyll fluorescence and other physiological characteristics on Wheat seedlings influence by iso-osmotic stress of PEG and NaCl and their relative mitigation effect of Se

Lan CY, Lin KH, Chen CL, Huang WD, Chen CC (2020). Comparisons of chlorophyll fluorescence and physiological characteristics of wheat seedlings influenced by iso-osmotic stresses from polyethylene glycol and sodium chloride. Agronomy, 10(3): 325

Abstract

This study aimed to distinguish the effects of the osmotic and ionic effect caused by salt stress and to evaluate the contribution of 22 μM Se treatment on both effects on Wheat (*Triticum aestivum* L.) cultivar Taichung SEL.2. We hypothesized that TCS2 is more vulnerable to the osmotic stress according to its' salt-tolerance property and that 22 μM Se exert a beneficial effect to both stresses. In order to test the hypothesis, the osmotic agents of polyethylene glycol (PEG) and NaCl were used in three iso-osmotic concentrations with final osmotic potentials (OP) of -1.05 MPa (24% (w/v) PEG and 200 mM NaCl), -1.33 MPa (26.5% (w/v) PEG and 250 mM NaCl) and -1.57 MPa (29% (w/v) PEG and 300 mM NaCl). The more-stabilized chlorophyll fluorescence (ChlF) parameters (maximal quantum yield of phothsystem II (F_v/F_m), effective quantum yield of photosystem II(Φ_{PSII}), non-photochemical quenching of PSII (NPQ), and the less-

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extensive degradation of photosynthetic pigments (total chlorophyll and carotenoids) were observed under NaCl-treated seedlings. Ascorbate peroxidase (APX) activity was performed better and the less accumulation of malondialdehyde (MDA) were identified on NaCl-treated seedlings. The elongation of shoots of NaCl-treated seedling was also preserved on NaCl-treated seedlings. The results above supported the hypothesis that TCS2 is more vulnerable to osmotic stress than the toxic effect of ion under 3 levels of iso-osmotic concentrations created by PEG and NaCl. Nevertheless, contrary to our hypothesis, a disadvantageous effect of 22 μM Se had been observed between Se treatments (No added Se and 22 μM Se) in the ChlF performances of F_v/F_m, Φ_{PSII} and NPQ and APX activity, photosynthetic pigments, resulting in a higher accumulation of MDA concentration in seedlings treated with 22μM Se. The effective treatment concentration of Se required furthering experimentation.

Keywords: wheat; salt stress; osmotic stress; selenium; chlorophyll fluorescence; APX activity

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Introduction

Salinity has impacted wheat (*Triticum aestivum* L.) production around the world with increasing frequency and intensity. It was estimated that more than 69% of the total wheat production had been seriously influenced by high salinity (Isayenkov, 2012). Therefore, it is crucial to understand the mechanisms by which wheat perceive and adapt to salt stress so that the situation can be adequately improved.

Plant response to salinity at different time scales called a two-phase growth response (Munns and Sharp, 1993). The first phase was water stress phase or osmotic phase, which happened within minutes to hours, caused by the salt outside the roots inhibited the water absorption of plant, resulting in "physiological drought" (Filek *et al.*, 2012). The second phase was the toxic effects of ions, which takes days to progress (Munns, 2002). As a result, salt stress could trigger many common reactions in a plant similar to that of drought stress. Therefore, it is difficult to distinguish between the effect of drought and salt stress and their relative importance to plant. Indeed, what is the primary mechanism which governed a crop's ability to cope with salt stress? Is it the capacity of crop to confront drought, or the ability to against toxic effects of ions in salt stress?

There are many studies aimed to answer this question. In those studies, the iso-osmotic potential of NaCl has been most commonly generated by polyethylene glycol

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(PEG), a widely used osmotica to induce water stress in order to simulate low water potential due to dry soil (Almansouri *et al.*, 2001), as it is a non-ionic, neutral and water-soluble polymer which does not penetrate the roots (Sayar *et al.*, 2010). The results of those studies vary depending on tested species/varieties and the development stages. In the results of some studies, PEG was more harmful than NaCl treatments. It occurred in the cases of germination stage of durum wheat (Almansouri *et al*., 2001) and *Hordeum* species (Huang and Redmann, 1995), and on seedling stage in wheat (Filek *et al*., 2012; Sayar *et al*., 2010). However, Muranaka *et al.* (2002a, b) have revealed that NaCl treatments were more harmful than that of PEG treatments on the seedling stage of wheat. Some studies compared between sensitive and resistant varieties and reported that the salt-resistant varieties are more vulnerable to water deficits while 'ion excess' might prevailing over in the sensitive types (Greenway and Munns, 1980; Sharma *et al.*, 1984). The salinity resistance also varies with the development stage of the plant (Borsani *et al.*, 2001; Lutts *et al.*, 1995). In the study of Sayar *et al*. (2010), the seedling stage was more susceptible to NaCl than to PEG under iso-osmotic potential treatments than germination.

It is obvious that there are species/genotypes/stages-specific responses to salinity (Shannon, 1997). Crops/varieties/developmental stages suffered more in NaCl treatment when they are unable to prevent salt entry (gene-related) (Chaves *et al.*, 2009; Munns, 2005), or to compartmentalize the salt in cell vacuoles or lack of ability to prevent salt

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from reaching the toxic levels in leaves (Munns, 2002). However, if crops/genotypes/developmental stages could tolerance the resulting ion concentrations caused by NaCl treatment (Yeo, 1983), Na^+ and Cl^- may further serve as an osmoticum (Hsiao *et al.*, 1976) to produce a rapid osmotic adjustment (Prat and Fathi-Ettai, 1990) so that the shoot turgor could be maintained. The osmoregulation created by ionic NaCl was much faster as well as less energy and carbon-demanding than organic solutes involved in osmotic adjustment created by PEG (Pérez-Alfocea *et al.*, 1993), which is the main reason why PEG treatments were more harmful than those of NaCl treatments in some varieties.

To evaluate the relative influence of osmotic and ionic stress caused by salt stress, the biochemical, physiological and morphological responses can be applied to get insight to the mechanisms involved (Slama *et al.*, 2008). Basically, the enzymatic and non-enzymatic defense system and the photosynthetic system will be inhibited, the lipid peroxidation products will be increased, and the growth will be reduced (Zörb *et al.*, 2019). The biochemical analysis provides information regarding the process above. Nevertheless, it is time-consuming and requires complicated technical support. However, the chlorophyll fluorescence (ChlF) of PSII can provide effective physiological changes as a simple and rapid procedure (Sayar *et al.*, 2008; Siringam *et al.*, 2009). In this study, the measurements of ChlF were conducted for early identification, following by growth

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analysis and the measurements of biochemical (APX activity, lipid peroxidation product: malondialdehyde (MDA) and photosynthetic pigments) analysis.

Selenium (Se) is considered a beneficial element to plants (Pilon-Smits *et al*., 2009). Previous studies indicated that Se can delay senescence (Djanaguiraman *et al*., 2005; Xue *et. al*.,2001) and promote the vegetative and reproductive growth of plants (Hajiboland *et al*., 2012). Studies reported that Se mitigated disadvantageous phenomena caused by various stressful situations, such as drought (Xiaoqin *et al*., 2009; Nawaz *et al*., 2015), and salt stress (Mona *et al*., 2017; Diao *et al*., 2014). Therefore, a positive effect of Se related to the effects of osmotic and ionic effect caused by salt stress was expected.

Wheat (*Triticum aestivum* L.) cultivar Taichung SEL. 2 (TCS2), one of the most widely cultivated cultivars in Taiwan, appeared to be a salt-tolerant cultivar (Lan *et al.*, 2019). However, the mechanisms that governed its ability to cope with such stress remained unclear. Hence, the purpose of this study was to understand the biochemical and physiological responses of TCS2 under iso-osmotic potentials created by PEG and NaCl in order to distinguish its abilities and mechanisms to cope with salt stress. We hypothesize that TCS2 is more vulnerable to osmotic stress than the toxic effect of ion under 3 levels of iso-osmotic concentrations created by PEG and NaCl and that 22 μM Se exert a beneficial effect to both stresses.

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Materials and methods

Plant and growth conditions

Wheat (*Triticum aestivum* L.) cultivar Taichung SEL. 2 (TCS2), one of the most widely cultivated wheat cultivars in Taiwan, was used in this study. Seeds used in the present study were obtained from the Department of Agronomy, National Taiwan University (Taipei, Taiwan). The seeds were sterilized with 1% hydrogen peroxide for 5 min, washed with distilled water, and germinated in Petri dishes on wetted filter paper at 25 °C in the dark. After 24 h of incubation, uniformly germinated seeds were selected and cultivated in 150 mL beakers containing complete Hoagland's nutrient solution (Hoagland and Arnon, 1950), which was replaced every 3 days. Hydroponically cultivated wheat seedlings were raised in growth chambers with fluorescent lamp lighting at 25 and 20 °C during the day and night, respectively, under a 12 h photoperiod. The photosynthetic photon flux density (PPFD) was uniformly set to 300 μmol m^{-2} s^{-1}.

Experimental treatments

Se was added in the form of Sodium selenite (Na_2SeO_3, Sigma-Aldrich Chemie GmbH, Taufkirchen, BY, Germany) to the nutrient solution (pH = 4.6) with the treated

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concentration of 22 μM once the germinated seeds were cultivated in 150 mL beakers. No treatment (Control) consisted of the nutrient solution without Se supplementation. Hydroponically grown seedlings that had reached stage Z1.0 (Zadoks *et al.*, 1974) on day 6 were treated with the osmotic agents of polyethylene glycol (PEG-6000, Merck) and NaCl (Sigma). These osmotic agents were used in three iso-osmotic concentrations based on the treatments of Almansouri *et al*. (2001) with final osmotic potentials of -1.05 MPa (24% (w/v) PEG and 200 mM NaCl), -1.33 MPa (26.5% (w/v) PEG and 250 mM NaCl) and -1.57 MPa (29% (w/v) PEG and 300 mM NaCl). The experiment was independently performed three times for a randomized design of growth conditions.

Growth analysis

Shoot height and root length were measured with a ruler before the measurements of chlorophyll fluorescence (ChlF) and sample collection.

Measurements of ChlF

Fluorescence parameters in seedling leaves were determined after PEG and salt treatments. ChlF was measured in the middle portion of the first leaf of each seedling

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taken at ambient temperature with Chl fluorometer imaging-PAM (Walz, Effeltrich, Germany). Actinic light and saturating light intensities were set to 185 and 7200 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), respectively. The minimal (F_0) and maximal (F_m) ChlF, the maximum quantum yield of PSII (F_v/F_m), the effective quantum yield of PSII (Φ_{PSII}) and the non-photochemical quenching of PSII (NPQ) were measured and calculated according to previously described methods (Kramer *et al.*, 2004; Van and Snel, 1990).

Measurement of APX activities

APX activities were measured base on the method of Nakano and Asada (1981). Briefly, 0.1 g of the latest newly expanded leaf was placed in 2 mL of sodium phosphate buffer (50 mM, pH 6.8) in an ice bath for extraction and centrifuged at 4°C and 12,000 rpm for 20 min. The supernatant (0.1 mL) was collected, followed by the sequential addition of 2.7 mL of potassium phosphate buffer (150 mM, pH 7.0), 0.4 mL of ethylenediaminetetraacetic acid (EDTA, 0.75 mM), 0.5 mL of H_2O_2 (6 mM), 0.5 mL of H_2O, and 0.5 mL of ascorbate (1.5 mM) and then mixed well. The absorbance at 290 nm of the sample solution was determined every 15 s for 1 min using a spectrophotometer

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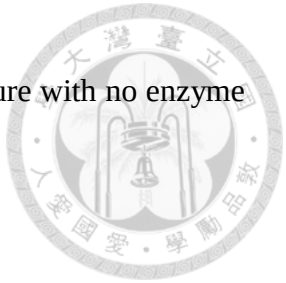
(Hitachi U3010, Tokyo, Japan). The blank containing the same mixture with no enzyme extract was also measured.



Determination of the photosynthetic pigment concentrations

The photosynthetic pigment concentrations were determined using the method of Yang *et al.* (1998). Briefly, 0.01 g of lyophilized sample powder was extracted with 12 mL of an 80% acetone solution, and then centrifuged at 4500 rpm for 5 min. The supernatant of the sample extract was tested to determine the absorbance extents of Chl *a*, Chl *b*, and carotenoids (Car) in acetone at 663.6, 646.6, and 440.5 nm, respectively.

Determination of the MDA concentration

MDA was determined using a previously described method of Heath and Packer (1968). Briefly, lyophilized sample powder (0.03 g) was mixed with 1 mL of 5% TCA, and then centrifuged at 10,000 rpm and 20 ˚C for 5 min. The supernatant (250 μL) was added to 1 mL of 0.5% thiobarbituric acid (TBA) which was made up with 20% TCA. The mixture was placed in a water bath at 95˚C for 30 min, and then immediately cooled in an ice bath. The reaction mixture was centrifuged at 3000 rpm and 20˚C for 10 min,

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and the absorbance was determined at 532 and 600 nm. The blank was the same reaction mixture with no sample extract.

Statistical analyses

All measurements were evaluated for significance using an analysis of variance (ANOVA) followed by a least significant difference (LSD) test and *t* - test at $p < 0.05$. All statistical analyses were conducted using R i386 3.5.1 software (https://cran.r-project.org/bin/windows).

Results

Growth analysis

There was no significant difference between Se treatments (No added Se and 22 μM Se) in all levels of three corresponding iso-osmotic potentials creating by PEG & NaCl in the performance of shoot height (Fig. 4-1A). Therefore, the results without Se treatment were further discussed in the following content (Fig. 4-1B).

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Shoot heights of seedling with PEG treatment declined dramatically from 24.3 to 12.6 cm with treatment concentration of 24% (w/v) PEG and then decreased gradually to 11.6 cm with the final treatment concentration of 29% (w/v) PEG. A similar declining trend was observed in NaCl-treated seedlings. However, NaCl-treated seedlings were always significantly longer ($p < 0.05$) than those of PEG-treated seedlings under iso-osmotic potential treatments.

ChlF

A disadvantageous effect of 22 μM Se had been observed between Se treatments (No added Se and 22 μM Se) in the ChlF performances of F_v/F_m (Fig. 4-2A), Φ_{PSII} and NPQ (Fig. 4-3A) , which was contrary to our hypothesis. Therefore, the results without Se treatment were further discussed in the following content (Fig. 4-2B; Fig. 4-3B).

F_v/F_m and Φ_{PSII} in leaves, determined after dark adaption and under illumination, are the indexes of the maximum and effective quantum yield of PSII, respectively. They are widely used to estimate the status of plant under stress (Sun *et al.*, 2006). F_v/F_m in leaves of seedlings with PEG-treatment declined dramatically with the increasing PEG concentrations from 0.781 to 0.019 ($p < 0.05$). However, F_v/F_m in leaves of seedlings with NaCl-treatment maintained stable until the NaCl concentration exceeded 250 mM. As a

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result, F_v/F_m valves in leaves of NaCl-treated seedlings were always significantly higher ($p < 0.05$) than those values in leaves of PEG-treated seedlings under iso-osmotic potential treatments ($p < 0.05$) (Fig. 4-2B). Φ_{PSII} in leaves of seedlings with PEG-treatment declined with the increasing PEG concentrations from 0.526 to 0.013 ($p < 0.05$). Φ_{PSII} in leaves of seedlings with NaCl-treatment showed the same trend with those of PEG-treated seedlings ($p < 0.05$), with NaCl-treated seedlings significant higher than that of PEG-treated seedlings under the highest osmotic potentials of -1.57 Mpa ($p < 0.05$).

NPQ represents the non-photochemical quenching of PSII. NPQ in leaves of seedlings with PEG-treatment declined dramatically with the increasing PEG concentrations from 0.22 to 0.00 ($p < 0.05$), while NPQ in leaves of seedlings with NaCl-treatment increased dramatically in 200 mM than gradually decreased to the similar value of CT. NPQ valves in leaves of NaCl-treated seedlings were always significantly higher ($p < 0.05$) than those values in leaves of PEG-treated seedlings under iso-osmotic potential treatments ($p < 0.05$) (Fig. 4-3B).

APX activities

A disadvantageous effect of 22 μM Se had been observed between Se treatments (No added Se and 22 μM Se) in APX activity (Fig. 4-4A), which was contrary to our

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hypothesis. Therefore, the results without Se treatment were further discussed in the following content (Fig. 4-4B).

The APX activities of PEG and NaCl-treated seedling both showed descending trends with significant higher activities in NaCl-treated seedling than those of PEG-treated seedlings under the same iso-osmotic potential ($p < 0.05$) (Fig. 4-4B).

Photosynthetic pigments

A disadvantageous effect of 22 μM Se had been observed between Se treatments (No added Se and 22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl in photosynthetic pigments (Fig. 4-5A) , which was contrary to our hypothesis. Therefore, the results without Se treatment were further discussed in the following content (Fig. 4-5B).

The Chl *a*, *b*, *a*+*b* concentrations of PEG and NaCl-treated seedling all showed descending trends with significant higher concentraions in NaCl-treated seedling than those of PEG-treated seedlings under the same iso-osmotic potential ($p < 0.05$) (Fig. 4-5B). The concentration of Car decreased significantly once PEG and NaCl were applied to seedlings, but there was no significant difference with an increase in the PEG and NaCl treatment concentrations. Nevertheless, the values of NaCl-treated seedlings were always

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significantly higher ($p < 0.05$) than those of PEG-treated seedlings under iso-osmotic potential treatments (Fig. 4-5B).

MDA concentration

Seedlings with 22 μM Se treatment accumulated more MDA concentration than the seedlings without Se treatment under three corresponding iso-osmotic potential creating by PEG & NaCl (Fig. 4-6A), which was contrary to our hypothesis. Therefore, the results without Se treatment were further discussed in the following content (Fig. 4-6B).

MDA concentration of seedlings with PEG treatments increased dramatically from 13.5 nmol g^{-1} DW (CT) to 116.7 nmol g^{-1} DW (29% (w/v) PEG) ($p < 0.05$), while the MDA concentration in NaCl-treated seedlings were just increase slightly from 13.5 nmol g^{-1} DW (CT) to 33.2 nmol g^{-1} DW (300 mM NaCl) with no significant difference between NaCl treatments. The MDA concentration of PEG-treated seedlings were always significantly higher ($p < 0.05$) than those of NaCl-treated seedlings under iso-osmotic potential treatments (Fig. 4-6B).

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Discussion

A disadvantageous effect of 22 μM Se had been observed between Se treatments (No added Se and 22 μM Se) in the ChlF performances of F_v/F_m (Fig. 4-2A), Φ_{PSII} & NPQ (Fig. 4-3A), APX activity (Figure 4-4A) and photosynthetic pigments (Figure 4-5A), resulted in a higher accumulation of MDA concentration in seedlings treated with 22μM Se (Figure 4-6A).

Although a beneficial micronutrient, Se exerts a dual effect in plants (Djanaguiraman *et al*., 2005): It can stimulate plant growth and provide beneficial effects at low concentration, but it is harmful to plants at higher concentrations. Positive effects of Se depend on its form, dose and the chosen plant genotype (Sieprawska *et al*., 2015). According to the results of our preliminary study for wheat (*T. aestivum* L.) cultivar Taichung SEL. 2, the growth rate of wheat seedlings was strongly retarded at 10 mg Se L^{-1} (294 μM), but was not promoted at 0.5 mg Se L^{-1} (14.7 μM). Se at 1 mg L^{-1} (29 μM) might exceed the plant's threshold, resulting in a slightly disadvantageous effect on the plant. Therefore, 0.75 mg Se L^{-1} (22 μM) was an appropriate concentration for Se treatment in this study. In Se & Salt exp. (chapter 3), the protective effect of 22 μM Se on the growth and physiological traits of wheat seedlings under salt stress had been proved. However, the toxic effect of 22 μM Se was observed in Se & Salt/PEG exp. (chapter 4).

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It is obvious that, apart from its form and dose, some other environmental factors may also contribute to the effects of Se. In fact, the experiments of Se & Salt exp. (chapter 3) and Se & Salt/PEG exp. (chapter 4) were conducted in different growth chamber. The seedlings of Se & Salt exp. (chapter 3) were incubated in the growth chamber 1 while the seedlings of Se & Salt exp. (chapter 4) were grown in the growth chamber 2. The conflicting results may be due to differences in the light source or microenvironment conditions between growth chambers 1 and 2. Therefore, a further experiment was needed to confirm the effective treatment concentration of Se.

Contrary to our hypothesis, a disadvantageous effect of 22 μM Se has been observed between Se treatments (No added Se and 22 μM Se). Therefore, the following discussion was focusing on the results of "No added Se" treatments.

In order to identify the relative influences between water deficits and ion toxic effects resulting from salt stress, comparisons between three levels of iso-osmotic concentrations of PEG and NaCl treatments have been done. The treatment concentrations of PEG and NaCl were chosen according to visual symptoms of osmotic and ion-toxic effect on wheat leaves. The noticeable sigh of the water deficits are leaf wilting and rolling due to stomatal closure created by PEG (Filek *et al*., 2012), while the ion excess symptom resulting from NaCl treatments are leaf yellowing or death of older leaves (Munns, 2002). The treatment

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period was set to allow the water relations and toxic effects of ions symptoms becoming apparent visible while causing no irreversible damage to wheat (Filek *et al*., 2012). The ChlF of PSII was than measured as an early identification.

The F_v/F_m, NPQ, Y(NPQ) and Y(NO) values were statistically more influenced by osmotic stress created by PEG than by NaCl treatment in all levels of iso-osmotic concentrations, while Φ_{PSII} values also showed the same trend in the highest iso-osmotic concentration of -1.57MPa. (Fig. 1B, 2B) The result of ChlF measurements indicating that the PSII efficiency were significantly affect and damage by the induced water stress. Drought and salt stress affect photosynthesis either by diffusion limitations through the stomata (stomatal closure) or by the influence of photosynthetic metabolism (Chaves *et al*., 2009; Muranaka *et al*., 2002a). It may be highly related with the osmoregulation process under drought and salt stress (Prat and Fathi-Ettai, 1990). Under osmotic stress, the osmotic adjustment was accomplished by synthesizing organic compatible solutes such as organic acids, amino acids, sugars and polyols (Hellebusi, 1976; Sayar *et al*., 2010), which take time to build up (Hsiao *et al*., 1976). Whereas under salt stress, turgor could be maintained by uptake of ions (Na^+ and Cl^-) (Sharma *et al*., 1984), and the cost is much lower than the ATP needed to synthesize organic solute (Munns, 2002). It might also be the results that the treatment time was not long enough to allow the organic solutes to build up. Φ_{PSII} are the effective quantum yield of PSII under illumination. The

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indifference values of Φ_{PSII} between iso-osmotic concentrations of PEG and NaCl in -0.58 and -1.33Mpa indicated that the capability of PSII under the absorbed irradiance of 185 μmol m^{-2} s^{-1} of PAR in the photochemical reaction were similar. The result was supported by Muranaka *et al*. (2002a) and Flagella *et al.* (1998) who had discovered that Φ_{PSII} is only influenced under extreme water deficit. The results of the ChlF parameters in our study showed that it can be used as potent indices to screen salt or drought-tolerance species.

Photosynthetic pigments (Chl *a*, Chl *b* and Car), also called light-harvest pigments, are essential for photosynthesis mechanism (Siringam *et al*., 2009). In addition, Car can also absorb excessive light, protected chlorophyll from excessive light-loading and damage, which is called photoprotection (Siefermann-Harms, 1987). Chl *a*, Chl *b* and Chl *a*+*b* are all sharply dropped on PEG and salt-treated seedlings with an increase in treating concentrations. However, values of Chl *a* , Chla *b* and their sum were statistically more influenced by osmotic stress created by PEG than by NaCl treatment in all levels of iso-osmotic concentrations ($p < 0.05$) (Fig. 4-5B), suggesting that the damage level was more severe at PEG-treated seedling. As for Car, the PEG and NaCl would strongly inhibit Car concentrations, even though there were no significant difference under decreasing levels of PEG or salt treatments, separately. PEG-treatments reduced Car concentrations significantly than that of NaCl-treated seedling, indicating that the photoprotection

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provided by NaCl-treated seedling were better than that of PEG-treated seedlings (Fig.4-5B). The results of Chl *a*, *b*, *a*+*b* and Car were in agreement with the results of ChlF. Our results were consistent with a study by Siringam *et al*. (2009), revealing that Chl *a* and total Chl were positively related to ChlF values. The opposite results could be found that ionic toxicity of salt stress resulting in more Chl degradation than water deficit effect (Suriyan and Chalermpol, 2009), which indicated that the mechanisms involved are highly species depend.

There are many enzymes which involved in the enzymatic antioxidant system. However, enzymes that required K^+ as a cofactor are more sensitive to high salinity (Chaves *et al*., 2009). One of them is ascorbate peroxidase (APX) which participating in ascorbate-glutathione pathway (Wang *et al.*, 2009), as it has a K^+ binding site essential for APX activity (Pandey *et al.*, 2017). The increasing Na^+ concentration in NaCl-affected plant will displaces K^+ from essential binding sites resulting in the inhibition of APX enzyme activity. However, in our study, APX activities were statistically more influenced by osmotic stress created by PEG rather than by NaCl treatment in all levels of iso-osmotic concentrations. This phenomenon indicated that as a salt-resistant variety, TCS2 are able to prevent salt from reaching the toxic levels in leaves, either by prevented salt entry, compartmentalized the salt in cell vacuoles, or some other mechanism involved. (Chaves *et al*., 2009; Munns, 2002, 2005). However, it could still allow little ions to entry

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to maintain the turgor pressure in NaCl-treated seedlings, while APX activities were more inhibited by PEG-treated seedlings because they could not maintain the turgor pressure properly under osmotic stress (Fig. 4-4B).

The free radicals induced by abiotic stress are prone to attack membrane lipids, resulting in the accumulation of MDA, a product of lipid peroxidation, has been regarded as a good indicator of oxidative stress (Pinto *et al.*, 2016). The result of MDA measurement in this study indicating that PEG-treatments would induced serious lipid peroxidation with an increase in the PEG concentration, indicating that seedlings were severely injured in oxidative stress caused by PEG treatment (Fig. 4-6B). However, the MDA concentrations remained stable under all levels of NaCl treatments, suggestion that TCS2 was able to control the lipid peroxidation even under acute salt-stress. Overall, the PEG-treated seedling resulted in significantly higher MDA level than the NaCl-treated seedling reflects that the status of osmotic-induced oxidative stress was much greater than that of toxic effect of ion. It might have been associated with higher efficiency of ChlF, higher Chl *a*, *b*, *a*+*b* and Car concentrations and higher APX activity in NaCl-treated seedlings.

The shoot height were statistically more influenced by osmotic stress created by PEG than by NaCl treatment in all levels of iso-osmotic concentrations. Cell-division

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enlargement is very important for the growth of plant, which are highly affected by water stress (Shao *et al.*, 2008). PEG treatments affected the maintenance of turgor resulting in the poor performance of shoot height. Nevertheless, the higher shoot height were observed in salt-treated seedlings because salt treatments could provide Na^+ and Cl^- as osmotica to reduce the osmotic stress in NaCl-treated seedlings (Huang and Redmann, 1995).

In general, there is no doubt that Wheat (*Triticum aestivum* L.) cultivar Taichung SEL. 2 (TCS2) appeared not to be a good water saver. TCS2 grown under PEG had visual symptoms of wilting revealing their disability to absorb water. However, they could remained turgid under NaCl treatments indicated their ability to cope with excess ion stress caused by NaCl applications. The phenomena above were supported by the results of ChlF, photosynthetic pigments, APX activity, MDA concentration and shoot length presented in the study. These results can contribute to breeder who are interested in breeding drought and/or tolerant wheat varieties.

Conclusion

The results of this studies supported our hypothesis that Wheat (*Triticum aestivum* L.) cultivar Taichung SEL. 2 (TCS2) was more vulnerable to the osmotic stress than the toxic

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effect of ion under 3 levels of iso-osmotic concentrations created by PEG and NaCl. As a salt-tolerance variety, NaCl-treated TCS2 seedlings showed higher ability to maintain the efficiency on ChlF and APX activity, preventing the photosynthetic pigments from degrading. Therefore, the accumulation of MDA concentrations was inhibited, resulting in better performance in shoot height seedling growth. However, a disadvantageous effect of 22 μM Se had been observed between Se treatments (No added Se and 22 μM Se). A further experiment was needed to confirm the effective treatment concentration of Se.

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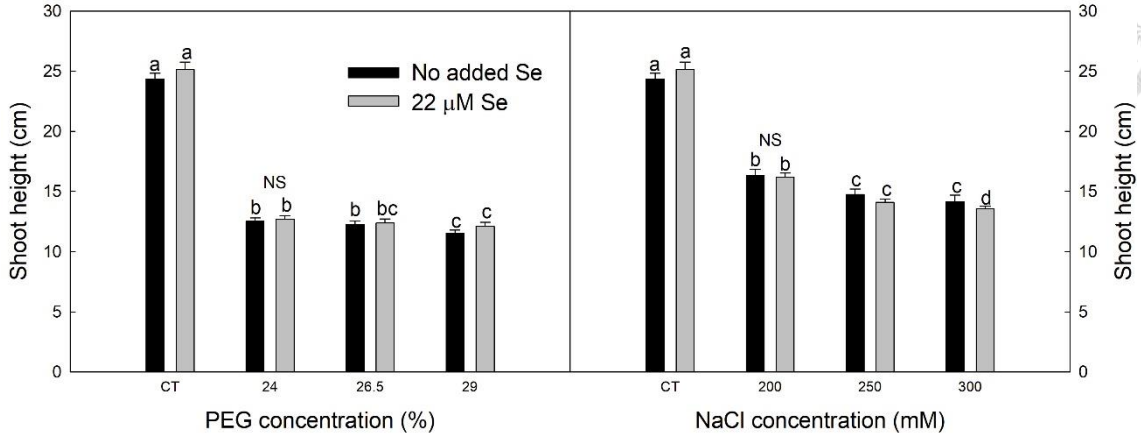

Figure 4-1A. Means ± SD (n=5) of shoot height of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl) . Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under the same osmotic potential.

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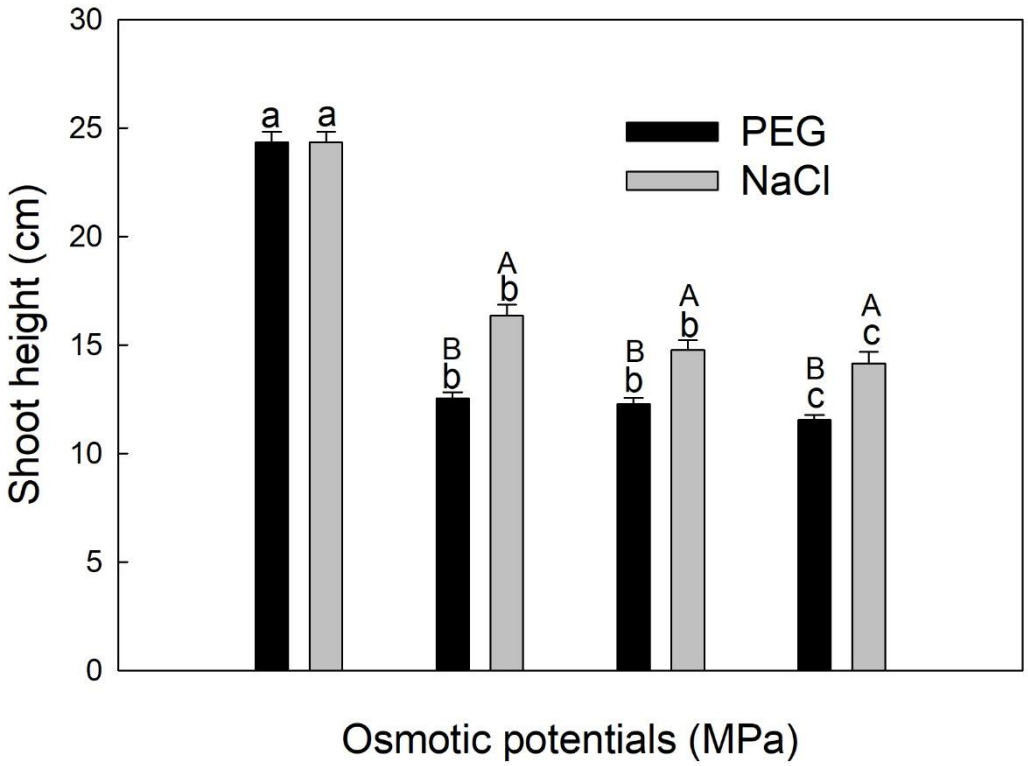

Figure 4-1B. Means ± SD (n=5) of shoot height of leaves under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl) . Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between PEG and NaCl concentration under the same osmotic potential.

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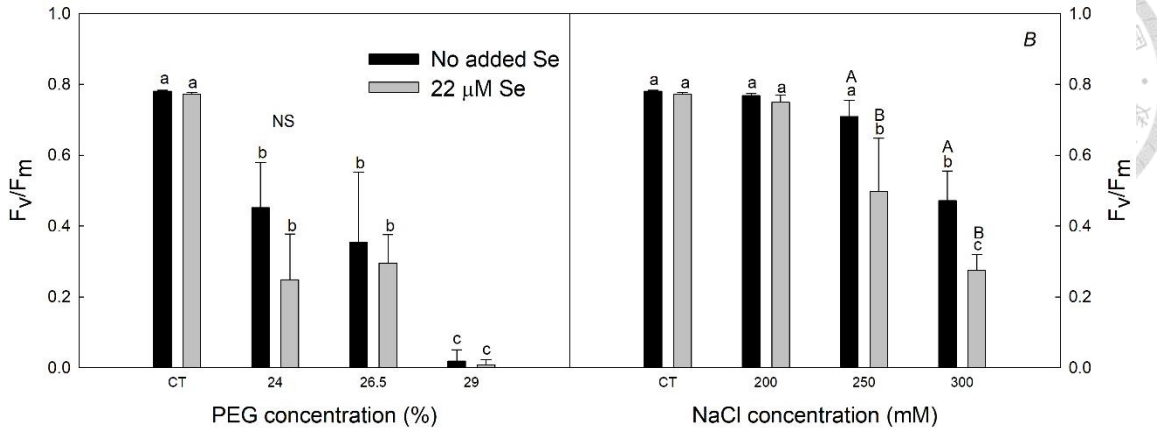

Figure 4-2A. the mean ± SD (n=3) of the maximum quantum yield of photosynthetic system II (Fv/Fm) of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). The false color code depicted on the top of the images ranges from 0.0 (black) to 1.0 (purple). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (*p*–value < 0.05). Means with different capital letters significantly differ by a *t*-test between untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under the same osmotic potential.

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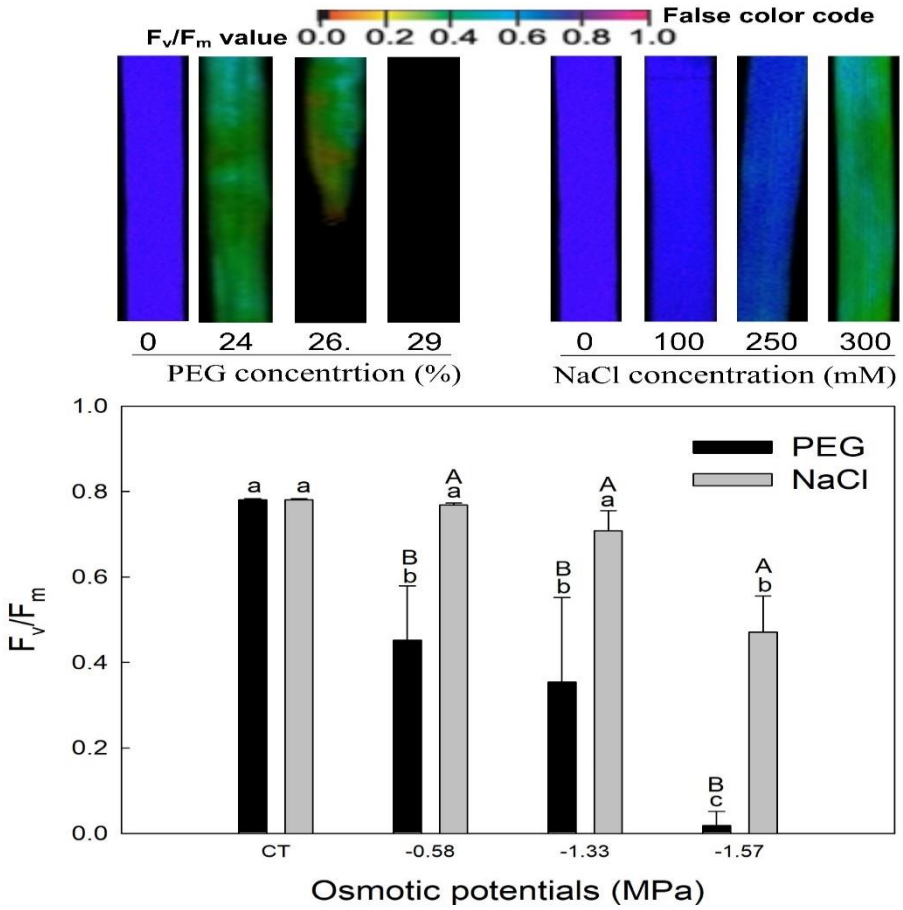

Figure 4-2B. Images and the mean ± SD (n=3) of the maximum quantum yield of photosynthetic system II (Fv/Fm) of leaves collected under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). The false color code depicted on the top of the images ranges from 0.0 (black) to 1.0 (purple). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between PEG and NaCl concentration under the same osmotic potential.

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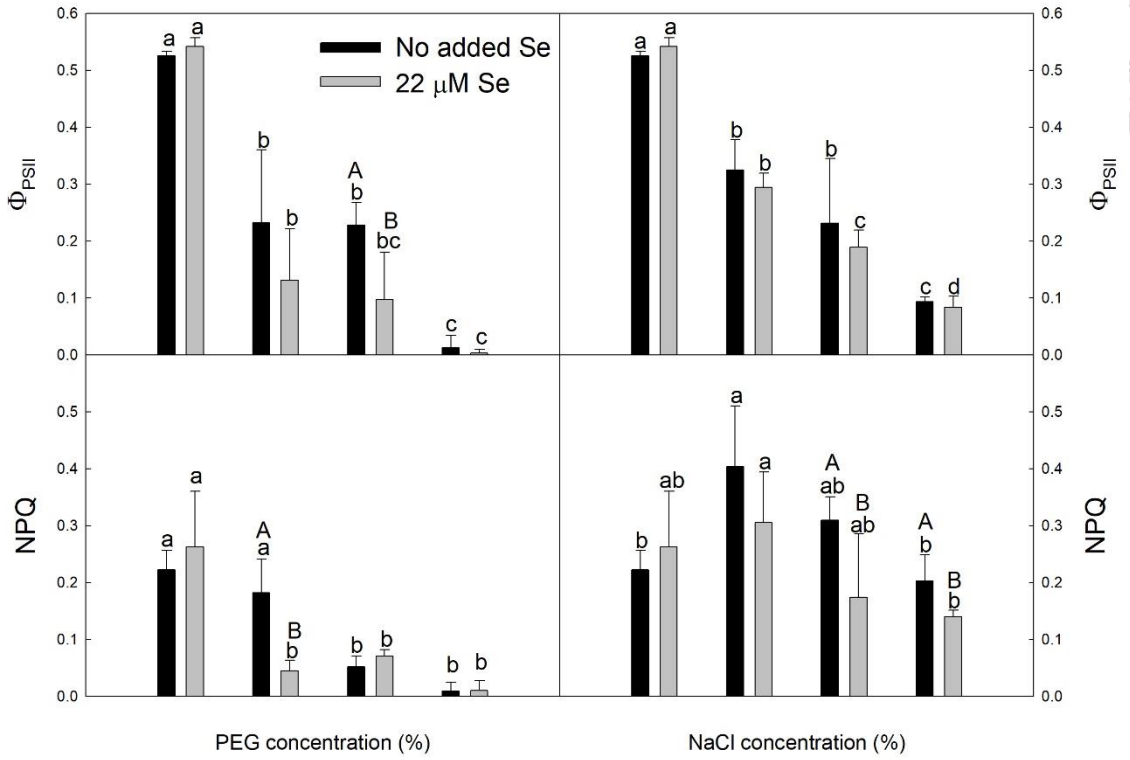

Figure 4-3A. Means ± SD (n=3) of the effective quantum yield of photosystem II under illumination (ΦPSII), non-photochemical quenching (NPQ) of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between PEG and NaCl concentration under the same osmotic potential.

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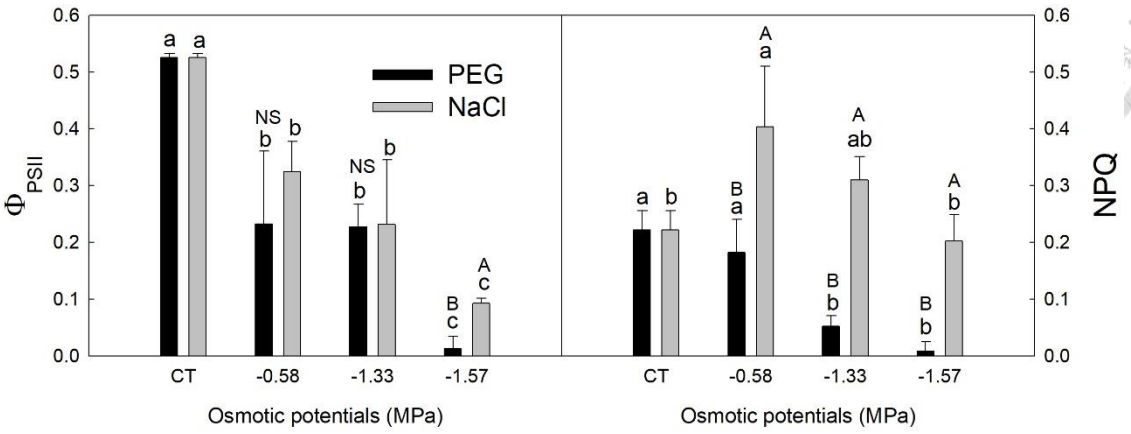

Figure 4-3B. Means ± SD (n=3) of the effective quantum yield of photosystem II under illumination (ΦPSII), non-photochemical quenching (NPQ) of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under the same osmotic potential.

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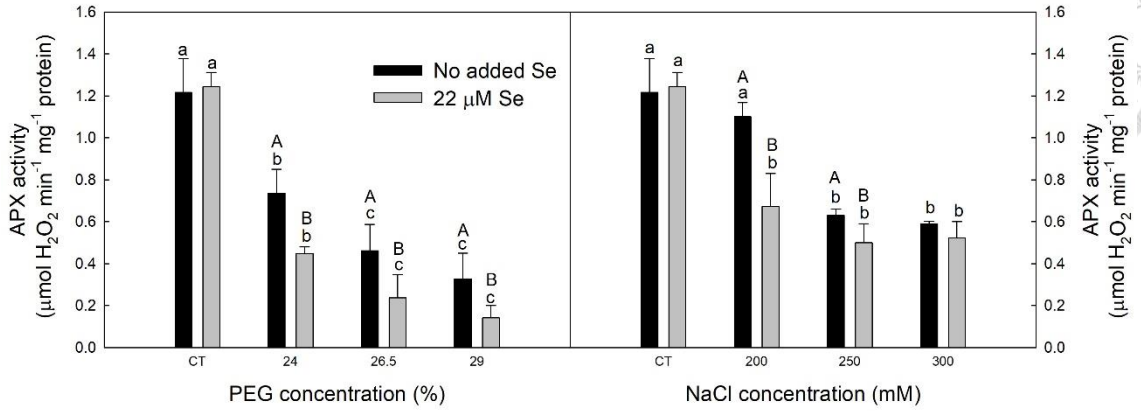

Figure 4-4A. Means ± SD (n=3) of APX activity of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under the same osmotic potential.

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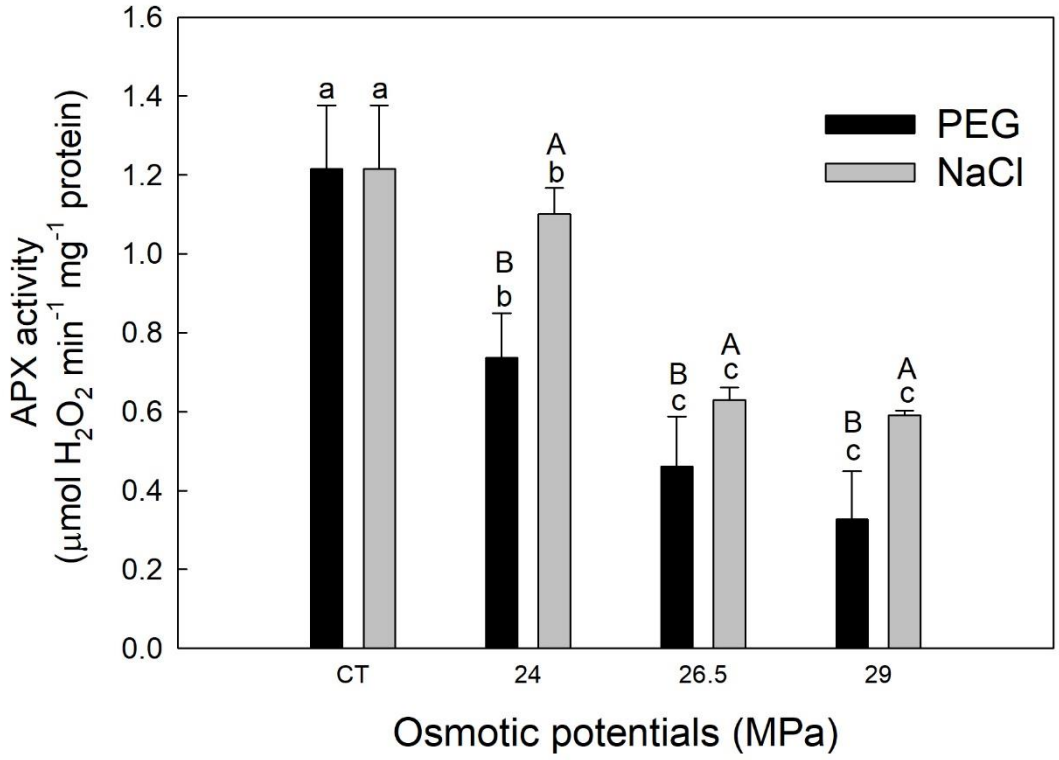

Fig 4-4B. Means ± SD (n=3) of APX activity of leaves collected from the seedlings treated with three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a t-test between PEG and NaCl concentration under the same osmotic potential.

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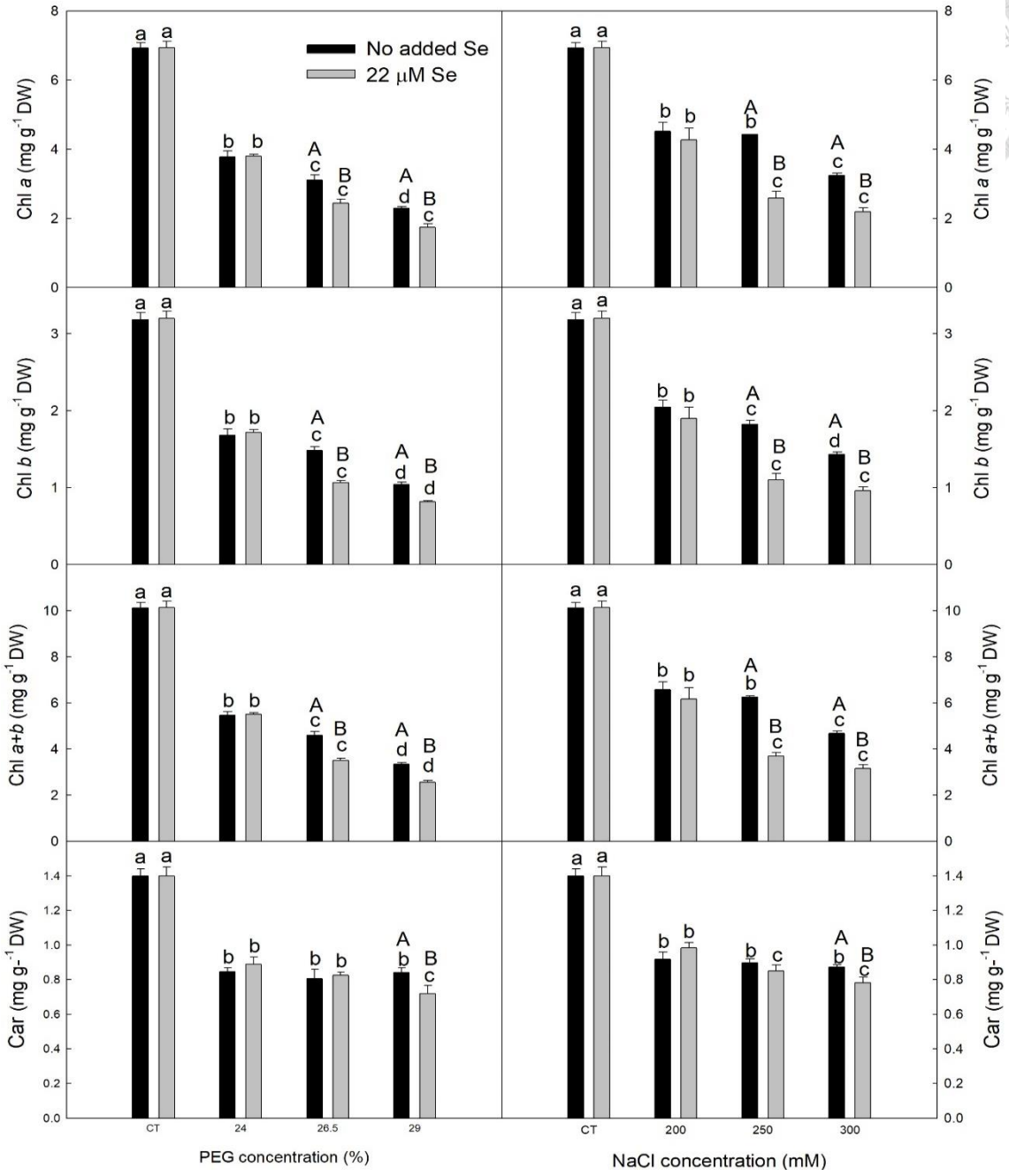

Figure 4-5A. Means ± SD of chlorophyll (Chl *a*, Chl *b*, Chl *a*+*b*) and carotenoids of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Values are the mean ± SD (n=3). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a *t*-test between untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under the same osmotic potential. OP, osmotic potential. CT, Control. Chl, chlorophyll. Car, carotenoids.

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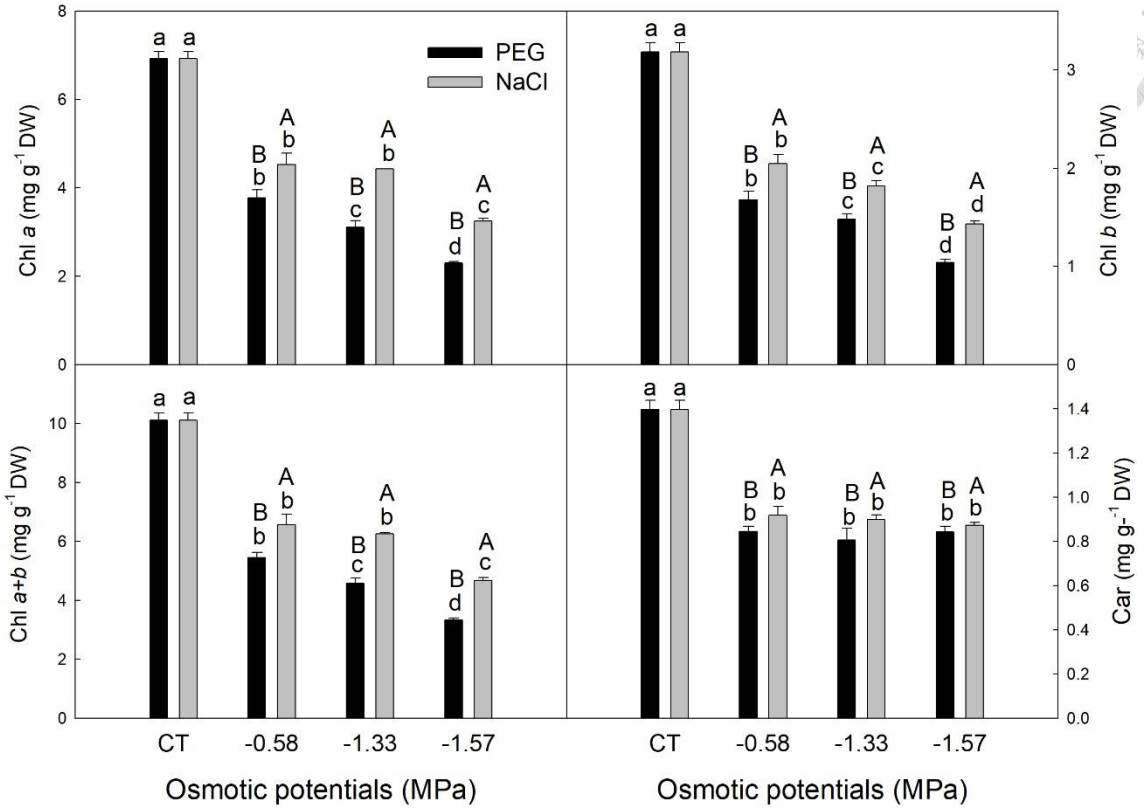

Figure 4-5B. Means ± SD of chlorophyll (Chl *a*, Chl *b*, Chl *a*+*b*) and carotenoids of leaves collected from three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Values are the mean ± SD (n=3). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a *t*-test between PEG and NaCl concentration under the same osmotic potential. OP, osmotic potential. CT, Control. Chl, chlorophyll. Car, carotenoids.

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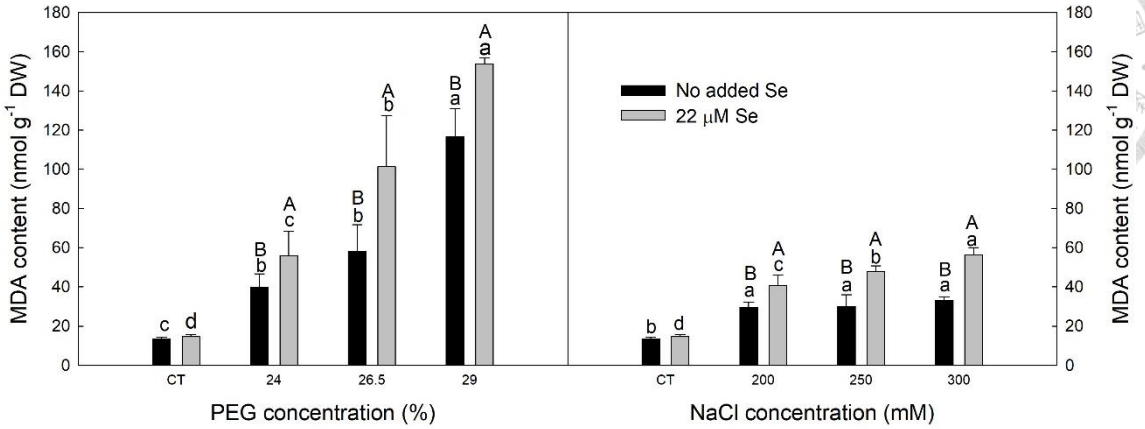

Figure 4-6A. Means ± SD (n=3) of Malondialdehyde (MDA) concentration of leaves collected from untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (*p*–value < 0.05). Means with different capital letters significantly differ by a *t*-test between untreated seedlings (No added Se) and Se-treated seedlings (22 μM Se) under the same osmotic potential.

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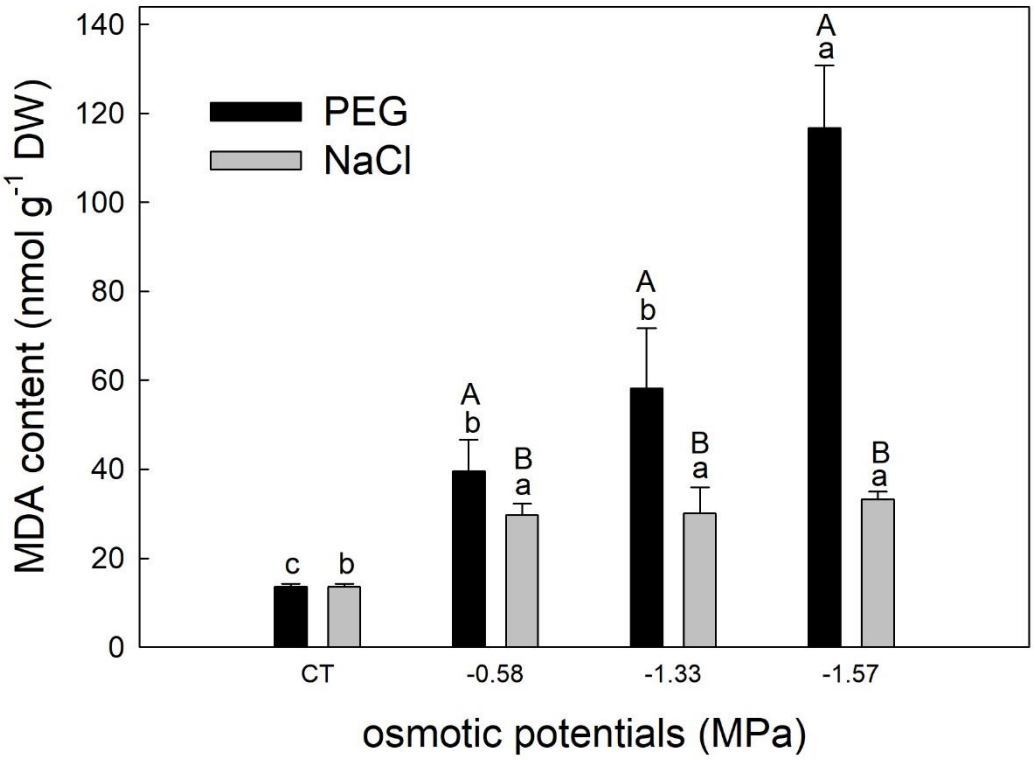

Fig 4-6B. Means ± SD (n=3) of Malondialdehyde (MDA) concentration of leaves collected from the seedlings treated with three corresponding iso-osmotic potential creating by PEG & NaCl (-0.58 Mpa: 24% (w/v) PEG, 200 mM NaCl; -1.33 Mpa: 26.5% (w/v) PEG, 250 mM NaCl; -1.57 MPa: 29% (w/v) PEG, 300 mM NaCl). Means with different lowercase letters significantly differ by an LSD test under the different PEG or NaCl concentration respectively (p–value < 0.05). Means with different capital letters significantly differ by a *t*-test between PEG and NaCl concentration under the same osmotic potential.

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Chapter 5. Conclusion

In this study, the functions and the effects of AZ and Se on wheat seedlings cultivar 'Taichung SEL.2' (TCS2) were investigated. In AZ exp., we proved that AZ provided limited support for the physiological functioning of TCS2 under Heat stress. In Se & salt exp., our results suggested that TCS2 cultivated with 22 μM Se treatment could prevent salt stress-induced disadvantageous effects. However, In Se & salt/PEG exp., a disadvantageous effect of 22 μM Se was observed. Nevertheless, we found out that TCS2 was more vulnerable to the osmotic stress than the toxic effect of ion under 3 levels of iso-osmotic concentrations created by PEG and NaCl. The results of this study provided a better understanding of the physiological mechanisms of AZ and Se on Wheat (*Triticum aestivum* L.) cultivar 'Taichung SEL.2' (TCS2) under heat and salt stress, respectively. Moreover, this result could also contribute to breeders who are interested in breeding drought or salt tolerant wheat varieties.

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