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Title: Melatonin enhances drought resistance by regulating leaf stomatal behaviour, root growth and catalase activity in two contrasting rapeseed (*Brassica napus* L.) genotypes

Running title: *Enhanced root growth and root catalase activity induced by melatonin may mitigate stomata closure*

Authors: Lulu Dai [1,2,3], Jun Li [1], Harry Harmens [4], Xiaodong Zheng [5], Chunlei Zhang [1*]

[1] Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, 430062, China

[2] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Shuangqing Road 18, Haidian Distract, Beijing, 100085, China

[3] College of Resources and Environment, University of Chinese Academy of Sciences, Shijingshan Disctrict, Beijing, 100049, China

[4] UK Centre for Ecology & Hydrology, Environment Centre Wales, Deiniol Road, Bangor, Gwynedd LL57 2UW, UK

[5] College of Horticulture, Qingdao Agricultural University, Changcheng Road 700, Chengyang District, Qingdao, 266109, China

*Corresponding author: zhangchunlei@caas.cn

Abstract Two contrasting rapeseed genotypes, Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant), were studied under drought stress with or without pretreatment with melatonin to (i) explore whether melatonin enhances drought resistance by regulating root growth and (ii) determine the relationship between the belowground and aboveground responses to melatonin under drought stress. Results show that the light-saturated rate of photosynthesis (P_n), stomatal conductance (g_s), water use efficiency (*WUE*) and chlorophyll content were decreased by drought for Qinyou 8, whereas drought only decreased P_n and chlorophyll content for Q2. Drought decreased actual photochemical efficiency in saturated light (F_v'/F_m'), actual photochemical efficiency (*PhiPS*Ⅱ), quenching of photochemical efficiency (*qL*) and electron transport rate (*ETR*) in Qinyou 8. However drought only decreased F_v'/F_m' and *qL* in Q2. Drought increased malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents in the roots of both genotypes. Melatonin had no significant additional effects on root guaiacol peroxidase (POD) and superoxide dismutase (SOD) activities, but enhanced root catalase (CAT) activity of droughted plants further. Melatonin promoted taproot and lateral root growth under drought stress. Melatonin also promoted stomatal opening resulting in enhanced photosynthesis in the two genotypes. The two mechanisms induced by melatonin synergistically enhance drought resistance of rapeseed as indicated by enhanced gas exchange parameters under melatonin pretreatment. The findings provide evidence for a physiological role of melatonin in improving drought resistance, especially in belowground parts.

Abbreviations

ABA, abscisic acid

CAT, catalase

C_i, intercellular CO_2 concentration

ETR, electron transport rate

F_v'/F_m', actual photochemical efficiency in saturated light

g_s, stomatal conductance

H_2O_2, hydrogen peroxide

MDA, malondialdehyde

P_n, light-saturated rate of photosynthesis

PhiPS Ⅱ, photochemical efficiency

POD, guaiacol peroxidase

qL, quenching of photochemical efficiency

ROS, reactive oxygen species

SEM, scanning electron microscopy

SOD, superoxide dismutase

T_r, transpiration rate

WUE, water use efficiency

Keywords: *Brassica napus*; drought; gas exchange; melatonin; root antioxidants; root structure; stomatal behaviour

1. Introduction

Drought is one of the biggest threats to global food security and is becoming a more serious problem due to scarce water resources and the increasing population (Wang et al., 2003). Human-caused climate change makes extreme weather conditions such as droughts and heat waves a more frequent occurrence (Mann et al., 2017). Drought stress inhibits plant growth by affecting various aspects of plant life, especially by inducing alterations in water relations within the rhizosphere and plant (Turner and Begg, 1981). Plant responses to water scarcity are complex, involving adaptive changes and/or deleterious effects (Chaves et al., 2002). Overall, plants respond to water deficit via different mechanisms including physiological, metabolic and defence systems. Responses include well-developed root systems, changes in plant hormones, enhanced antioxidant enzymatic systems, stomatal closure and production of low molecular osmolytes, such as glycine betaine, proline and other amino acids (Zhu, 2003). In recent years, genetic engineering and conventional breeding techniques have been proven promising for enhancing tolerance to various abiotic stresses; however, these methods are either expensive, complicated, and time-consuming, or even unacceptable in many countries around the world (Moshelion et al., 2015). In addition, many investigators gradually improved plant drought resistance by exogenously applied or endogenously available drought-resistance chemicals such as abscisic acid (ABA) (e.g. Li et al., 2011; Sharp et al., 2000; Wang et al., 2003). However, applications of ABA are equally limited in actual agricultural practice due to its rapid catabolism by relevant enzymes, chemical instability and high production costs (Naeem et al., 2016; Xiong et al., 2018). Hence, it is necessary to explore some alternative drought-resistance chemicals and investigate corresponding mechanism of drought resistance to mitigate harmful effects of drought on food production in the future.

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring compound found in invertebrates, mammals, birds, reptiles, amphibians and fish (Arnao, 2014).

As a well-known animal hormone, melatonin has many important biological functions regarding circadian rhythms, mediating changes in seasonal reproduction, tumour inhibition, immuno-enhancement and reducing oxidative stress (Arnao et al., 2014; Demas and Nelson, 1998; Tan et al., 2012). Since 1995, melatonin has been detected in the roots, leaves, seeds and fruits of a considerable variety of plant species (Dubbels et al., 1995; Hattori et al., 1995). Numerous studies have demonstrated that melatonin plays an important role in regulating growth and development of vascular plants, and as an antioxidant protecting plants against (a)biotic stress (e.g. Lei et al., 2013; Shi and Chan, 2014; Tal et al., 2011; Zhang et al., 2013). The antioxidative effect of melatonin has been reported for several plant species, for example rice (Park et al., 2013), maize (Jiang et al., 2016), apple (Wang et al., 2012) and grape (Vitalini et al., 2013). Li et al. (2012) reported that exogenous melatonin application to *Malus hupehenis* decreased the oxidative damage caused by reactive oxygen species (ROS) via directly scavenging hydrogen peroxide (H_2O_2) and enhancing antioxidant enzyme activities. Melatonin application enhanced tolerance to salt and drought stress in soybean by up-regulating the expression of genes that were inhibited by salt stress (Wei et al., 2015). Melatonin is now known to alter many plant characteristics including germination (Zhang et al., 2013), seedling growth, flowering time, grain yield and senescence (Byeon and Back, 2014; Wang et al., 2013). Another unique function of melatonin in plants is its auxin-like activity, thereby promoting plant growth. Chen et al. (2009) found that melatonin application promotes root growth in *Brassica juncea*, and Sarropoulou et al. (2012) reported the promotion of adventitious root regeneration in shoot tip explants of *Prunus cerasu*s L. Therefore, melatonin appears to play an important role in abiotic stress resistance, especially drought and salt stress.

However, most studies on melatonin-enhancing stress resistance have been limited to leaf responses so far (e.g. Li et al., 2012; Vitalini et al., 2013; Wang et al., 2012), few studies focused on root responses to melatonin under stress conditions. Zhang et al. (2013) only investigated melatonin effects on root growth for a very

limited number of parameters in cucumber, excluding antioxidant responses. The impact of melatonin on root growth and root antioxidant responses under drought stress are still unclear to date. In addition, systematic studies on belowground and aboveground responses to melatonin under drought stress are very limited. Hence, studies exploring the internal relationships between the belowground and aboveground responses of plants to melatonin under drought stress are needed.

Rapeseed (*Brassica napus* L.) is the most important oil crop in China (Wang et al., 2010). It is very susceptible to water deficit during the entire growth period (Zhang et al., 2014b). In this study, two rapeseed genotypes, Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant), were subjected to drought stress after pretreatment with or without exogenous melatonin. We hypothesized that drought significantly inhibits the growth of rapeseed and that melatonin will improve drought tolerance in roots. The objectives of this study were to (1) explore how melatonin improves drought resistance via regulating root growth; and (2) investigate the relationship between the belowground (root growth and root antioxidant response) and aboveground (stomatal structure and gas exchange) responses to melatonin under drought stress.

2. Materials and methods

2.1. Plant materials and experimental conditions

Seeds of two contrasting rapeseed (*Brassica napus* L.) genotypes, Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant), were obtained from the Oil Crops Research Institute (OCRI), Chinese Academy of Agricultural Sciences (CAAS), Wuhan, China. The two genotypes were chosen because of their significant difference in drought resistance (Naeem et al., 2016; Xiong et al., 2018). The seeds were surface-sterilized with mercury (Ⅱ) chloride for 10 min, washed in running tap water for 15 min, and germinated in an incubator at 25 °C. Three germinated seeds were planted per pot (height 15 cm, width 12 cm) filled with a mixture of vermiculite, soil

and sand (2:1:1, v/v/v); 5-g slow-release fertilizer containing 13% N, 10% P and 14% K was added to each pot.

Three-leaf stage rapeseed seedlings were irrigated with 100 μM melatonin solutions (M^+) or distilled water (M^-) for 7 days (7 d, 200 ml per pot each day), respectively. The melatonin (Sigma–Aldrich, St. Louis, MO, USA) solutions were dissolved in ethanol followed by dilution with MilliQ water [ethanol/water (v/v) D 1/10000]. After melatonin pretreatment, the seedlings were subjected to drought (W^-) or well-watered (W^+) for another 7 d. Hence, there were four treatments in total (1) M^-W^+ (distilled water pretreatment + well-watered); (2) M^-W^- (distilled water pretreatment + drought); (3) M^+W^+ (100 μM melatonin pretreatment + well-watered); (4) M^+W^- (100 μM melatonin pretreatment + drought). The melatonin concentration (100 μM) applied in this study was based on Li et al. (2014). There were 16 replications per treatment per genotype, so 128 plants in total, as lots of leaf samples were needed for the analyses. The seedlings were grown under a movable rain shelter to avoid rain affecting the experiment. The parameters described below were determined after 14 d after initial melatonin application (7 d of drought stress treatment).

Soil moisture content (%) was monitored daily using a Soil Moisture Kit (HH2-ML3, DELTA-T, CO, UK).

2.2. Photosynthetic gas exchange and chlorophyll content

Gas exchange was measured with a portable photosynthesis system (Licor-6400XT, LI-COR Inc., Lincoln, NE, USA) as described by Feng et al. (2011) and Dai et al. (2017). The following parameters were determined: light-saturated rate of photosynthesis (P_n, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}), intercellular CO_2 concentration (C_i, μmol mol^{-1}), and transpiration rate (T_r, mol m^{-2} s^{-1}). Leaf water use efficiency (*WUE*, μmol CO_2 $mmol^{-1}$ H_2O) was calculated as the ratio of P_n and T_r. Two fully expanded upper leaves from one plant were randomly selected in each pot. Photosynthetic Photon Flux Density (PPFD) was controlled at

1200 μmol m^{-2} s^{-1} (light-saturation), block temperature at 25 °C, the CO_2 concentration in air entering the leaf chamber at 400 μmol mol^{-1} and the relative humidity at 50-70%. The measurements were conducted between 09:00-12:00 h. After the gas exchange measurements, chlorophyll content was determined at the same leaves as photosynthesis measurement using SPAD-502 (PLUS, KONICA MINOLTA, Japan).

2.3. Chlorophyll *a* fluorescence

Fully expanded leaves at the top of plants were randomly selected to measure actual photochemical efficiency of photosystem II (PSⅡ) in saturated light (F_v'/F_m'), actual photochemical efficiency of PSⅡ in light (*PhiPS*Ⅱ), quenching of photochemical efficiency of PSⅡ (*qL*) and electron transport rate (*ETR*) using MINI-IMAGING-PAM (Walz Company, Germany) according to Naeem et al. (2016).

2.4. Stomatal structure, density and aperture

Leaf stomata were observed using scanning electron microscopy (SEM). Briefly, three leaves were randomly selected per treatment for each genotype in the fourth position from the top of each plant and samples were immediately fixed with a 4% glutaraldehyde solution in 0.1 M phosphate-buffered saline (PBS; pH 6.8). After being rinsed five times with PBS (each 5, 10, 15, 20 and 30 min respectively), samples were dehydrated using a graded ethanol series, vacuum-dried and gold-coated. SEM was performed using a JSM-SU8010 microscope (JEOL Ltd., Tokyo, Japan). Stomata were counted at random in 20 visual sections on the abaxial epidermis, and final tallies were used to compute their densities (mm^2). Lengths (μm), widths (μm) and apertures (μm) were measured randomly from 20 stomata on the same specimens using *Image J* software (Li et al., 2014).

2.5. Root structure

Root structure was measured using a root scanner (Epson Expression 11000XL, EPSON, Nagano, Japan) and analyzed using WinRHIZO software (LC4800-Ⅱ

LA2400, Saint foy, Canada). Total root length (cm), taproot length (cm), a lateral root length (cm), root diameter (mm), root surface area (cm^2) and volume (cm^3) of each sample were determined (Zhang et al., 2013).

2.6. Oxidation products and antioxidants in roots

Roots were sampled between 12:00 and 14:00 h and quickly wrapped in tinfoil, immediately frozen in liquid N and stored at -80 °C until analysis. Frozen root tissues were ground into power in liquid N with a mortar and a pestle for the biochemical assay. Malondialdehyde (MDA, μmol g^{-1} FW) content was assessed to estimate lipid peroxidation by 2-thiobarbituric acid-reactive metabolite (TBARS) according to the method of Heath and Packer (1968) and calculated by the equation of C_{MDA} (m mol L^{-1}) = $6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$. The hydrogen peroxide (H_2O_2, μmol g^{-1} FW) content was determined as a H_2O_2–molybdate complex resulting from the reaction of tissue–H_2O_2 with molybdate; 0.1 g roots were ground in liquid N in 1.0 mL saline solution. The samples were centrifuged at 2300 *g*, 4 °C for 20 min, the supernatant was collected and the H_2O_2 content was determined according to the method described by Sengupta et al. (2013).

Antioxidant enzyme extraction and analysis

To avoid potential differences of the content of antioxidant enzymes in different root positions, all samples were collected from taproot tips. Approximately 0.05 g fresh roots tissue were ground in liquid N and extracted with 2 mL of 50 mM sodium phosphatebuter (pH 7.0, containing 1% vinyl pyrrolidone). The samples were centrifuged at 18, 000 *g*, 4 °C for 20 min and the supernatant was collected. All extractions were carried out on ice. Peroxidase (POD, U^{-1} g FW) activity was measured as follows: 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.2 mM guaiacol and 10 mM H_2O_2. The reaction was initiated by adding 100 μL of enzyme extract and the oxidation of guaiacol was measured by the increase in absorbance at 470 nm. Superoxide dismutase (SOD, U^{-1} g FW) activity was determined by estimating its ability of inhibiting the photochemical reduction of nitro

blue tetrazolium (NBT) and the absorbance was read at 560 nm. The amount of enzyme that inhibited 50% NBT reduction was defined as one unit of SOD activity. Catalase (CAT, U^{-1} g FW) activity was determined according to the description by McKee et al. (1997). The changes of absorbance at 240 nm were monitored after the enzyme extract was mixed with 30 mM KH_2PO_4/K_2HPO_4 (PH 7.0) containing 10 mM H_2O_2. The unit of CAT and POD activity was defined as the decrease and increase of 0.01 ΔOD per minute at 240 nm and 470 nm, respectively. All measurements were conducted using spectrophotometric methods.

2.7. Biomass

Five intact plants per treatment were randomly collected and sampled for the determination of biomass components, i.e. leaves, stems and roots. The dry weight of different plant organs was determined after drying in an oven at 65 °C for 7d until constant weight.

2.8. Statistical analysis

All original data passed Shapico-Wilk and Levene's tests for normality and homogeneity of variance. Analysis of variance (ANOVA) for the variables was performed using the SPSS 20.0 for Windows statistical software package (SPSS, Inc., Chicago, IL, USA). Turkey's Honestly Significant Difference (HSD) test was applied to identify significant differences for each genotypes, respectively. $P \leq 0.05$ was considered as statistically significant.

3. Results

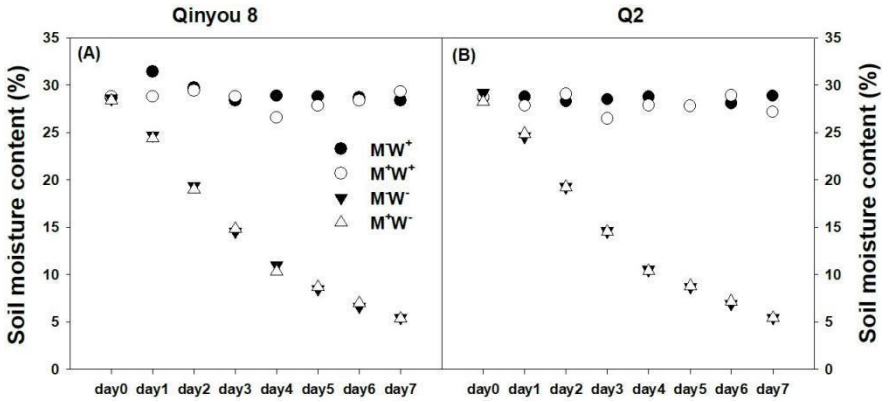

Fig. 1. Soil moisture contents in the pots in Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes for each drought day (averaged values, n = 5). M^-W^+: distilled water pretreatment + well-watered, M^-W^-: distilled water pretreatment + drought; M^+W^+: 100 μM melatonin pretreatment + well-watered, M^+W^-: 100 μM melatonin pretreatment + drought.

3.1. Biomass and chlorophyll content

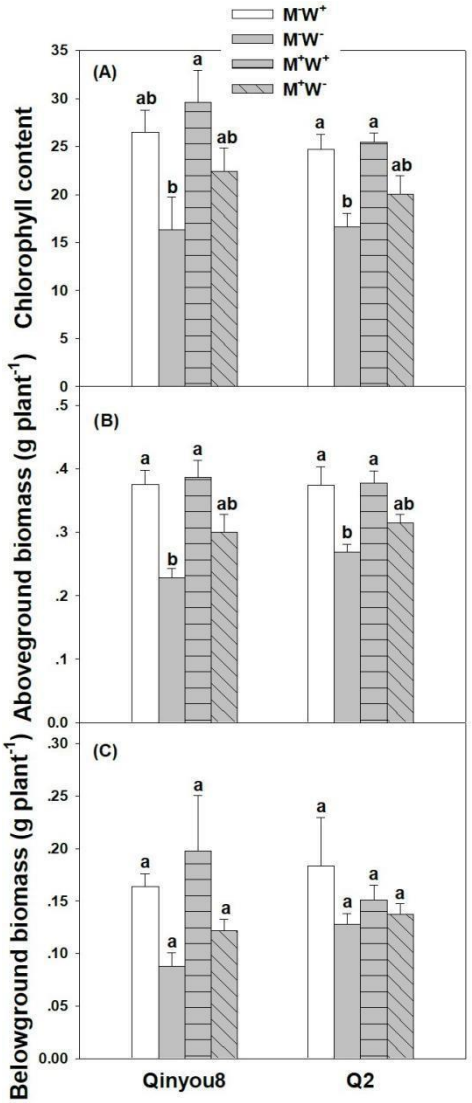

Fig. 2. Effects of drought and melatonin on chlorophyll content (A), aboveground (B) and belowground biomass (C) in Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes. M^-W^+: distilled water pretreatment + well-watered, M^-W^-: distilled water pretreatment + drought; M^+W^+: 100 μM melatonin pretreatment + well-watered, M^+W^-: 100 μM melatonin pretreatment + drought. Different letters indicate per genotype significant differences between treatments (mean ± SD, Tukey test, $P \leq 0.05$, n = 5).

Drought decreased chlorophyll content by 30.8 and 26.9% in Qinyou 8 and Q2, regardless of melatonin treatment. However, there was no effect of melatonin on chlorophyll content when averaged for drought treatments. No significant interaction between drought and melatonin on chlorophyll content was found in both genotypes

(Fig.2 A).

For Qinyou 8, drought reduced aboveground and belowground biomass by 30.6 and 41.9%, respectively, when averaged for drought treatments. However melatonin had no significant effects on biomass (+14.2 & 28.3% for aboveground and belowground biomass, respectively), regardless of drought. For Q2, drought decreased only aboveground biomass (-22.9%), but it had no significant effects on belowground biomass (-19.9%), regardless of melatonin treatment. Neither aboveground nor belowground biomass was affected by melatonin, when averaged for drought treatments (Fig.2 B&C).

3.2. Gas exchange

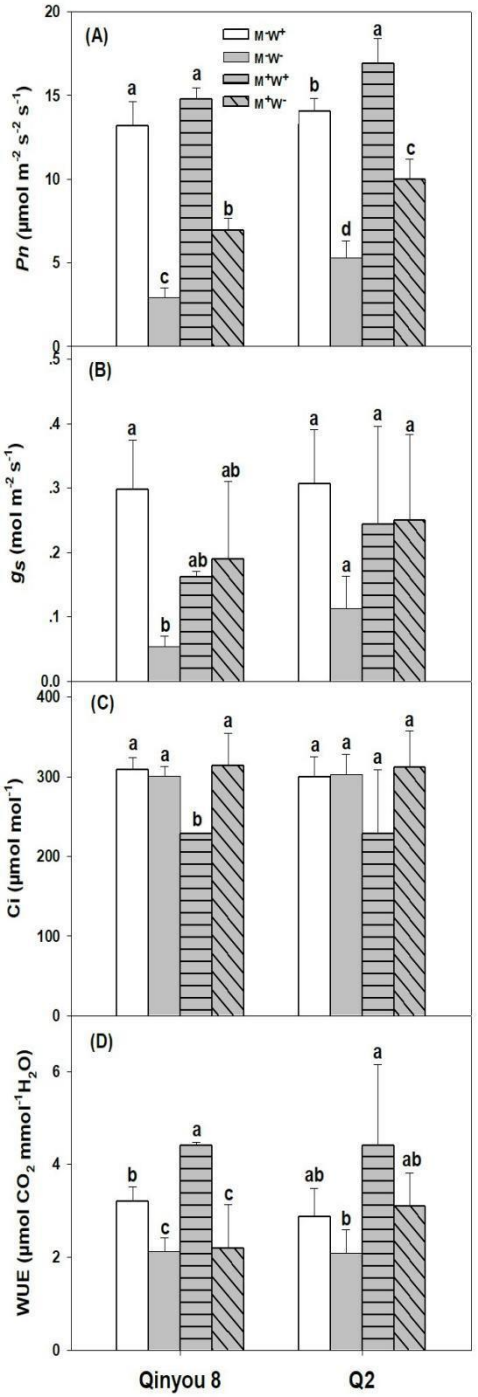

Fig. 3. Effects of drought and melatonin on light-saturated photosynthesis (P_n, A), stomatal conductance (g_s, B), intercellular CO_2 concentration (C_i, C), and water use efficiency (*WUE*, D) in Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes. For details of treatments and the meaning of different letters, see Fig. 2 (n = 3).

Compared to M^-W^+, P_n under drought (M^-W^-) decreased by 78.0 and 62.3% in

Qinyou 8 and Q2, respectively. However, M^+W^- increased P_n by 138.5 and 88.3% in Qinyou 8 and Q2, respectively, compared to M^-W^- (Fig.3 A). g_s was decreased by drought (M^-W^-) in Qinyou 8 (-82.0%), but it was not affected in Q2 (-63.3%) (Fig.3 B). Drought (M^-W^-) had no effect on C_i compared to M^-W^+ in both genotypes. However, M^+W^- treatment increased C_i in Qinyou 8 (+37.3%) compared to M^+W^+ (Fig.3 C). Compared to M^-W^+, *WUE* was decreased by drought (M^-W^-) in Qinyou 8 (-33.7%), but not in Q2 (-26.9%). However, M^+W^+ treatment increased *WUE* by 37.5 and 53.6% in Qinyou 8 and Q2, respectively, compared to M^-W^+(Fig.3 D).

3.3. Chlorophyll *a* fluorescence

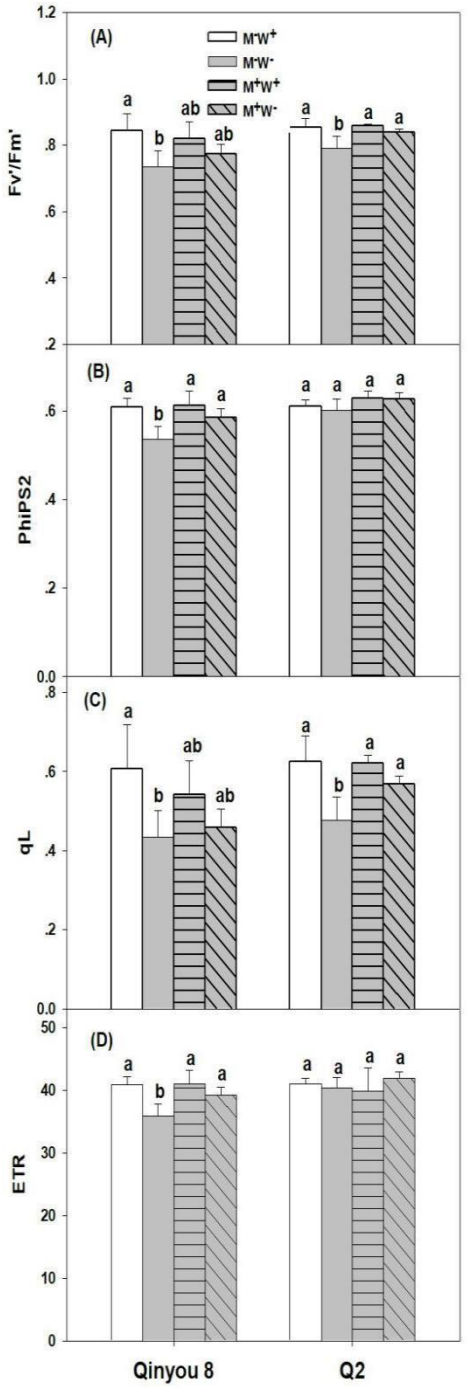

Fig. 4. Effects of drought and melatonin on actual photochemical efficiency of PS Ⅱ in saturated light (F_v'/F_m', A), actual photochemical efficiency of PS Ⅱ in light (*PhiPS* Ⅱ, B), quenching of photochemical efficiency of PS Ⅱ (*qL*, C) and electron transport rate (*ETR*, D) in Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes. For details of treatments and the meaning of different letters, see Fig. 2 (n = 3).

Compared to M^-W^+, F_v'/F_m' for Qinyou 8 and Q2 was decreased by 13.0% and

7.3%, respectively, when subjected to drought (M^-W^-). However, M^+W^- had no effect on F_v'/F_m' in Qinyou 8 (-5.6%) and Q2 (-2.3%), respectively, compared to M^+W^+. M^+W^- increased F_v'/F_m' in Q2 (+6.0%) but not in Qinyou 8 (+5.4%), compared to M^-W^- (Fig.4 A). M^-W^- decreased *PhiPS*Ⅱ in Qinyou 8 (-12.1%), but it had no effect on Q2 (-1.5%), compared to M^-W^+. M^+W^- treatment had no effect on *PhiPS*Ⅱ in Qinyou 8 (-4.4%) and Q2 (-0.3%), compared to M^+W^+ (Fig.4 B). M^-W^- decreased *qL* by 28.7 and 23.7% in Qinyou 8 and Q2, respectively, compared to M^-W^+. M^+W^- treatment did not affect *qL* in Qinyou 8 (-15.4%) and Q2 (-8.2%), compared to M^+W^+. M^+W^- increased *qL* by 19.3% only in Q2 (Fig.4 C). *ETR* was decreased by M^-W^- in Qinyou 8 (-12.1%) only, compared to M^-W^+. No effect was found of M^+W^- on *ETR* in Qinyou 8 (+4.3%) and Q2 (-5.4%), compared to M^+W^+.

3.4. Stomatal structure, density and aperture

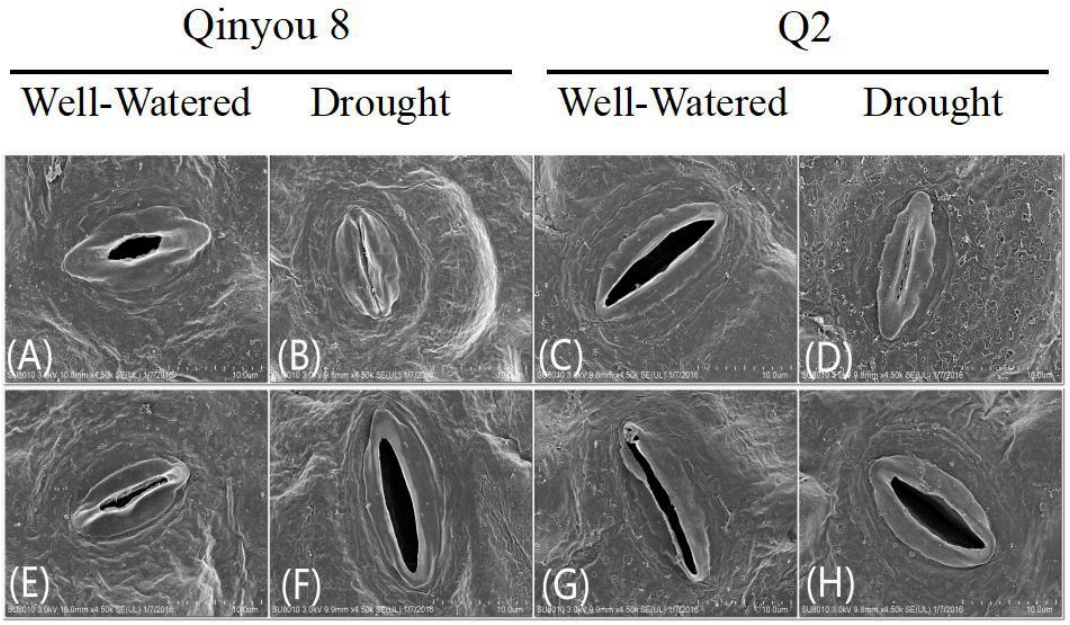

Fig. 5. SEM images of stomata in leaves of Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes, respectively: stomata in leaves of well-watered plants (A and C); stomata in drought treatment for 7 d (B and D); stomata from well-watered plants pre-treated with 100 μM melatonin (E and G); and stomata in drought treatment pre-treated with 100 μM melatonin (F and H). Magnification × 4500, scale

bars = 10.0 μm.

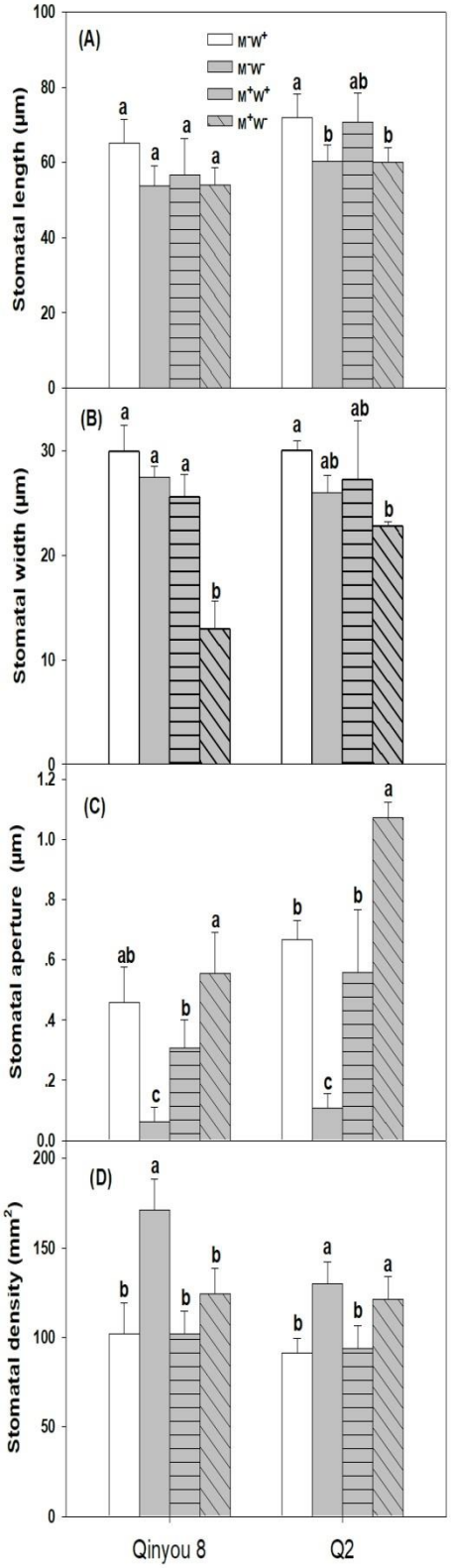

Fig. 6. Effects of drought and melatonin on stomatal length (A), width (B), aperture (C) and density (D) in leaves of Qinyou 8 (drought-sensitive) and Q2

(drought-tolerant) genotypes. For details of treatments and the meaning of different letters, see Fig. 2 (n = 20).

Compared with well-watered plants (Fig.5 A, C), drought caused nearly all stomata to close in both genotypes (Fig.5 B, D). Correspondingly, stomatal aperture was reduced in M^-W^- by 86.4 and 84.0% for Qinyou 8 and Q2, respectively compared with M^-W^+ (Fig.6 C). In the melatonin pretreatment, stomata remained partially open in the two genotypes under drought conditions (Fig.5 F, H). Moreover, stomatal aperture was increased in the M^+W^- treatment by 80.1 and 92.0% for Qinyou 8 and Q2, respectively compared with M^+W^+ condition, and increased in M^+W^- for Qinyou 8 (+7.82-fold) and Q2 (+9.04-fold), respectively compared with M^-W^- (Fig.6 C). Stomatal length and width were not affected by M^-W^- compared with M^-W^+ except that stomatal length was decreased by M^-W^- in Qinyou 8 (-17.6%) (Fig.6 A, B). M^+W^- treatment had no effect on stomatal length and width except that stomatal width was decreased in Qinyou 8 (-49.4%) compared with M^+W^+ (Fig.6 A, B). Stomatal density was increased by M^-W^- in Qinyou 8 (+67.6%) and Q2 (42.4%), respectively compared with M^-W^+. M^+W^- treatment decreased stomatal density in Qinyou 8, but it had no effect on stomatal density in Q2 compared with M^-W^- (Fig.6 D).

3.5. Root structure

Drought (M^-W^-) only increased lateral root length of Q2, compared to M^-W^+, whereas drought had no effect on any root structure parameters of Qinyou 8. However, melatonin pretreatment (M^+W^-) increased lateral root length of Qinyou 8, compared to M^+W^+. Taproot length and lateral root length of Q2 were increased by M^+W^-, compared to M^-W^+. No significant effects of drought or melatonin pretreatment on the total root length, root diameter, root surface area and root volume were found (Table 1).

Table 1 Effects of melatonin on root structure for Qinyou 8 and Q2 genotypes under well water and drought conditions. M^-W^+: distilled water pretreatment + well-watered; M^+W^+: 100 μM melatonin pretreatment + well-watered; M^-W^-: distilled water pretreatment + drought; M^+W^-: 100 μM melatonin pretreatment + drought. Values are means of three replicates ± SD. The letters are based on the Tukey test and are only shown for parameters that show significant differences between treatments.

genotypes	treatments	total root length (cm)	taproot length (cm)	a lateral root length (cm)	root diameter (mm)	root surface area (cm^2)	root volume (cm^3)
Qinyou 8	M^-W^+	1215 ± 20.17	44.95 ± 11.28a	24.27 ± 2.209ab	3.197 ± 0.190	389.3 ± 12.38	96.87 ± 5.458
	M^+W^+	1076 ± 141.9	38.87 ± 9.592a	19.05 ± 1.517b	3.611 ± 0.783	391.4 ± 10.47	111.0 ± 26.44
	M^-W^-	1141 ± 72.32	41.15 ± 2.735a	24.98 ± 3.651ab	3.122 ± 0.314	376.8 ± 20.27	88.56 ± 8.373
	M^+W^-	1119 ± 108.6	42.06 ± 10.93a	29.12 ± 1.435a	3.531 ± 0.567	381.1 ± 20.00	102.5 ± 16.22
Q2	M^-W^+	973.6 ± 181.6	31.96 ± 4.083b	17.79 ± 6.052b	3.813 ± 0.628	394.3 ± 14.92	117.2 ± 22.91
	M^+W^+	1049 ± 64.07	38.86 ± 6.501ab	22.09 ± 3.223ab	3.561 ± 0.330	392.1 ± 2.065	116.1 ± 13.57
	M^-W^-	1131 ± 141.6	46.27 ± 10.67ab	28.49 ± 0.086a	3.500 ± 0.553	378.0 ± 7.064	105.1 ± 18.94
	M^+W^-	1243 ± 102.4	51.39 ± 2.048a	27.04 ± 0.728a	3.416 ± 0.125	380.2 ± 4.053	99.59 ± 0.048

3.6. Oxidation products and antioxidants in roots

Compared with M^-W^+, MDA and H_2O_2 content for M^-W^- was increased for both genotypes. The increase was 143 & 109% for MDA, 95.4 & 120% for H_2O_2 in Qinyou 8 and Q2 genotypes, respectively. However, melatonin pretreatment reduced the production of MDA and H_2O_2 as suggested by the lower increases induced by M^+W^-, i.e. 100% for MDA in Q2, 56.6 & 86.5% for H_2O_2 in Qinyou 8 and Q2, respectively compared with M^+W^+. Furthermore, M^+W^- had no effect on MDA and H_2O_2 contents in Qinyou 8 (-23.6 & -14.6%) and Q2 (-14.6 & -15.6%), respectively compared with M^-W^- treatment (Fig.7).

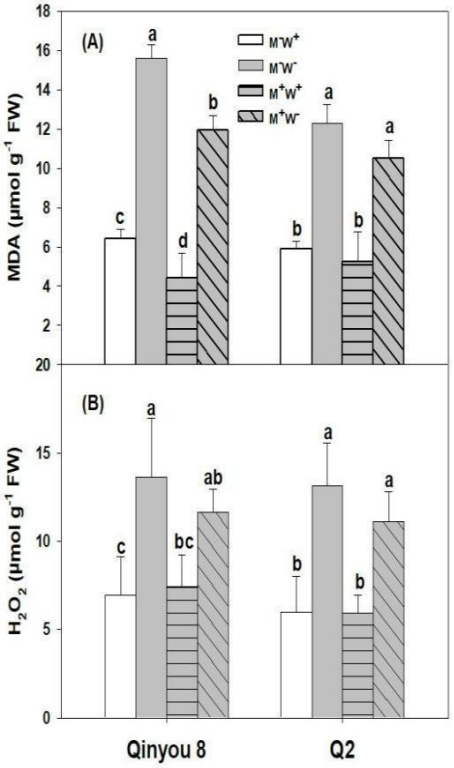

Fig. 7. Effects of drought and melatonin on malondialdehyde (MDA, A) and hydrogen peroxide (H_2O_2, B) content in the taproot tips of Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes. For details of treatments and the meaning of different letters, see Fig. 2 (n = 3).

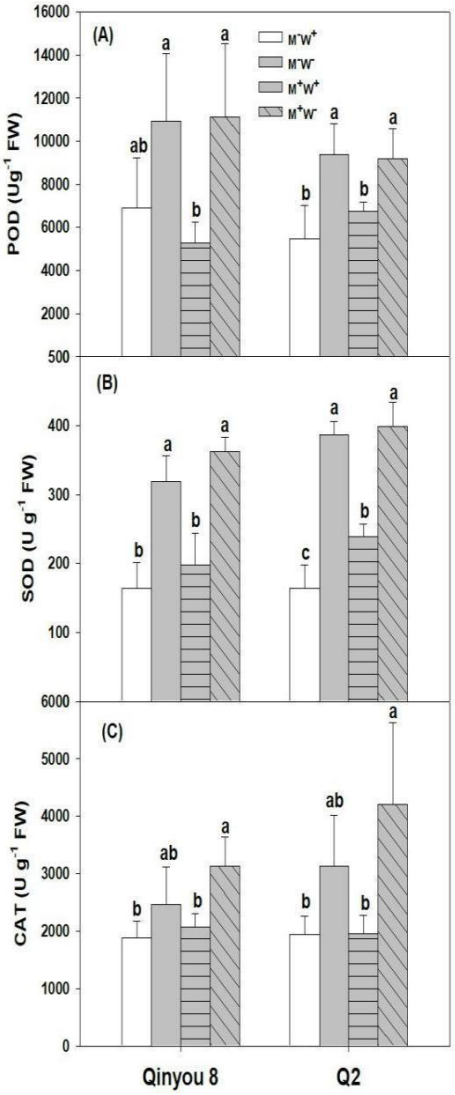

Fig. 8. Effects of drought and melatonin on peroxidase (POD, A), superoxide dismutase (SOD, B) and catalase (CAT, C) activities in the taproot tips of Qinyou 8 (drought-sensitive) and Q2 (drought-tolerant) genotypes. For details of treatments and the meaning of different letters, see Fig. 2 (n = 3).

Antioxidant enzyme activities were stimulated by drought (M^-W^-) in both genotypes compared with M^-W^+, except for POD in Qinyou 8 and for CAT in both genotypes. The increases were 57.8 & 71.4% in POD, 94.5 & 136% in SOD, and 30.6 & 60.9% in CAT for Qinyou 8 and Q2, respectively. Melatonin pretreatment had no significant additional effects on POD and SOD activities in droughted plants, but it stimulated CAT activity further

because M^+W^- treatment significantly increased CAT activity, compared to M^+W^+, whereas M^-W^- had no effect on CAT activity, compared to M^-W^+ (Fig.8).

4. Discussion

The beneficial effects of melatonin in mitigating abiotic stress have been documented in many studies, e.g. UV radiation (Afreen et al., 2006), extreme temperatures (Lei et al., 2013; Shi and Chan, 2014), heavy metals (Posmyk et al., 2008; Tan et al., 2007), salt stress (Li et al., 2012; Tal et al., 2011; Zhang et al., 2014a) and drought stress (Li et al., 2014; Wang et al., 2013; Zhang et al., 2013). However, the way in which melatonin improves drought tolerance via effects on roots has not been investigated systematically before. Here, we investigated the belowground (root growth and root antioxidant metabolism) and aboveground (stomatal structure and gas exchange) responses of rapeseed to melatonin under drought stress. The results show that melatonin not only promoted the growth of lateral roots and the taproot, but also enhanced the activity of CAT in the taproot tip. In addition, melatonin promoted stomatal opening (i.e. increases stomatal aperture) and therefore enhanced gas exchange in leaf of drought-sensitive and tolerant genotypes.

Various experiments have shown that stomatal responses are often more closely linked to soil moisture content than to leaf water status (e.g. Davies and Zhang. 1991; Stoll et al., 2000). Plant water status adjustments induced by drought have a direct impact on photosynthesis and in turn affect physiological processes in plants (Robredo et al., 2007). In our study, P_n and g_s were decreased by drought in the two genotypes, but exogenous melatonin application mitigated the reduction in P_n. Higher P_n under M^+W^- (melatonin and drought) treatment indicates higher carbon fixation and photosynthesis for optimum growth compared with the M^-W^- (distilled water and drought) treatment. Although the higher P_n under M^+W^- seems to be due to a higher g_s, melatonin did not significantly affect g_s in the drought treatment because of the high variation in g_s. However, a significant positive linear relationship between P_n and chlorophyll content ($R^2 = 0.64$, $P < 0.0001$, data not shown),

indicating that variation in P_n induced by drought maybe ascribed to changes in chlorophyll content. Chlorophyll *a* fluorescence is an important indicator for quantifying the photosynthetic activities in plants (Carvalho et al., 2001). In this study, a decline in F_v'/F_m', *PhiPS*Ⅱ, and *qL* by drought suggests that drought stress may have induced photo-inhibitory or photo-oxidative processes in the two genotypes to some extent. Higher reductions in *PhiPS*Ⅱ in Qinyou 8 indicated that Qinyou 8 had a weaker capacity for carbon metabolism and/or a lower utilization of ATP and NADPH than Q2. Melatonin may protect the plants from photo-inhibitory or photo-oxidative induced by drought. No significant difference was found between M^+W^- and M^+W^+ treatments among the four fluorescence parameters. In accordance with our findings, Ding et al. (2017) also found that exogenous melatonin mitigates photo-inhibition by accelerating non-photochemical quenching in tomato seedlings exposed to moderate light. Additionally, the chloroplasts ultra-structure of cucumber leaves under PEG treatment (PEG simulated drought) was improved by melatonin treatment (Zhang et al., 2013), supporting the conclusion that pretreatment with melatonin might protect plants from photo-inhibition or photo-oxidation induced by drought further.

In addition, SEM stomatal images showed that drought stress caused stomata to close. Many studies have shown that closing of stomata is an effective way to reduce water loss in *vivo*, enabling plants to adapt to drought stress (e.g. Chaves et al., 2002; Naeem et al., 2016). However, in our study, we found that stomata stayed partially open under drought stress after melatonin pretreatment. It is suggested that melatonin maintains water and CO_2 transport through stomata to some extent to maintain photosynthesis under drought condition (Jarvis et al., 1999). Stomatal aperture was increased by M^+W^- (drought and melatonin) treatment for Qinyou 8 and Q2 genotypes compared with M^-W^-, which further supports the conclusion. Other studies show that melatonin could improve the functions of stomata by enabling stomata to re-open under osmotic stresses such as drought (Li et al., 2014) or salt stress (Ye et al., 2016). Our study shows that there is no significant difference in soil moisture content with and without melatonin pretreatments under drought (data not shown), suggesting that partially opening of stomata induced by melatonin did not cause more water loss. It may be a

trade-off strategy between enhanced photosynthesis and potential water loss due to partially open of stomata. Further studies are needed to explore the effects of melatonin on stomata, especially under more severe or longer drought treatments. Stomatal width was decreased by melatonin compared with drought stress, indicating that stomata were narrower than those under drought stress. Overall, melatonin significantly increased stomatal aperture, decreased stomatal width, but had no effect on stomatal length in our study. However, Li et al. (2014) showed that melatonin significantly increased stomatal aperture and width, whilst reducing stomatal density of *Malus* species under drought conditions. Reported differences in the effect of melatonin on stomatal size (length and width) and density may be ascribed to species-specific difference, the time/concentrations of melatonin treatments and/or the magnitude of drought stress. A review by Hernández-Ruiz (2014) concluded that the effects of melatonin are dependent on its concentrations.

Root structure determines the ability of plants to capture water and nutrients, which is critical for drought tolerance (Markesteijn and Poorter, 2009). In the present study, drought increased lateral root length of Q2 only, compared to M^-W^+, whereas it had no effect on any root structure parameters of Qinyou 8, suggesting that drought promoted lateral root growth to absorb moisture from greater depth only for Q2. The mechanisms underlying the sustained root growth under drought stress include osmotic adjustment and an increase in the loosening capacity of the cell wall (Chaves et al., 2002). However, melatonin pretreatment significantly increased lateral root length of Qinyou 8 compared to M^+W^+, and significantly increased taproot length and lateral root length of Q2 compared to M^-W^+. Therefore, we conclude that melatonin enhances drought tolerance in rapeseed primarily by stimulating taproot and lateral root growth. Zhang et al. (2013) reported that melatonin only promotes lateral root growth of cucumber under drought. The promotion of root growth may contribute to stomatal re-opening through maintaining plant water potential. Li et al. (2014) found that re-opening of stomata may also result from regulation of ABA levels by melatonin.

Our study reported for the first time the impact of melatonin on root antioxidant

enzymes (SOD, POD and CAT) activity of drought-stressed plants, showing that only the activity of CAT was increased by melatonin. Previous studies only reported impacts of melatonin on leaves, showing that it can enhance the activity of antioxidant enzymes under abiotic stresses, such as cold (Shi et al., 2014), drought (Ye et al., 2016) and salt (Jiang et al., 2016). Oxidative stress occurs in plants exposed to a variety of abiotic stresses, including drought, and might result in peroxidation of essential macromolecules and the disruption of cellular redox homeostasis and signalling (Mirzaee et al., 2013). Although MDA and H_2O_2 contents in roots were increased by drought for both genotypes in the current study, melatonin pretreatment mitigated the increase in root MDA content, suggesting that drought-induced cellular damage and the subsequent loss of membrane integrity were alleviated in pretreated with melatonin. The detected ROS are involved in cellular signaling processes and can activate induce many genes and induce proteins involved in stress defense mechanisms (Mittler et al., 2004).

Overall, we conclude that melatonin not only plays an important role in enhancing drought tolerance at the leaf level, it also regulates root growth (taproot and lateral root) and CAT activity in roots to protect rapeseed from drought. Melatonin promotes stomata opening (i.e. increases stomatal aperture) and thus enhances gas exchange of the two genotypes. Melatonin also promotes lateral root and taproot formation, and enhances CAT activity in roots. Hence, melatonin protects oil seed rape from photo-inhibition or photo-oxidation induced by drought. The response of melatonin to drought stress may be genotype-specific. In our study, the magnitude of mitigating drought stress by melatonin seems to be stronger for Qinyou 8 than Q2 as suggested by a higher increase in P_n and decrease in MDA in Qinyou 8 than Q2 under drought with melatonin pretreatment, compared to drought treatment alone. The increase in root growth by melatonin may contributes to the stomatal re-opening. The mechanism of the signaling transduction pathway between roots and shoots, induced by melatonin under drought stress, requires further investigation.

5. Conclusions

For the first time, melatonin effects on gas exchange, chlorophyll *a* fluorescence and stomatal structure in leaves, and the morphological structure and antioxidant enzymes in roots of two rapeseed genotypes under drought stress were investigated. Melatonin regulates root growth, i.e. promotes taproot and a lateral root growth to absorb moisture from greater depth in the soil, and thus promotes stomatal opening (i.e. increases stomatal aperture) resulting in enhanced net rate of photosynthesis in the two genotypes. Melatonin increased CAT activity, whereas it had no effects on the activities of POD and SOD in the root of droughted plants. Pretreatment of oil seed rape with melatonin and subsequent exposure to drought stress stimulated the growth of taproot and lateral roots and prevented stomatal closure under drought conditions. The findings provide further evidence for a physiological role of melatonin and a theoretical basis for melatonin application on improving drought resistance in agricultural practice. Future studies should investigate the molecular mechanisms of melatonin's functions and the possible effects of melatonin application on the oil yield and appropriate oil compositions of oil seed rape.

Competing interests

The authors declare no competing interests.

Author contributions

CLZ and JL proposed the experiment. LLD designed and conducted the experiment, collected and analysed the data and wrote the manuscript. CLZ, HH and XDZ revised the manuscript. All of authors approved the final manuscript.

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