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EFFECTS OF PARAQUAT-INDUCED OXIDATIVE STRESS ON ANTIOXIDANTS AND CHLOROPHYLL FLUORESCENCE IN STAY-GREEN WHEAT (*TRITICUM AESTIVUM* L.) FLAG LEAVES

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Abstract

Paraquat, a highly toxic herbicide, induces the production of reactive oxygen species in the cell. Paraquat resistance in stay-green wheat (*Triticum aestivum* L.) was investigated. Two cultivars, CN12 and CN17, were found to have a higher photo-oxidative resistance than that of common wheat MY11, as measured with antioxidant indexes and chlorophyll *a* fluorescence. The flag leaves of both CN12 and CN17 maintained a relatively high antioxidant enzyme activity, chlorophyll content, and photosynthetic index and a low level of lipid degradation after 24 h of treatment with 10 μM paraquat. The Stay-green wheat cultivars CN12 and CN17 had a high resistance to paraquat-induced photo-oxidative stress, which can potentially alleviate some of the damage induced by environment pollution.

Introduction

The herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) has been continuously used as efficient herbicide in agriculture for over 50 years since it was invented in 1956 (Akinloye *et al.* 2011). Paraquat is a non-selective and fast-acting herbicide that affects all green tissues. It is also very strongly absorbed into the soil and thereby biologically deactivated (Roberts *et al.* 2002).

In light, paraquat affects the chloroplast by intercepting electrons from photosystem I (PSI) on the thylakoid membrane and creating a radical anion that is reoxidized by molecular oxygen to form superoxide ($^{-}O_2^{\bullet}$) and other reactive oxygen species (ROS) (Chiang *et al.* 2008). The addition of 1 mM paraquat to illuminated chloroplast suspensions increases the production of $^{-}O_2^{\bullet}$ by up to ten-fold (Chia *et al.* 1981). Paraquat was used to simulate the ROS-induced damage caused by other environment stresses, such as drought, heat and salinity (Chia *et al.* 1981, Bhatt *et al.* 2011). The development of paraquat-resistant varieties could be an effective strategy to counteract various environmental stresses and ensure high crop yields.

Some stay-green plants (which delay senescence) were found to have high resistance to paraquat, and the mechanism of resistance seems to be similar in different plants (Chia *et al.* 1981, Falk *et al.* 2002). Superoxide dismutase (SOD) dismutate the superoxide to H_2O_2, which is removed by ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) (Nehnevajova *et al.* 2012). Malondialdehyde (MDA) and chlorophyll levels are used as an indicator of lipid peroxidation and chloroplast damage, respectively (Khayatnezhad *et al.* 2011). The major mechanism by which stay-green plants adapt to various stresses such as drought, heat, and salt can be attributed to the ROS degradation system and the protection of the membrane (Allen 1995, Jena *et al.* 2009), but it is unknown how the stay-green plants adapt to paraquat stress.

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The effects of paraquat stress on antioxidants and chlorophyll fluorescence in stay-green wheat was not reported. It is reported that the stay-green wheat lines Chuannong12 (CN12) and Chuannong17 (CN17) are highly resistant to paraquat-induced photo-oxidative stress.

A world-wide food crisis remains today due to the rapidly growing world population (Hodges 2005). Increasingly serious environmental damage can induce sharp reductions in crop productivity (Wang *et al.* 2010). The development of new cultivars with a high tolerance to environmental stress can ensure plant growth and yield. Some stay-green plants have been reported to resist various environment stresses that are associated with ROS. In this study, the resistance of stay-green wheat to environmental stress using paraquat treatment was investaged.

Materials and Methods

Wheat (*Triticum aestivum* L.) cultivars CN12 and CN17 exhibiting the stay-green phenotype based on several years of observation were grown in clay soil on the farm at the Ya'an Agricultural Research Station of Sichuan Agricultural University (27° 17' N, 120° 16' E) in Southwest China in the wheat growing season of 2010. Mianyang11 (MY11), an agronomic parent of both CN12 and CN17, was used as the control. Sufficient nutrients and water were supplied, and diseases were controlled throughout the growing season.

Flag leaves (10 cm length) from heading wheat of CN12, CN17 and MY11 were incubated with various concentrations (0 - 500 μM) of paraquat and then illuminated at 400 μmol/m^2/sec light intensity for 24 h before investigation.

Leaf samples were pulverized in liquid N_2 using a mortar and pestle and then resuspended in 3 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM ethylenediaminetetraacetic (EDTA), 50 mM NaCl, 1% polyvinyl polylpyrrolidone (PVPP) and 1 mM ascorbic acid. The homogenate was centrifuged at 16000 × g for 15 min at 4ºC. Enzyme activity was determined spectrophotometrically: SOD at 560 nm, APX at 290 nm and CAT at 240 nm, as previously described (Lima *et al.* 2002, Lascano *et al.* 2003).

Leaf samples were pulverized in liquid N_2 and homogenized with 2 ml of cold 80 % (v/v) aqueous acetone and centrifuged twice at 16000 × g for 10 min at 4ºC. The combined supernatants were diluted ten-fold with 100 % acetone, and the absorbance was measured at 645 and 663 nm (Spano *et al.* 2003). Chlorophyll *a* and *b* concentrations were calculated using the equations of Hill *et al.* (1985).

Leaf samples were pulverized in liquid N_2, quartz sand and 3 ml of 50 mM potassium phosphate buffer (pH 7.5), and an equal volume (v) of 0.5 % thiobarbituric acid was added. The complex was kept in boiling water for 30 min and centrifuged at 3000 × g for 10 min. Absorbance was measured at 532 and 600 nm, and the MDA content was determined using the absorption coefficient (Behera *et al.* 2002).

Chlorophyll *a* fluorescence from PSII in flag leaves was measured with a modulated fluorescence meter (Li-6400-40 LCF, Li-Cor, USA). To ensure maximum photochemical efficiency of PSII, leaves were dark-adapted for at least 30 min. The minimum fluorescence (F_o) and maximum fluorescence (F_m) were measured in the dark-adapted leaves. The air temperature was 25ºC, the vapor pressure deficit (VPD) was 0.6 k Pa, and the actinic light was 1000 μmol/m^2/s. To ensure efficient excitation capture of open PSII reaction centers, leaves were then measured in the light. Minimum fluorescence (F'_o) and maximum fluorescence (F'_m) values under light were read as for F_o and F_m (Genty *et al.* 1989, Chen *et al.* 2010). The maximum photochemical efficiency of PSII in the dark-adapted leaves was calculated as the ratio of variable fluorescence ($F_v = F_m - F_m$) to F_m ($F_v/ F_m = F_m - F_o/ F_m$). The efficiency of excitation capture by open PSII reaction centers was calculated as $F'_v / F'_m = F'_m - F'_o / F'_m$.

Results and Discussion

The malondialdehyde (MAD) content exhibited the same trend in MY11, CN12 and CN17 at different paraquat concentrations and decreased sharply after treatment with 0 - 5 µM paraquat and then increased acutely with 10 µM (Fig.1 A). There were minimal differences in MAD production between the cultivars at low paraquat concentrations (0, 5, 10 µM). MY11 showed a higher increase than CN12 and CN17. The differences between MY11 and both CN12 and CN17 were significant. The MAD level of MY11 was 1.25 and 1.50 times higher than that of CN12 and CN17, respectively at the highest paraquat concentration (500 µM). Although MY11 showed a dose-dependent loss of chlorophyll, CN12 and CN17 maintained a high level of chlorophyll at paraquat concentrations lower than 100 µM, with the highest levels being observed with 5 µM paraquat (Fig. 1B).

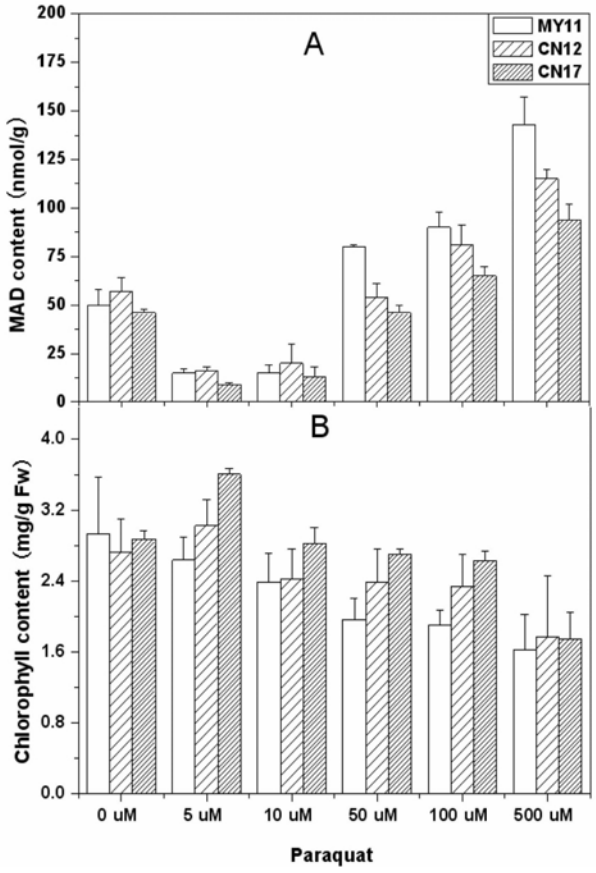

Fig. 1. The effect of paraquat treatment on (A) malondialdehyde (MAD) production and (B) chlorophyll content in flag leaves of the wheat cultivars MY11, CN12 and CN17. Duration of paraquat treatment and standard errors of the means are indicated. (n = 3).

Although the SOD activities of CN12 and CN17 flag leaves follow the same trend as that in MY11 at with various paraquat concentrations, the SOD activities remain relatively higher in CN12 and CN17. A decrease in activity with increasing paraquat concentration, except for a slight increase with 5 µM paraquat was observed (Fig. 2 A).

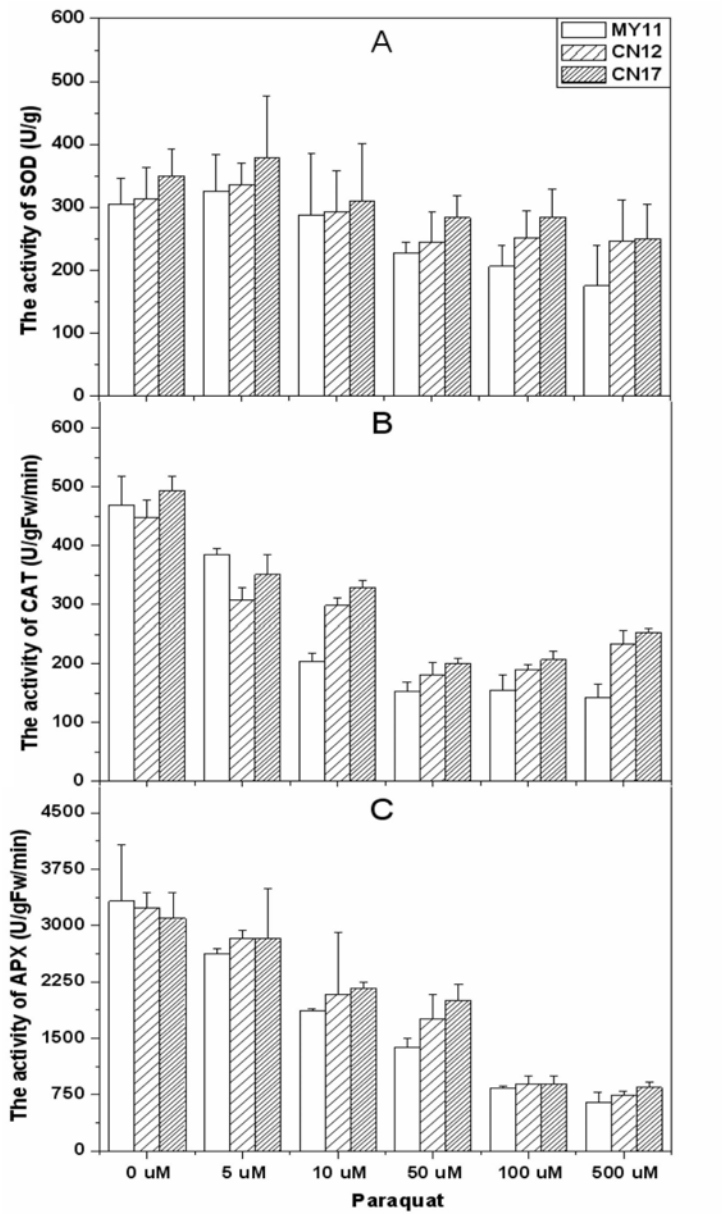

Fig. 2. Comparison of the effect of paraquat treatment on (A) superoxide dismutase (SOD), (B) catalase (CAT), and (C) ascorbate peroxidase (APX) from the flag leaves of the wheat cultivars, MY11, CN12 and CN17. Duration of paraquat treatment and standard errors of the means are indicated, (n = 3).

The catalase (CAT) activity of MY11 was found to decrease sharply with 5 µM versus 10 µM paraquat treatment, while the decrease in CN12 and CN17 mainly occurred with 10 µM versus 50 µM paraquat treatment (Fig. 2 B). Although MY11 had slightly higher CAT activities than CN12 at 0 µM and 5 µM and CN17 at 5 µM, the activity of MY11 decreased to 204 U/gFw/min, while

CN12 and CN17 maintained high levels of 298 and 329 U/gFw/min, respectively. A dose-dependent decrease of APX activity is in all genotypes (Fig. 3B). MY11 had slightly higher APX activity at 0 µM paraquat and lower activities at all other paraquat concentrations.

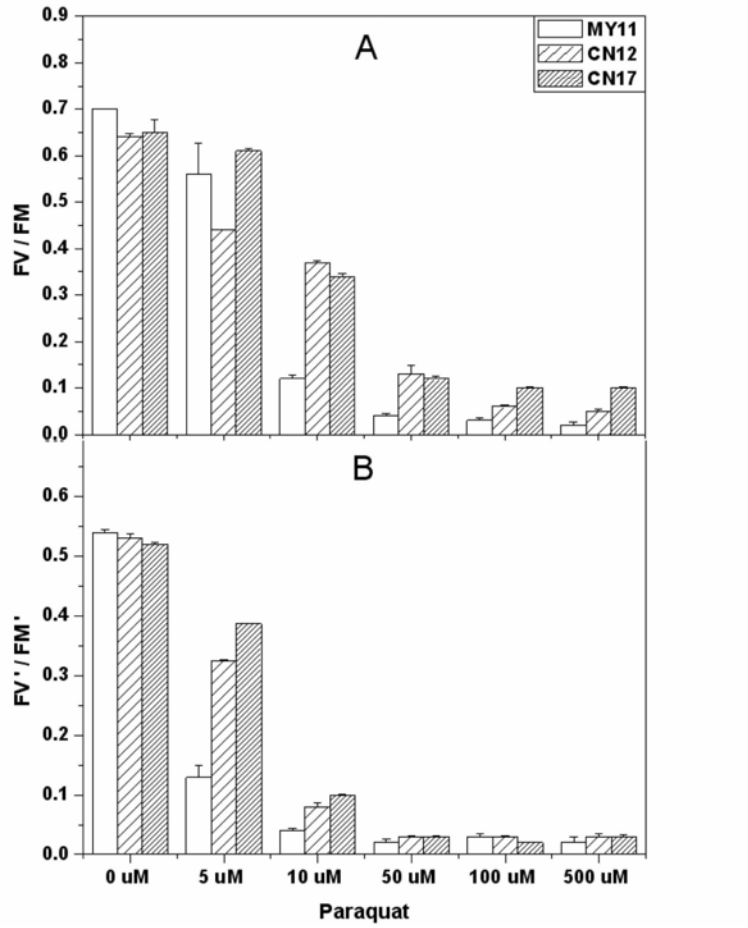

Fig. 3. The effect of paraquat treatment on (A) the maximal efficiency of PSII photochemistry (*Fv*/ Fm) and (B) the efficiency of excitation capture by open PSII reaction centers (F'v/F'm) of flag leaves of the wheat cultivars, MY11, CN12 and CN17. Duration of paraquat treatment and standard errors of the means are indicated. (n = 3).

Antioxidant enzymes are present in the cell to protect against ROS that are induced by biotic and abiotic stresses (Razinger *et al.* 2007). The activities of these enzymes have been used to indicate the level of oxidative resistance (Allen 1995, Kuk *et al.* 2006). In general, CN12 and CN17 exhibited higher levels of SOD, CTA and APX activities at all paraquat concentrations (Fig. 2), indicating once again that these strains are more resistant to paraquat than MY11. SOD activity was found to decrease more slowly than that of APX and CAT. Because SOD dismutates the superoxide radical to H_2O_2 and APX and CAT metabolize H_2O_2 to H_2O (Kuk *et al.* 2006), we can infer that the main ROS damage induced by paraquat comes from H_2O_2.

The efficiency of PSII photosynthetic activity was measured with F_v/F_m and F'_v/F'_m. The F_v/F_m and F'_v/F'_m ratios for MY11, CN12 and CN17 were not significantly different in 0 μM paraquat, and MY11 was the most sensitive to paraquat (Fig.3). The F_v/F_m ratio in MY11 was approximately 56% in 5 μM paraquat and only 12% in 10 μM. In CN12 and CN17, this ratio was approximately 1.5 times higher in 10 μM (Fig.3 A). The F'_v/F'_m ratio in MY11 in 5 μM was only 24% of that in 0 μM, whereas this ratio in CN12 and CN17 only decreased a little (Fig.3 B). All the F_v/F_m and F'_v/F'_m ratios were very small with little variation at the 50, 100, and 500 μM paraquat concentrations for all three genotypes. Paraquat first affects PSI, then damages the surrounding area and PSII (Lascano *et al.* 2003).

The stay-green cultivars CN12 and CN17 were found to exhibit significantly higher resistance to paraquat-induced photooxidative stress than their common agronomical parent MY11. In this study, CN17 performed better than CN12. Both strains harbor the wheat-rye 1BL/1RS translocated chromosome, and thus paraquat resistance may be associated with 1RS of rye.

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