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Physiological responses of *Brassica napus* to fulvic acid under water stress: Chlorophyll a fluorescence and antioxidant enzyme activity

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ABSTRACT

The ameliorative effect of fulvic acid (0, 300, and 600 mg L^{-1}) on photosystem II and antioxidant enzyme activity of the rapeseed (*Brassica napus* L.) plant under water stress (60, 100, and 140 mm evaporation from class A pan) was studied using split plots in a randomized complete block design with three replications. Results indicated that application of fulvic acid (FA) improved the maximum quantum efficiency of PSII (F_v/F_m) and performance index (PI) of plants under both well-watered and limited-water conditions. The time span from F_o to F_m and the energy necessary for the closure of all reaction centers was significantly increased, but the size of the plastoquinone pool was reduced with increasing water stress levels. Plants treated with FA had higher peroxidase and catalase activities under all irrigation conditions. Activities of ascorbate peroxidase and superoxide dismutase in plants increased with increasing water stress. Malondialdehyde increased under severe water stress, but application of FA significantly decreased lipid peroxidation. Production of reactive oxygen species (ROS) is a common phenomenon in plants under stress. Under this condition, the balance between the production of ROS and the quenching activity of antioxidants is upset, often resulting in oxidative damage. In this study, application of FA significantly increased fluorescence of chlorophyll a, inhibiting ROS production and enhancing antioxidant enzymes activity that destroyed ROS. Thus, ROS in plant cells was reduced under water stress by application of FA and consequently lipid peroxidation was reduced.

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; FA, fulvic acid; MDA, malondialdehyde; MWS, moderate water stress; POD, peroxidase; SWS, severe water stress; WWC, well-watered condition; PI, performance index; PSII, photosystem II; SOD, superoxide dismutase.

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1. Introduction

Growth and development of plants depend on environmental conditions. Water stress is one of the major factors that affect crop production. Up to 45% of the world agricultural lands are subject to continuous or frequent drought stress [1]. To survive under water stress, plants have evolved morphological, physiological, and biochemical responses. Photosynthesis and cell growth are the primary processes affected by stress [2]. Water stress in higher plants affects PSII and antioxidant activities.

Water stress may result in photo-inhibition that includes photo-damage to the photosynthetic apparatus, causing irreversible inactivation of PSII. Water stress also reduces photosynthetic efficiency or increases the dissipation of excess excitation energy as heat, and these effects may lead to changes in fluorescence parameters [3]. Chlorophyll a fluorescence, though corresponding to a very small fraction of the dissipated energy from the photosynthetic apparatus, is generally accepted as providing access to understanding of its structure and function [4]. PSII is responsible for chlorophyll a fluorescence and the rate of PSII photochemical conversion. Chlorophyll a fluorescence allows us to study the different functional levels of photosynthesis indirectly. It is useful to investigate the effects of environmental stresses on plants, given that photosynthesis is often reduced in plants experiencing adverse conditions. Thus, analysis of chlorophyll a fluorescence is considered an important approach for evaluating the health or integrity of the internal apparatus during photosynthesis [5].

Water stress is one type of oxidative stress that, at the cellular level, enhances the generation of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. ROS in plant cells are controlled by enzymatic and non-enzymatic scavenging mechanisms. Numerous studies have shown that the activity of antioxidant enzymes is correlated with plant tolerance to abiotic stresses, including responses to drought stress in wheat (*Triticum aestivum* L.) [6,7], alfalfa (*Medicago sativa* L.) [8], rice (*Oryza sativa* L.) [9], and chickpea (*Cicer arietinum* L.) [10]. Superoxide dismutase (SOD), considered to be the first defense against ROS, is responsible for the dismutation of O_2^- to H_2O_2 and O_2. CAT, APX, and POD are enzymes that catalyze the conversion of H_2O_2 to water and O_2 [11]. The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signaling and/or damage will occur [12].

Soil organic matter consists mainly of humic and fulvic acids, which are called humin materials [13]. Application of humin materials to plants affects cell membranes, leading to enhanced transport of minerals, improved protein synthesis, plant hormone-like activity, promoted photosynthesis, modified enzyme activities, solubilization of micro and macro elements, reduction of active levels of toxic minerals, and increased microbial populations [14]. Rapeseed (*Brassica napus* L.) is the third largest oilseed crop in the world, following oil palm (*Elaeis guineensis*) and soybean (*Glycine max* Merr.) [15]. It is unknown whether the application of fulvic acid can improve PSII and antioxidant activities of plants under water-deficit conditions. The present study aimed to investigate this question in rapeseed.

2. Materials and methods

2.1. Plant material and treatments

A split plot experiment (using RCB design) with three replications was conducted in 2012 and repeated in 2013 at the University of Payame Noor, Iran, to determine the effects of foliar application of fulvic acid on PSII and antioxidant enzyme activity of rapeseed (*B. napus*) under water stress. Three irrigation treatments, including well-watered conditions (WWC), moderate water stress (MWS), and severe water stress (SWS) (60, 100, and 140 mm evaporation from class A pan, respectively), represented main plots and three concentrations of fulvic acid, including 0 (control), 300, and 600 mg fulvic acid L^{-1}, were allocated to subplots. Seeds of rapeseed were treated with 2 g kg^{-1} benomyl and sown on May 22, 2013 at 3 cm depth in a sandy loam soil. Seeding density was 90 seeds m^{-2}. Each plot consisted of 4 rows of 4 m length, spaced 25 cm apart. All plots were irrigated immediately after sowing, and after seedling establishment, plants were thinned to 65 plants m^{-2}. Subsequent irrigations were applied according to the treatments. The two levels of fulvic acid were sprayed on plants at vegetative growth and early flowering stages.

2.2. Chlorophyll a fluorescence measurements

Induction of chlorophyll a fluorescence was monitored with a handy-PEA portable fluorometer (Hansatech, UK) at the flowering stage. Fluorescence emission from the upper surface of the leaves was monitored. Leaves dark-adapted for 30 min and then were exposed to the saturated white light to estimate the initial (F_o) and maximum (F_m) fluorescence values, respectively. The performance index (PI) parameter (photosynthesis relative vitality), T_{FM} (ms; time taken to reach F_m, an indicator of quinone (Q_A), the reduction rate of the PSII acceptor; thus, the rate of PSII electron transport) and *Area* (the area above the fluorescence induction curve between F_o and F_m, a measure of the size of the plastoquinine pool in PSII) were also monitored. According to Kalaji et al. [16],

$$F_v/F_m = (F_m - F_o)/F_m$$

(a value related to the maximum quantum yield of PSII)

$$S_m = Area/(F_m - F_o)$$ (representing energy necessary for the closure of all reaction centers).

2.3. Enzyme assays

At the flowering stage, young leaves of rapeseed plants were collected and enzyme activities were assayed. Leaf samples were collected in an ice bucket. Leaves were washed with distilled water and surface moisture was wiped off. Leaf samples (0.5 g) were homogenized in ice-cold 0.1 mol L^{-1} phosphate buffer (pH 7.5) containing 0.5 mmol L^{-1} EDTA, with a pre-chilled mortar and pestle. Homogenates were transferred to centrifuge tubes and centrifuged at 4 °C in a refrigerated centrifuge for 15 min at 15,000 ×*g*. The supernatant was used for enzyme assay.

2.3.1. Catalase

Catalase (CAT) activity was determined according to Aebi [17]. The reaction mixture contained 30 mmol L^{-1} H_2O_2 in a 50 mmol L^{-1} phosphate buffer (pH 7.0), and 0.1 mL enzyme in a total volume of 3 mL. CAT activity was estimated by decreased absorbance of H_2O_2 at 240 nm.

2.3.2. Peroxidase

The reaction mixture contained 25 mmol L^{-1} phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mmol L^{-1} H_2O_2, and enzyme extract. Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation [ε = 26.6/(mmol L^{-1} cm)] [18].

2.3.3. Ascorbate peroxidase

The reaction mixture contained 50 mmol L^{-1} potassium phosphate (pH 7.0), 0.2 mmol L^{-1} EDTA, 0.5 mmol L^{-1} ascorbic acid, 2% H_2O_2, and 0.1 mL of enzyme extract in a final volume of 3 mL. A decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated using the extinction coefficient ε = 2.8/(mmol L^{-1} cm). One unit APX activity was defined as 1 mmol ascorbate oxidized/mL per min at 25 °C [19].

2.3.4. Superoxide dismutase

SOD activity was determined according to Dhindsa and Dhindsa [20] and one unit (U) of SOD was the amount causing 50% inhibition of nitro blue tetrazolium (NBT) reduction in light. Enzyme activity was expressed in terms of specific activity (U mg^{-1} protein) [21].

2.4. Determination of lipid peroxidation rate

Lipid was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated following Cakmak and Horst [18] with some modifications. Fresh leaf samples (0.2 g) were ground in 5 mL of 0.1% (*w*/*v*) trichloroacetic acid (TCA) at 4 °C. Following centrifugation at 12,000 ×*g* for 5 min, 1 mL of the supernatant was added to 4 mL of 0.5% (*w*/*v*) thiobarbituric acid (TBA) in 20% (*w*/*v*) TCA. Samples were heated at 90 °C for 30 min and the reaction was stopped in an ice bath. After centrifugation at 10,000 ×*g* for 5 min, absorbance of the supernatant was recorded at 532 nm with a spectrophotometer and was corrected for nonspecific turbidity by subtraction of the absorbance at 600 nm.

2.5. Statistical analysis

All the data were analyzed according to the experimental design, using SAS 9.1.3 software. Trait means were compared by Duncan's multiple range test at $P \leq 0.05$.

3. Results

3.1. Chlorophyll a fluorescence

Application of fulvic acid (FA) significantly improved maximum efficiency of PSII activity (F_v/F_m) under all irrigation treatments. Treated plants with 600 mg L^{-1} of FA (FA2) had more F_v/F_m than those in other treatments under both well-watered and limited-water conditions. However, difference between F_v/F_m in FA-treated and untreated plants under moderate water stress (MWS) and severe water stress was not significant (Fig. 1-A).

Photosynthesis relative vitality of plants decreased with increasing water stress levels, but application of FA increased PI of plants under both well-watered condition (WWC) and water stress. The highest and lowest PI were recorded for FA_2-treated plants and the control, respectively, under all irrigation conditions. However, under WWC, there was no significant difference in PI either between NFA and FA_1 or between FA_1 and FA_2. Difference in PI between FA-treated and NFA was not significant under MWS and SWS (Fig. 1-B).

T_{FM} and energy necessary for the closure of all reaction centers (S_m) increased, but the size of the plastoquinine pool in PSII (Area) decreased significantly with increasing water stress levels (Table 1).

T_{FM} was significantly affected by application of FA. FA_2-treated plants had lower T_{FM} than did plants under other treatments. No significant difference in T_{FM} of FA_1-treated and NFA plants was observed (Fig. 2).

3.2. Antioxidant activity

3.2.1. Peroxidase (POD)

The POD activity of plants under water stress was higher than that of WWC plants. Application of FA significantly increased POD activity under all irrigation conditions. Maximum and minimum activity of POD were observed in plants treated with FA_2 under SWS and NFA plants under WWC, respectively. POD activity in FA_1-treated plants was similar ($P \leq 0.01$) to that of NFA and FA_2-treated plants under well-watered and limited-water conditions (Fig. 3-A).

3.2.2. Catalase activity (CAT)

CAT activity of rapeseed plants increased as a result of water stress and application of FA. CAT activity increased with application of FA under all irrigation treatments. There was no significant difference in CAT activity in plants treated with different concentrations of FA (300 and 600 mg L^{-1}). Also, under MWS and SWS, CAT activity in plants sprayed with FA_1 was similar ($P \leq 0.01$) to that of plants with NFA and FA_2 (Fig. 3-B).

3.2.3. Ascorbate peroxidase (APX) and superoxide dismutase (SOD)

Ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities of rapeseed plants were significantly affected by different levels of water stress. However, application of FA had no effect on activity of these enzymes. APX and SOD activities of plants increased with water stress. Maximum and minimum activity of APX and SOD were observed in plants under SWS and WWC, respectively (Table 2).

3.3. Malondialdehyde (MDA)

The effects of different levels of water stress and FA on malondialdehyde (MAD) were significant. MAD of plants was

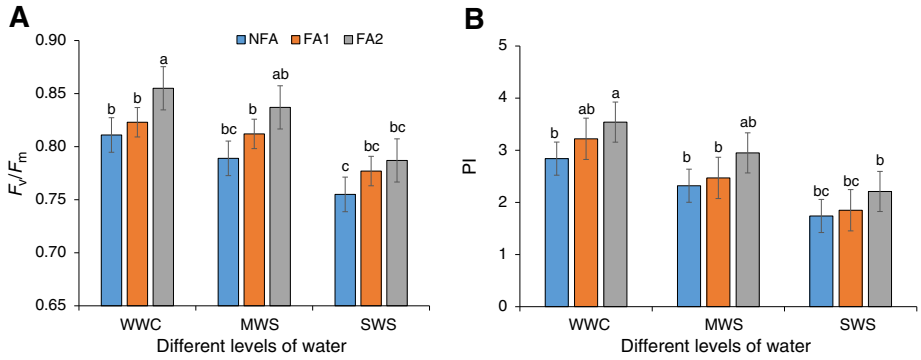

Fig. 1 – Means of maximum efficiency of PSII activity (F_v/F_m) and photosynthesis relative vitality (PI) in response to fulvic acid under different water stress conditions. Each value is the mean ± SE of three replicates (Duncan's multiple range test; $P \leq 0.05$). WWC, MWS, and SWS: 60, 100, and 140 mm evaporation from class A pan, respectively. NFA, FA_1, and FA_2: control, 300, and 600 mg L^{-1} of fulvic acid, respectively.

greater under SWS than under MWS or WWC. However, there was no significant difference in MDA of plants under WWC and MWS (Table 2). Application of FA significantly reduced MDA in rapeseed plants (Fig. 4).

4. Discussion

Drought severely impaired PSII activity in leaves of rapeseed plants and led to sustained decline in the F_v/F_m value (Fig. 1-A). This finding suggests the occurrence of photo-damage to PSII under SWS conditions, whereas no photo-inhibitory or comparatively little photo-damage to the photosynthetic apparatus under MWS was observed. A significant decrease in F_v/F_m also suggested a decrease in the maximum quantum efficiency of open PSII centers as well as an increase in energy dissipation as heat and increase of photo-damage to the photosynthetic apparatus. This result is in agreement with Kauser et al. [22] in rapeseed (*B. napus* L.). Foliar application of FA increased chlorophyll a fluorescence by increasing F_v/F_m under WWC and, in particular, under MWS and SWS (Fig. 1-A). This result may have been sustained by a decline in F_o and increase in F_m under these conditions. Changes in F_o value under water stress affect the probability of energy transfer from light harvesting complex to the PSII reaction center [23]. Application of FA may have several effects on PSII activity, one of which may be decreasing the number of inactive reaction centers where electrons can be transferred out of reduced Q_A and this lead to increasing in F0 and as a result of this F_v/F_m increased (Fig. 1-A).

Exogenous foliar application of FA significantly improved the performance index (PI) of rapeseed plants under both well-watered and limited-water irrigation conditions (Fig. 1-B). PI is an indicator of the vitality of photosynthesis [16]. Increasing PI may be associated with the effects of FA on the number of reaction centers per PSII antenna chlorophyll, the maximum quantum yield of primary photochemistry, and the quantum yield of electron transport. Water stress impaired both light and dark reactions of photosynthesis as a result of reduction of PI (Fig. 1-B).

The present study showed that T_{FM} and S_m significantly increased with water stress, but that Area was reduced under these conditions (Table 1). Water stress reduced the total electron accepting capacity of leaves, as indicated by increasing Sm, which measures the pool size of electron transporters between PSII and the acceptor side of PSI [24] and consequently T_{FM} increased. *Area* is proportional to the pool size of the electron acceptors Q_A on the reducing side of PSII. If

Table 1 – Means of time taken to reach F_m (T_{FM}), the energy necessary for the closure of all reaction centers (S_m) and the size of the plastoquinine pool in PSII (Area) of rapeseed plants under different water stress conditions.

Treatment	T_{FM} (ms)	S_m	*Area*
WWC	315 c	22.68 c	68,000 a
MWS	489 b	25.64 b	52,000 b
SWS	741 a	27.85 a	41,000 bc

Different letters in each column for each factor indicate significant difference at $P \leq 0.05$. WWC, MWS, and SWS: 60, 100, and 140 mm evaporation from class A pan, respectively.

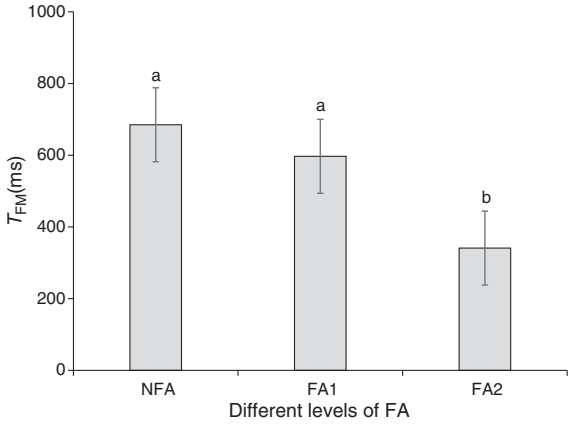

Fig. 2 – Changes in time taken to reach F_m (T_{FM}) of rapeseed plants in response to fulvic acid (FA) application. Each value is the mean ± SE of three replicates (Duncan's multiple range test; $P \leq 0.05$). NFA, FA_1, and FA_2: control, 300, and 600 mg L^{-1}, respectively, of fulvic acid.

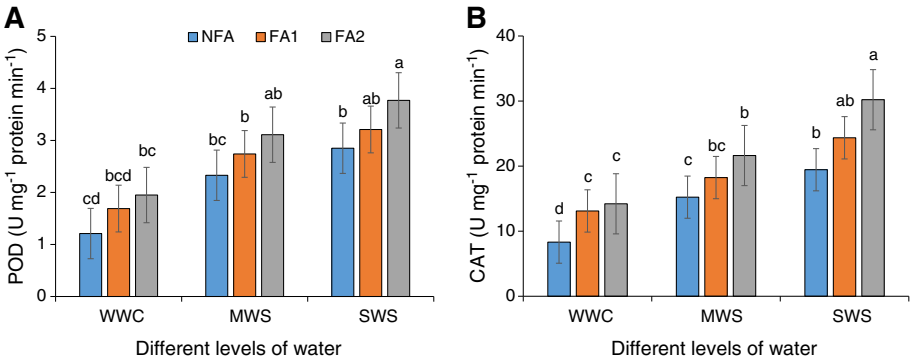

Fig. 3 – Peroxidase (POD) and catalase (CAT) activities in rapeseed plants in response to fulvic acid under different water stress conditions. Duncan's multiple range test; $P \leq 0.05$. WWC, MWS, and SWS: 60, 100, and 140 mm evaporation from class A pan, respectively. NFA, FA_1, and FA_2: control, 300, and 600 mg L^{-1}, respectively, of fulvic acid.

electron transfer from the reaction centers to the quinone pool is blocked, *Area* will be dramatically reduced [25]. Reduction in T_{FM} with application of FA led to enhancement of the average redox state of Q_A in the time span from 0 to T_{FM} (Fig. 2).

Many of the degenerative reactions associated with environmental stresses including water deficit result in the production of ROS in plants, causing additional oxidative stress. Plants can protect themselves by synthesizing antioxidant enzymes (POD, CAT, and APX). Water stress increased POD, CAT (Fig. 3-A and B), APX, and SOD (Table 2) activities in rapeseed plants. In this study, application of FA and particularly FA_2 significantly improved POD and CAT activity under both limited-water and well-watered irrigation conditions (Fig. 3-A and B). Higher activity of these antioxidant enzymes in plants with increasing water stress and application of FA suggests more effective H_2O_2 removal in these plants under water stress. CAT converts H_2O_2 to water in peroxisomes. Increase in POD activity under various stress conditions has been linked to protection from oxidative damage, lignification, and crosslinking of cell walls for protection from such adverse conditions [21,26]. Increased POD activity during water stress has been reported for wheat and other rapeseed cultivars [27]. Increased POD activity with application of FA was observed in plants under both well-watered and water-stressed conditions (Fig. 1). Foliar application of humin materials to turfgrasses significantly enhanced the amounts of various antioxidants in leaves. Higher activities of CAT and APX decrease H_2O_2 levels in the cell and increase the stability of membranes and CO_2 fixation, given that several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. Yamazaki et al. [28] found that FA can be used as a growth regulator, improving plant growth and enhancing stress tolerance.

MDA in rapeseed plants increased under water stress (Table 2), and application of FA significantly decreased MDA (Fig. 4) and decreased lipid peroxidation. The rise in MDA content under water stress conditions suggests that drought stress induces membrane lipid peroxidation by means of ROS [26]. The reduction in MDA may be attributed to increases in antioxidant enzymes such as POD and CAT (Fig. 3-A and B), APX, and SOD (Table 2) in rapeseed plants.

Table 2 – Ascorbate peroxidase (APX; mmol ascorbate oxidized mg^{-1} min^{-1}), superoxide dismutase (SOD; U mg^{-1} protein) and malondialdehyde (MDA; nmol g^{-1} FW) of rapeseed plants under different levels of water stress.

Treatment	APX	SOD	MAD
WWC	37 c	4.32 c	1.85 bc
MWS	55 b	12.44 b	2.67 b
SWS	81 a	21.15 a	4.66 a

Different letters in each column for each factor indicate significant difference at $P \leq 0.05$. WWC, MWS, and SWS: 60, 100, and 140 mm evaporation from class A pan, respectively.

5. Conclusions

Rapeseed plants under water stress produce ROS, which affects plant metabolism such as by lipid peroxidation in membranes. Application of FA significantly improved

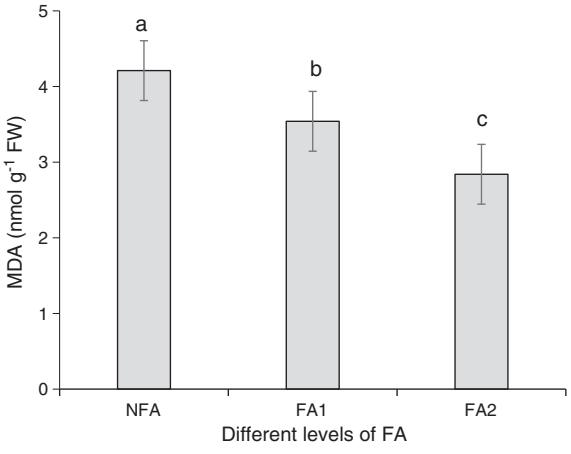

Fig. 4 – Malondialdehyde (MDA) of rapeseed plants in response to fulvic acid (FA) application. Duncan's multiple range test; $P \leq 0.05$. NFA, FA_1, and FA_2: control, 300, and 600 mg L^{-1} of fulvic acid, respectively.

fluorescence of chlorophyll a by reducing the number of inactive reaction centers and increasing the quantum yield of electron transport and the average redox state of Q_A, thereby inhibiting ROS production. Enhanced antioxidant enzyme activities as a result of FA treatment destroyed ROS. Thus, ROS in plant cells under water stress was reduced by application of FA and consequently lipid peroxidation of plants was reduced.

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