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Triadimefon increases drought tolerance through the regulation of photosynthesis and carbohydrate metabolism in rapeseed at bolting stage

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Abstract

Drought is a major abiotic factor limiting agricultural crop production. The focus of this study was the effect of triadimefon (TDM) on rapeseed photosynthesis and carbohydrate metabolism in response to drought stress. Results showed that TDM increased plant dry mass per plant and reduced the damage to photosynthetic processes by regulating stomatal and nonstomatal factors, inducing photosynthetic pigment synthesis, and improving photosynthetic activity. Chloroplast degradation and senescence was reduced in rapeseed leaves with TDM under drought stress. TDM restored structural connections between chloroplasts and cell membranes and modified the chloroplasts – from slightly elongated ellipses to archetypical ellipses. TDM also regulated enzymatic activity of sugar metabolism. These results indicate that TDM improved drought tolerance through the regulation of photosynthesis and carbohydrate metabolic pathways in rapeseed at the bolting stage, thereby increasing biomass under drought stress.

Additional key words: *Brassica napus* L.; chlorophyll fluorescence; photosynthetic parameters; soluble sugar; ultrastructure.

Introduction

Global climate change caused by rising atmospheric CO_2 concentrations reduces the quality of available agricultural land (Peters *et al*. 2012). According to the Intergovernmental Panel on Climate Change 2007 (IPCC 2007), the number of warm days and nights and the frequency of heat waves have increased in large parts of the world (Mittler and Blumwald 2010). Together with increases in the land areas experiencing drought, the frequency and severity of drought are predicted to increase as a result of climate change (Jury and Vaux 2005, IPCC 2007).

Drought stress can directly inhibit seedling establishment, resulting in lower plant densities and reduced yield. Rapeseed (*Brassica napus* L.), also known as rape, is cultivated mainly for its oil-rich seeds, and is very sensitive to drought stress. China is one of the largest suppliers of rapeseed oil in the world. With the drought as a major yield-limiting factor for this crop, improving drought tolerance is of critical importance. The Yangtze River Basin is the main distribution area of winter rapeseed in China, where the distribution of annual rainfall is uneven, highlighted by frequent seasonal drought succeeded by abundant rainfall. This variable climate situation causes huge losses in the rapeseed production in China (Zhang *et al*. 2014).

Drought causes chloroplast senescence and structural damage leading to a reduction in photosynthesis (Fahad *et al*. 2017). Drought stress thereby disrupts carbohydrate metabolism by decreasing the photosynthetic rate (P_N) in leaves (Mak *et al*. 2014), which is expected to reduce available sugars for assimilation in sink organs. An inhibition of growth processes is also likely (Liu *et al*. 2004). The bolting stage of rapeseed is an important period when vegetative growth is exuberant, with higher requirements for carbohydrates assimilation. Therefore, the bolting stage is a period relatively sensitive to water, and is directly related to the overall growth of rapeseed (Zhang *et al*. 2014).

Increasing plant tolerance to stress is of utmost importance in rapeseed maintenance. Application of plant growth regulators is considered as an effective way to increase plant resilience in this respect (Tuna 2012). One such augmenter,

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Abbreviations: AI – acid invertase; C_i – intercellular CO_2 concentration; Chl – chlorophyll; DAT – days of treatment; E – transpiration rate; FBP – fructose-1,6-bisphosphatase; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; NI – neutral invertase; NPQ – nonphotochemical quenching; P_N – photosynthetic rate; q_p – photochemical quenching coefficient; SEM – scanning electron microscopy; SPS – sucrose phosphate synthase; TDM – triadimefon; Φ_{PSII} – actual photochemical efficiency of PSII.
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triadimefon [TDM, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-l)butan-2-one] is a triazole compound with both fungicidal and plant growth regulatory properties (Jaleel *et al*. 2008a). Given that rapeseed is susceptible to fungal diseases during the bolting stage, triadimefon application is a preferred method for protecting rape plants from fungal diseases in China. Additionally, triadimefon protects plants from several types of abiotic stresses, such as salinity (Jaleel *et al*. 2008b), heat, cold (Feng *et al*. 2003), and drought (Manivannan *et al*. 2008). Such protection is accomplished by influencing hormonal balances, which inhibit gibberellin (GA) synthesis, thereby reducing ethylene content and increasing cytokinines (Jaleel *et al*. 2008a). Under normal water conditions, TDM treatment of white yam increases starch, soluble sugars content, and biomass (Jaleel *et al*. 2007b). In white yam, TDM increased also chlorophyll (Chl) content, stimulated rooting, increased antioxidant potentials, and enhanced alkaloid production (Jaleel *et al*. 2007a). In our previous study, we reported that triadimefon induced C and N metabolism and root ultrastructural changes under drought stress in soybean at flowering stage (Wu *et al*. 2013, Zhou *et al*. 2015). This indicated TDM could be used to improve rapeseed drought tolerance. However, the mechanism, by which TDM changes carbohydrate metabolism through manipulation of photosynthetic parameters, is not clearly understood.

In this study, we identified the effects of TDM on morphological growth indices, photosynthesis, and carbohydrate metabolism. Pot experiments were carried out at the bolting stage in rapeseed under drought-stress conditions. We proposed a potential mechanism for TDM-regulated photosynthesis and carbohydrate metabolism in rapeseed.

Materials and methods

Plant material, growth conditions, and treatments: A pot experiment was conducted at the Pailou Research Station, Nanjing, Jiangsu Province, China (32°02'N, 118°50'E) from October 2014 to May 2015. Rapeseed (*Brassica napus* L.), cv. Nannong 4, was used in the experiment. The soil used in this experiment was a yellow-brown loam with 23.4 mg(organic matter) kg^{-1}, 11.6 g(total N) kg^{-1}, 83.6 mg(available N) kg^{-1} , 13.7 mg(available P) kg^{-1}, and 91.7 mg(available K) kg^{-1}. Seeds of the Nannong 4 were sown in plastic pots (25 × 20 cm) containing 8.5 kg of soil in October 2014, and were grown under natural weather conditions.

At the bolting stage (March and April 2015), the seedlings were transferred to the greenhouse with an air relative humidity of 65–80% and PPFD of 900 μmol m^{-2} s^{-1} at noon. To minimize evaporation from the soil and protect the soil from direct sunlight, all pots were covered with 2-cm layer of cotton.

The experimental setup resulted in three treatments: CK [well-watered, relative water content (RWC) = 70%], D (drought, RWC = 45%), and DT (drought, sprayed with TDM, RWC = 45%). Pots were arranged in completely randomized block design with three replicates for each treatment. At the bloting stage, rapeseed plants were sprayed with 240 mg(TDM) kg^{-1} (DT treatment; spraying concentration was based on our preliminary experiment), and treatments of CK and D were sprayed with the same amount of water on 10 March. After the spraying, drought-stress treatments were realized by watering plants with limited water every day. Soil relative water content in D and DT treatments was kept at 45% every day by weighing the pots at dusk. After 20 d of drought stress, soil was rewatered to 70% RWC. The plants for CK treatments remained under normal water conditions. The top third fully expanded leaves were collected at 9:00 h at 0, 5, 10, 15, 20 d after drought stress (DAT) and 5 d after rewatering. The samples were frozen in liquid nitrogen, and then stored at –40°C until used for analyses of carbohydrate contents and enzyme activities of carbohydrate metabolism. The top third fully expanded leaves were sampled at 15 d after rewatering for morphology and structure observation with transmission electron microscopy and photographing with a camera, respectively.

Photosynthetic parameters: Photosynthetic rate (P_N), transpiration rate (E), intercellular CO_2 concentration (C_i), and stomatal conductance (g_s) were determined using an infrared gas analyzer (*IRGA*) portable photosynthesis system (*LI-6400XT*, *Li-COR Biosciences*, USA). Photosynthesis measurements were performed between 9:00–11:00 h with a saturating PPFD of 1,200 μmol(photon) m^{-2} s^{-1} on top third fully expanded leaves at 0, 5, 10, 15, and 20 DAS and 5 d after rewatering.

Chl *a* fluorescence measurement: Chl *a* fluorescence parameters were obtained using a portable photosynthesis system (*LI-6400*, *Li-COR Inc.*, USA) and an LED-based fluorescence source (*6400-40 LCF*). A brief period of saturating light [> 7,000 μmol(photon) m^{-2} s^{-1}] was used on dark-adapted leaves (F_m). The maximum quantum yield of PSII photochemistry (F_v/F_m), quantum efficiency of PSII photochemistry (Φ_{PSII}), nonphotochemical quenching (NPQ), and photochemical quenching coefficient (q_p) were measured for top third fully expanded leaves at 0, 5, 10, 15, and 20 DAS and 5 d after rewatering.

Photosynthetic pigment determination: For the Chl content of the leaves, Chl was extracted from 4–5 pieces of fresh leaves with 80% acetone extract, and the absorbance of the resulting extracts was measured by ultraviolet-visible spectrophotometer (*DU-730*, *Beckman*, USA) at wavelengths of 645 and 663 nm. The Chl concentration was then calculated following the method of Arnon (1949).

Stomatal structure and chloroplast ultrastructure: Scanning electron microscopy (SEM) images of stomata were obtained from leaves detached and fixed in glutaraldehyde (2.5%); images were obtained by environmental SEM (*S-3000N*, *HITACHI*, Japan) (Gao *et al*. 2018). Stomatal densities, lengths, and apertures were measured randomly using *Image J* software.

Transmission electron microscope (TEM) images of chloroplasts from leaves were obtained by a following procedure. Briefly, 0.5-cm^2 samples of leaves were kept in 2.5% glutaraldehyde (solved in 0.1 mol L^{-1}, pH 7.2, phosphoric acid buffer), fixed with 1% osmium tetroxide, dehydrated with graded concentrations of ethanol (50, 70, 80, 90, 95, and 100%), embedded with *Epon-812* resin, and sliced using an ultramicrotome (*EM-UC7*, *Leica*, Germany). The slices were observed and photographed with TEM (*H-7650*, *HITACHI*, Japan) (Torrance *et al*. 2006).

Determination of contents of carbohydrates: The soluble sugar, glucose, fructose, sucrose, and starch content were estimated as described by Durand *et al*. (2018). Tissue of each sample (100 mg) was ground with a glass rod and extracted in a sealed container in 5 ml of 20:80 (v/v) aqueous ethanol. The supernatant and pellet were used for soluble sugar measurements. For each sample, 50 mg of dry leaf mass was extracted in 2 ml of 80% (v/v) methanol in an 80°C water bath for 40 min. The extract was centrifuged at 5,000 × *g* for 10 min to get the supernatant for the sugar measurements. The reaction mixture was heated to 100°C in a water bath for 10 min (1 ml of supernatant, 4 ml of 98% sulfuric acid, and 1 ml of 5% phenol). The cooled reaction mixture was determined at 490 nm using spectrophotometer (*DU-730*, *Beckman*, USA) and D-glucose as standard.

Enzyme activities of carbohydrate metabolism: For the extraction of enzymes, 0.2-g samples were ground in 5 ml of solution containing 50 mM Hepes-NaOH buffer (pH 7.5), 1 mM EDTA, 0.5 mM $MgCl_2$, 2 mM diethyldithiocarbamic acid, 2% polyvinylpyrrolidone, 1% bovine serum albumin, and 2.5 mM dithiothreitol. After centrifuging for 20 min at 12,000 × *g*, the supernatant was collected and added into reaction solution to analyze the activity of sucrose synthase (synthesis direction)/ sucrose synthase (decomposition direction) (EC 2.4.1.13), sucrose phosphate synthase (SPS, EC 2.4.1.14), fructose-1,6-bisphosphatase (FBP, EC 3.1.3.11), acid invertase (AI, EC 3.2.1.26), and neutral invertase (NI, EC 3.2.1.26). Activities were determined by using the method described in Zhou *et al*. (2015). Enzyme activities were expressed in mg(substrate) min^{-1} mg^{-1}(protein).

Statistical analysis: All data were analyzed with *SPSS v. 18.0* for *Windows* (*SPSS Inc.*, Chicago, IL, USA). All analyses were carried out in biological triplicates (three different plants). Main effects of plant biomass, agronomic characteristics, yield, photosynthesis, Chl fluorescence, and C metabolism were compared by the *Duncan*'s multiple range tests at the 5% level of significance using data at 20 DAT. Significance of differences between mean numbers of open stomata and photosynthetic pigments were compared by *Duncan*'s multiple range tests at the 5% level of significance using data at 15 DAT.

Results

Plant biomass: Under drought stress, the shoot and root dry mass decreased by 47 and 36%, respectively, at 20 DAT compared with CK (Fig. 1). After pretreatment with TDM, the shoot dry mass increased by 20% compared with D, while root dry mass increased by 18%.

Photosynthesis and Chl *a* fluorescence: Drought decreased rapeseed photosynthesis significantly. At 20 DAT, P_N, g_s, C_i, and E were only 8.1, 6.7, 27, and 32% compared with CK (Fig. 2). After TDM treatment, P_N, g_s, C_i, and E increased 2.9, 1.0, 1.9, and 1.1 times at 20 DAT under drought stress, respectively. After rehydration, the recovery rate of each photosynthetic parameter after DT was faster than that of D, so that the plants were closer to normal growth.

Drought stress caused a significant reduction in F_v/F_m, Φ_{PSII}, and q_p. At 20 DAT, F_v/F_m, Φ_{PSII}, and q_p fell by 28, 36, and 38%, respectively. NPQ increased first and then declined, but the values were always higher than those of CK (Fig. 3). TDM could alleviate the decline of F_v/F_m, Φ_{PSII}, and q_p caused by drought stress. At the 20 DAT, DT promoted F_v/F_m, Φ_{PSII}, q_p, and NPQ, which increased by

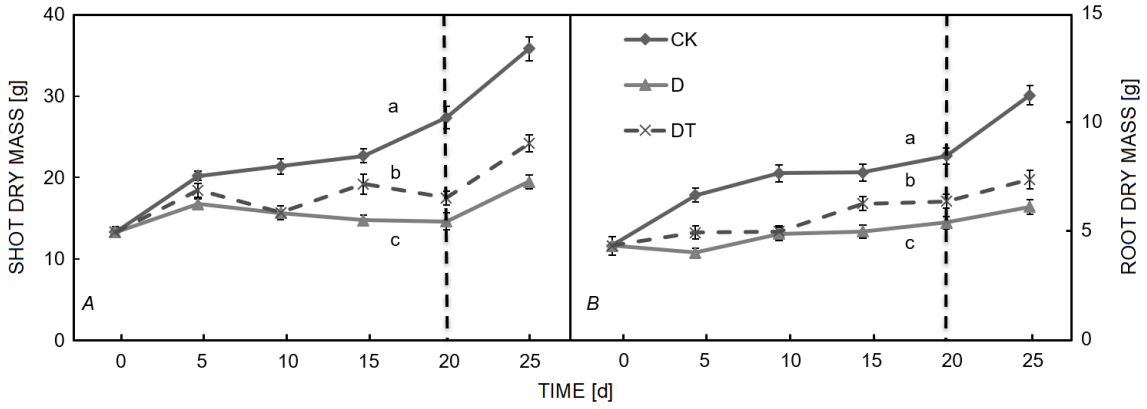

Fig. 1. Effects of triadimefon (TDM) on shoot and root dry mass in rapeseed under drought stress and after rewatering at bolting stage. *Vertical line* is the division between drought and rewatering. Values are means ± SD, $n = 4$. Values with *different letter* are significantly different according to *Duncan*'s test ($P<0.05$). CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

25, 30, 21, and 13%, respectively. After rehydrating, F_v/F_m, Φ_{PSII}, q_p, and NPQ could return to normal level.

Stomatal closure: The stomata of CK plants were in an open state and the individual pores were larger. Drought treatment led to stomatal closure, the number of open

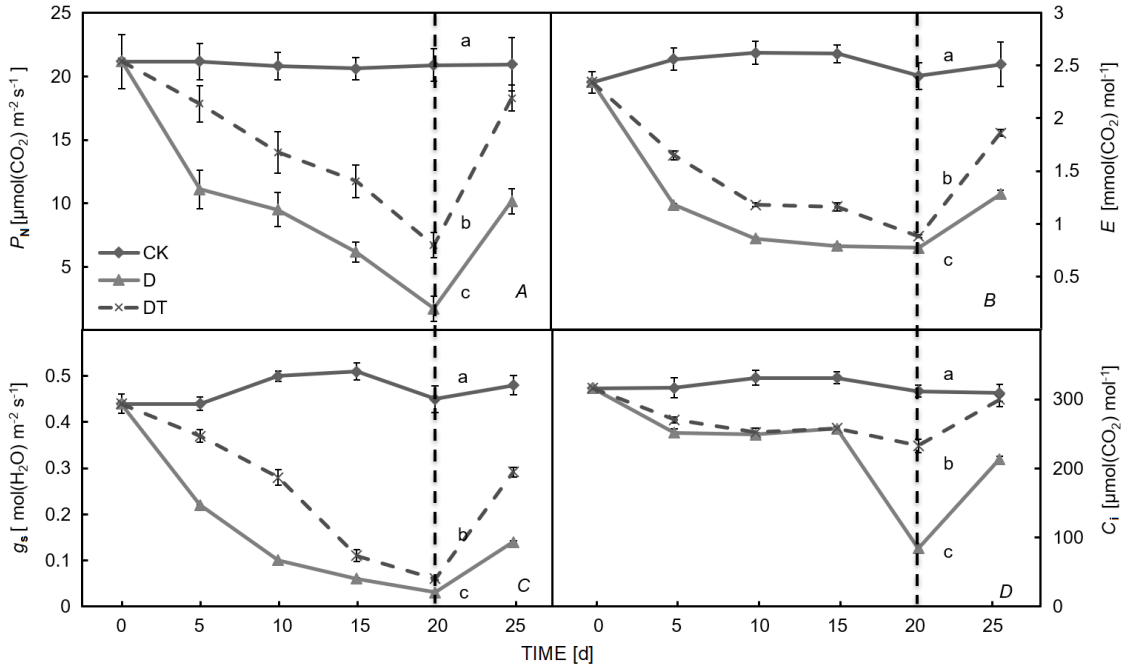

Fig. 2. (*A*) Effects of triadimefon (TDM) on net photosynthetic rate (P_N), (*B*) transpiration rate (*E*), (*C*) stomatal conductance (g_s), and (*D*) intercellular CO_2 concentration (C_i) in rapeseed leaves under drought stress and after rewatering at bolting stage. *Vertical line* is the division between drought and rewatering. Values are means ± SD, $n = 4$. Values with *different letter* are significantly different according to *Duncan*'s test ($P<0.05$). CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

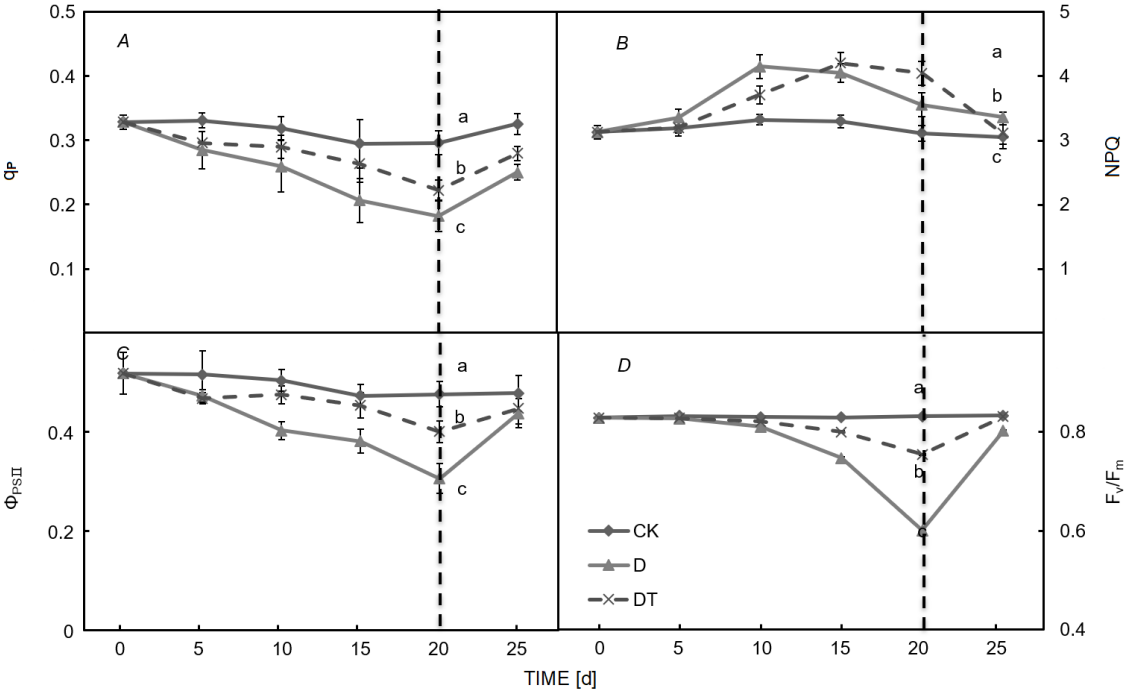

Fig. 3. Effects of triadimefon (TDM) on photosynthetic fluorescence in rapeseed leaves under drought stress and after rewatering at bolting stage. The maximum quantum yield of PSII photochemistry (F_v/F_m), quantum efficiency of PSII photochemistry (Φ_{PSII}), photochemical quenching coefficient (q_p), nonphotochemical quenching (NPQ). *Vertical line* is the division between drought and rewatering. Values are means ± SD, $n = 4$. Values with *different letter* are significantly different according to *Duncan*'s test ($P<0.05$). CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

stomata at 15 DAT was reduced to 25% of that of CK (Fig. 4). After TDM pretreatment, the number of open stomata increased by 21% compared to the D treatment, and the stomata were surrounded by a small amount of wax.

Photosynthetic pigments content: The Chl content was relatively stable in well-watered CK plants. Drought treatment resulted in a decline in the Chl content. We found that the green color of the leaves was bleached under drought, being then restored after TDM spraying obviously (Fig. 5*A*). The content of Chl was 34% lower than that of DT at 20 DAT. After pretreatment with TDM, the content of Chl showed a gradual decline, but was always higher than that of D. The Chl content of DT increased by 21 and 29% at 15 and 20 DAT, respectively. After rehydration, the Chl content in plants treated by DT could recover faster to normal level than that of plants after D treatment (Fig. 5*B*).

Chloroplast ultrastructure: Under nonstressed conditions, the chloroplasts in the leaves were elongated ellipses that contained well-arranged grana and smooth thylakoid membranes. Under drought stress, chloroplasts showed obvious shrinkage, had an increased number and size of osmiophilic particles (Fig. 6). Far from the cell wall, the chloroplast envelope was partially ruptured, and the thylakoid membranes were loose and disrupted, whereas the thylakoids were overly disorganized. TDM restored the connection between the chloroplasts and cell membranes in the drought-stressed rapeseed leaves, and the shape of the chloroplasts changed slightly from elongated ellipses to ellipses. TDM improved internal lamellar system and reduced number of osmiophilic particles.

Total soluble sugar, sucrose, fructose, glucose, and starch: Drought influenced the content of all kinds of soluble sugars. They showed the highest concentration at 10 DAT. Compared with CK, the total soluble sugars, sucrose, and fructose content of D increased by 15, 15, and 91%, respectively. The content of soluble sugars, sucrose, glucose, fructose, and starch in D declined by 18, 29, 22, 29, and 26%, respectively, at 20 DAT. During the drought period and recovery days, leaf fructose and sucrose content increased significantly with TDM treatment. At 10 DAT, the content of soluble sugars, sucrose, glucose, fructose, and starch under DT treatment increased by 8.5, 29, 7.6, 45, and 11%, respectively (Fig. 7).

Enzyme activities of carbohydrate metabolism: Under drought stress, the enzyme activity of SPS and sucrose synthase reached a maximum at 5 DAT showing an increase of 52 and 26% compared to CK, respectively (Fig. 8). At 20 DAT, these enzyme activities decreased by 30 and 61% compared with CK, respectively. TDM treatment increased the SPS and sucrose synthase enzyme activity under drought stress significantly and delayed the reduction of their activity at 15 and 20 DAT. DT increased blade SPS and SS (synthesis direction) activity by 46 and 62% at 15 DAT and by 92 and 92% at 20 DAT, respectively. Under drought stress, FBP activity increased first and then decreased, reaching its maximum at 10 DAT (26% increase compared to CK) and minimum at 20 DAT

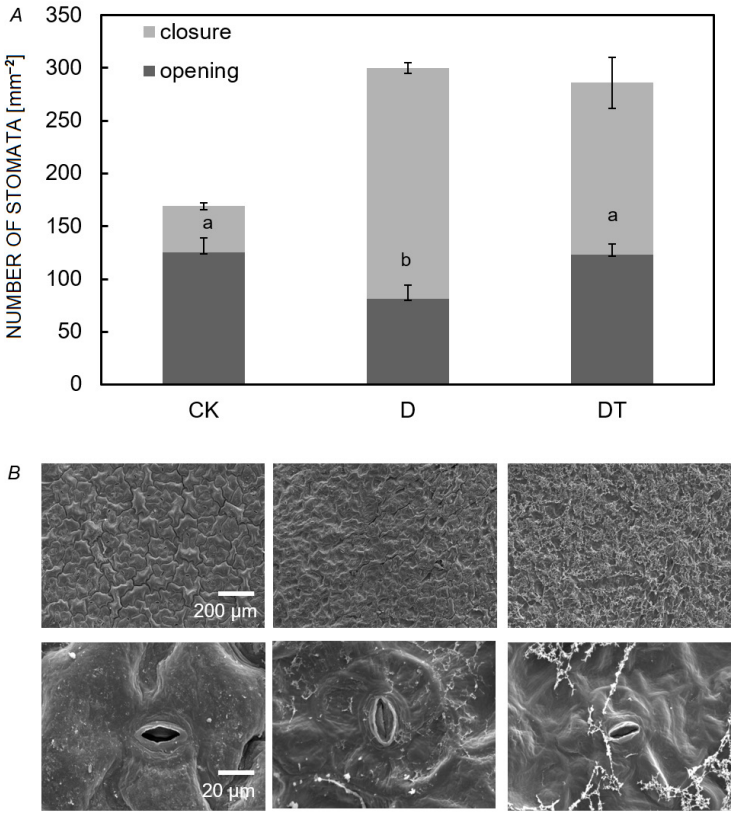

Fig. 4. Effects of triadimefon (TDM) on stomatal characteristics in rapeseed leaves under drought stress at bolting stage (15 DAT). (*A*) The number of closed and open stomata under different treatments. Values are means ± SD, n = 50. Values with *different letter* are significantly different according to *Duncan*'s test (P<0.05). (*B*) The photos of stomata under drought stress. CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

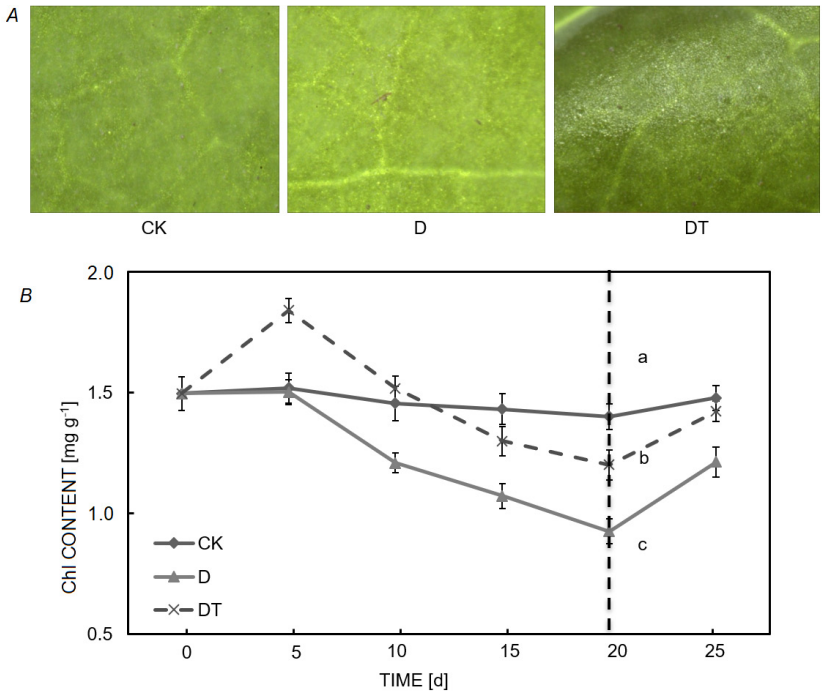

Fig. 5. Effects of triadimefon (TDM) on photosynthetic pigments (Chl) of rapeseed leaves subjected to drought stress and after rewatering at bolting stage. (*A*) The photos of top third fully expanded leaves were taken at 15 d after treatment. (*B*) Chlorophyll content. Values are means ± SD, $n = 4$. Values with *different letter* are significantly different according to *Duncan*'s test ($P<0.05$). CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

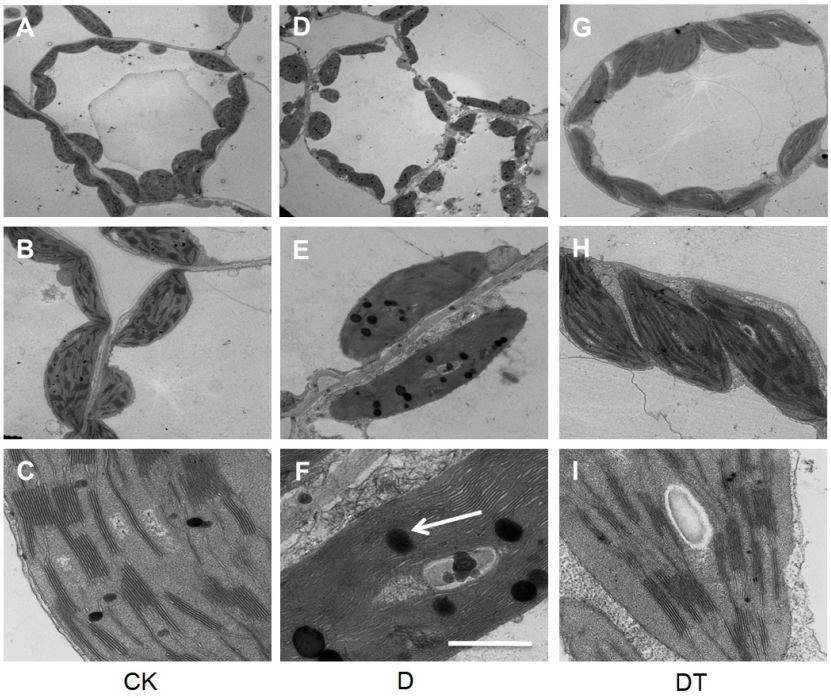

Fig. 6. Effects of triadimefon (TDM) on utrastructure of chloroplast under drought conditions in rapeseed at bolting stage. Leaves were sampled 15 d after treatment. *Note*: Arrows point to osmiophilic granules. Scale bars equal 2 μm. CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

(25% decrease compared to CK). TDM could promote the increase and reduce the decrease of FBP activity.

The sucrose synthase (SS, decomposition direction), acid invertase (AI), and neutral invertase (NI) are the main carbohydrate metabolism enzymes in plant leaves. Drought stress first increased and then decreased their activities. NI activity reached its maximum at 5 DAT of drought, which was 15% higher than that of CK, while AI and SS (decomposition direction) activity reached its maximum at 10 DAT (26 and 29% increase compared to CK, respectively). At 20 DAT of drought, the activity of AI, NI, and SS (decomposition direction) decreased by 72, 25, and 16%, respectively. TDM could promote the increase and reduce the decrease of SS (decomposition direction), AI, and NI.

Discussion

Water deficit limits the growth and decreases the biomass of plants (Estrada-Campuzano *et al*. 2008). TDM has been reported to improve the drought-stress tolerance (Manivannan *et al*. 2008, Zhou e *et al*. 2015) and increase

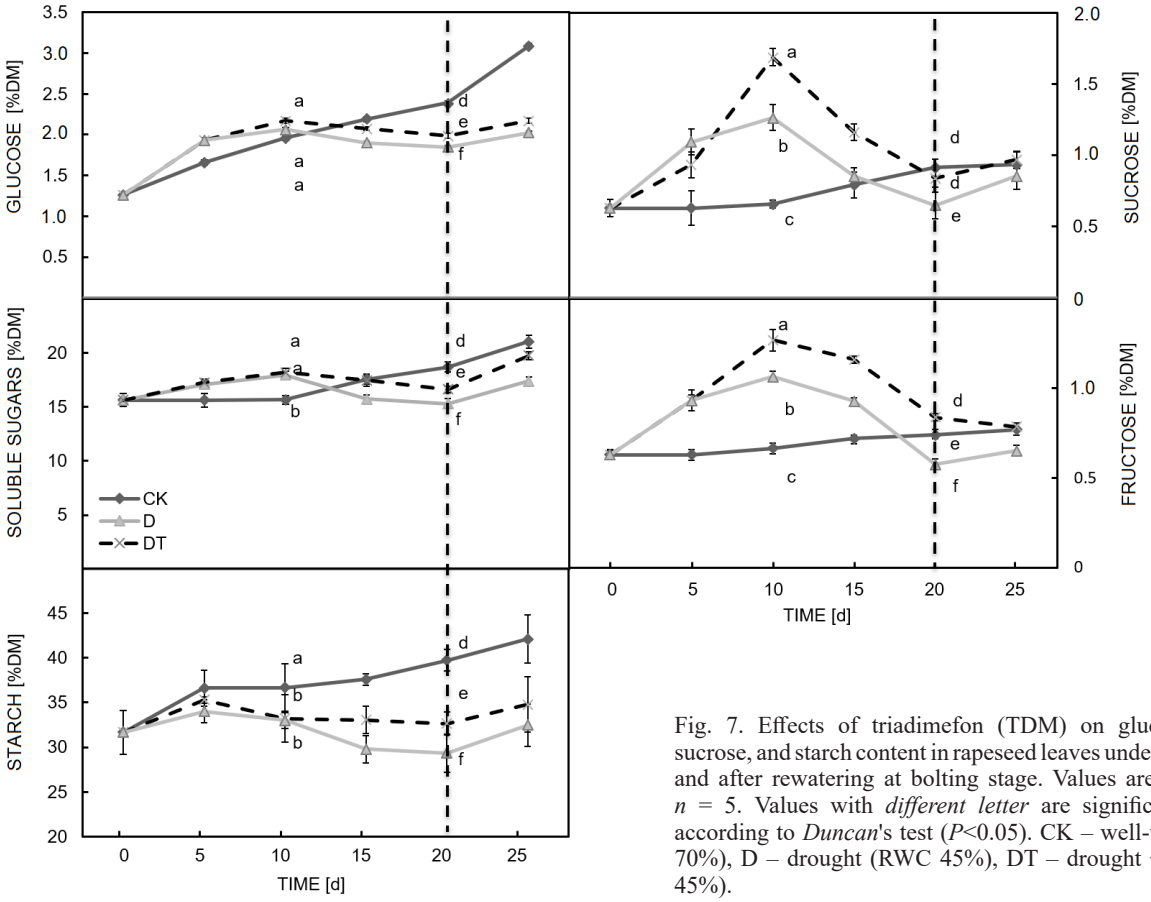

Fig. 7. Effects of triadimefon (TDM) on glucose, fructose, sucrose, and starch content in rapeseed leaves under drought stress and after rewatering at bolting stage. Values are means ± SD, n = 5. Values with *different letter* are significantly different according to *Duncan*'s test (P<0.05). CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

the contents of starch and sucrose, manifested as fresh and dry mass, in white yam (Jaleel *et al*. 2007b). Our results showed that TDM reduced the adverse effects of drought on growth and development of rapeseed. TDM increased the shoot and root dry mass.

Yield and dry mass are complex integrations dependent on the strength of source, plant's nutrient sources, assimilate sinks, streaming capabilities, and stress resistance (Fahad *et al*. 2017). Photosynthesis and carbohydrate metabolism are important factors affecting plant growth in this regard. Drought affects rapeseed at all stages of development, even more so at the bolting stage, which results in significant yield reduction (Zhang *et al*. 2014). The bolting stage in rapeseed is sensitive to water deprivation and requires more carbohydrate assimilates than other developmental stages. To achieve high growth rates, the bolting stage must overcome environmental stresses. The reduction of growth in rapeseed induced by drought might be due to a decrease in the rate of photosynthesis (Flexas *et al*. 2004) and disturbances in assimilate partitioning (Farooq *et al*. 2009). The F_v/F_m ratio is a sensitive indicator of plant photosynthetic performance (Björkman and Demmig 1987). It reflects the maximum efficiency of photosynthetic apparatus converting the absorbed light energy into chemical energy, and has been widely used for the detection of photoinhibition (Herppich and Peckmann 2000, Gitelson *et al*. 2003). Drought stress mainly harms PSII and regulates the electron transfer rate and photochemical efficiency which can cause the reduction of CO_2 assimilation ability (Huber *et al*. 1984).

In our study, TDM pretreatment improved the rapeseed F_v/F_m, Φ_{PSII}, and q_p values, and increased the light energy utilization rate. Together, these improved indices provide evidence for the TDM-dependent improvement in photosynthesis under drought stress. Carbon fixation is mainly accomplished through photosynthesis which is affected by stomatal closure, photosynthetic machinery damage, and leaf senescence under drought stress (Jones and Rawson 2006, Farooq *et al*. 2009). Stomatal closure is the foremost response to drought stress in order to cut down water loss through transpiration (Ludlow and Muchow 1990, Cornic *et al*. 2000). The change of g_s leads to the reduction of C_i, which limits the internal concentration used for carbon fixation (Keller and Ludlow 1993, Lawlor and Cornic 2002). In order to decrease water loss and reduce the effect of stomatal closure on photosynthesis, plants under drought stress increase the number of stomata and thicken the wax cover on the leaf surface (Wahid *et al*. 2007). Water deprivation also reduces the Chl content and damages the photosynthetic structures, such as thylakoid membranes (Fu and Huang 2001, Din *et al*. 2011). This destruction is attributed to the variation in the activities of enzymes, Chl biosynthesis and leaf senescence (Jaleel *et al*. 2008a, Anjum *et al*. 2011). The senescence of leaves and the destruction of grana lamellae are also important factors leading to the decrease of photosynthetic enzyme activity

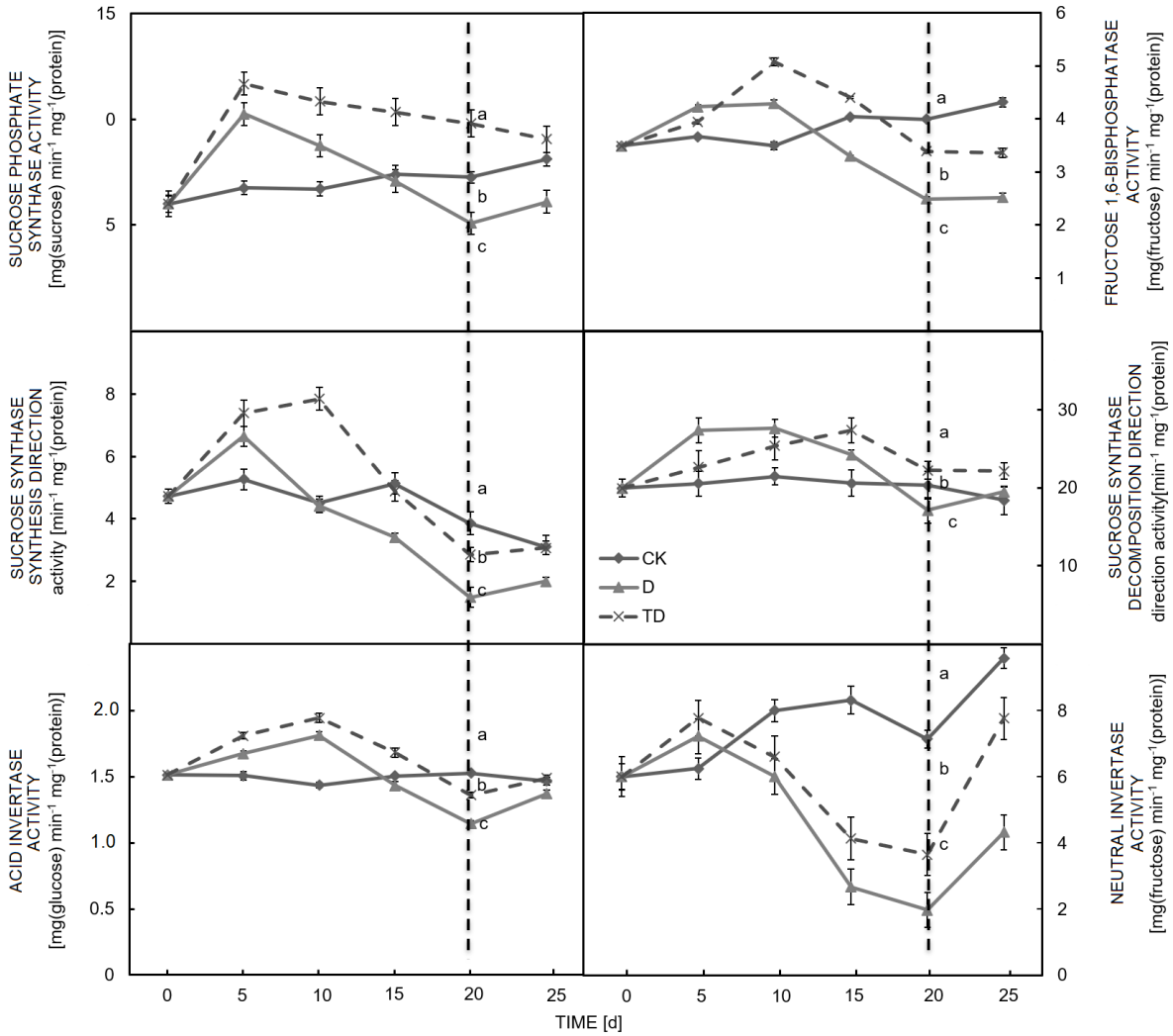

Fig. 8. Effects of triadimefon (TDM) on enzymes activities of carbohydrates metabolism in rapeseed leaves under drought stress and after rewatering at the bolting stage. Values are means ± SD, $n = 5$. Values with *different letter* are significantly different according to *Duncan*'s test ($P<0.05$). CK – well-watered (RWC 70%), D – drought (RWC 45%), DT – drought + TDM (RWC 45%).

and chloroplast degradation (Farquhar and Sharkey 1982).

Triazole-treated plants have been reported to increase Chl contents, showing enlarged chloroplasts, thicker leaf tissue, and increased root-to-shoot ratio (Asami *et al*. 2003, Manivannan *et al*. 2008). In our study, we found that the number of open stomata increased significantly after TDM treatment. Examination of leaf surface by SEM revealed that TDM increased the wax covering on the surface of the leaf blade and more stomata transformed into spongy mesophyll cells. We observed the ultrastructure of chloroplasts and determined that after drought treatment, rapeseed leaf chloroplasts displayed obvious shrinkage, plasmolysis, and the numbers of osmiophilic particles increased in leaf mesophyll cell; these are the characteristics of leaf senescence. However, TDM pretreatment delayed leaf senescence significantly, as indicated by full spindle superposition of chloroplasts alignment of grana lamella, and increase in overall Chl content. It has been reported that TDM can induce the increase of cytokinin and auxin content (Jaleel *et al*. 2007a). Cytokinin and auxin can delay leaf senescence (Peleg *et al*. 2011, Wang and Blumwald 2014), and as such, these hormonal changes may lead to the reduction of chloroplast degradation. Indeed, these findings support the idea that TDM can reduce damage to rapeseed photosynthesis brought about by drought stress by regulating the stomatal conductance, reducing chloroplast senescence, and improving photosynthetic enzyme activity. TDM treatment provides a material basis for the improvement of rape dry mass by increasing carbohydrate metabolism under drought stress.

Carbohydrate metabolism and associated transport from source to sink is also of great importance in order to enhance the production. The decrease in water availability induces a variety of physiological and metabolic changes in C metabolism (Reddy *et al*. 2004). Soluble sugars, especially sucrose, are the main form of sugar transported over long distances (Taiz and Zeiger 2006). Furthermore, an increase in soluble sugar content is indicative of

improved osmotic potential in leaves. Drought increased the content of sucrose, glucose, fructose, and the total soluble sugars in rapeseed leaves at the onset of the study. With prolonged drought conditions, the content of soluble sugars decreased gradually. TDM treatment significantly reduced the observed decrease of soluble sugars, especially sucrose and fructose. This phenomenon is caused by the increase of carbon assimilation by photosynthesis and may be related to the improvement of carbohydrate metabolic enzyme activity. Activation of SPS, as one of the first sites at which spinach leaves respond to drought stress, has been proposed and this regulation is likely important for osmotic regulation (Quick *et al*. 1989). Interestingly, TDM treatment maintained SPS activity at high levels even after 15 d of drought stress. As mentioned, photosynthesis remained highly efficient under TDM treatment, and can thus provide enough carbon to synthesize sucrose. The increase of sucrose-decomposition enzyme, and concomitant AI and NI activity, also ensured a certain concentration of fructose and glucose in the leaves. Therefore, we suggest a mechanism whereby TDM promotes carbon fixation and metabolism under drought stress in rapeseed at the bolting stage, leading to an eventual increase in biomass.

Conclusions: TDM increased biomass, photosynthesis, and carbohydrate metabolism under drought stress in rapeseed at the bolting stage. TDM reduced the drought stress-associated damage to photosynthesis by regulating stomatal structure and reducing chloroplast senescence. TDM also increased the content of key soluble sugars. Increases in sugar contents in the rapeseed leaves were accomplished due to maintenance of relatively high activities of carbohydrate metabolic enzymes under drought stress.

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