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Application of Azoxystrobin Fungicide Improves Drought Tolerance in Tomato, via Enhancing Physio-Biochemical and Anatomical Feature

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Abstract. To investigate whether the fungicide Azoxystrobin improves the potential to maintain physio-biochemical functions, tomato plants were grown under either well-watered and deficit irrigation conditions. Drought-stressed tomato plants showed significant reductions in cell membrane stability (CMS), relative water content (RWC), relative water loss (RWL) and chlorophylls, growth attributes and leaflet and main stem anatomical features, while exhibited increases in contents of proline and total phenols, activities of catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO), fresh (FW) and dry (DW) weights of roots, and leaflet spongy tissue thickness compared to well-watered control plants. Under full irrigation, Azoxystrobin treatment significantly increased RWC and chlorophyll content, POD and PPO activities, root DW, number of fruits per plant and many features of leaflet and main stem, while significantly decreased CMS and RWL, root, shoot and plant lengths, shoot and plant FW, and stem xylem tissue thickness compared to the control plants sprayed with water. However, Azoxystrobin treatment ameliorated drought stress in tomato plants and significantly increased CMS and free proline content, activities of CAT, POD and PPO, and contents of free and total phenols, and root DW and number of fruits per plant, in addition to spongy tissue thickness of leaflet, but not affected chlorophylls and carotenoids contents, root FW, plant DW and most of anatomical features compared to the stressed plants without Azoxystrobin treatment. These results support that Azoxystrobin foliar application may have a positive effect on well-watered and drought-stressed tomato plants.

1. Introduction

Tomato is an annual plant transplanted and grown in greenhouses, gardens and often in open fields. Tomato production passed through two critical growth periods, i.e. foliage growth and fruiting stages, during which any dereliction will continue its effect on final yield qualitatively and quantitatively. It is one of the most water demanding crops [1], especially in dry (arid and semi-arid) regions.

Early blight disease of tomato caused by *Alternaria solani* and *A. alternate,* is reported to be destructive of Solanaceae plants. Management of this disease is achieved mainly through protective fungicide applications with different mode of actions [2]. Strobilurins are one of the most important classes of agricultural fungicides. Their invention was inspired by a group of fungicidally active natural products [3]. Azoxystrobin is a systemic and broad-spectrum fungicide, which inhibits spore germination and mycelial growth in a wide range of phytopathogenic fungi [4]. Azoxystrobin provides a major alternative to ergosterol biosynthesis inhibitors with broad-spectrum control of cereals, fruits and vegetable diseases [5]. It represents a new option to manage fungicide resistance [6].

Tomato plants treated with Azoxystrobin are looked more vigorous than plants with only conventional fungicides with positive effect on yields, particularly when fungal infection had caused stress [7]. While under drought stress conditions, little information is available on the possibility to use this fungicide for mitigating drought stress conditions, especially for tomato. Application of

Azoxystrobin is reported to induce a positive influence on water status, water use efficiency (WUE) and yield response in tomato [8,9].

Variation in plant tolerance comes from the diversity of the fungicide detoxification mechanism, varying metabolites and their rate which called physiological selectivity. The metabolism of Azoxystrobin in different plants was similar and complex, resulting in the formation of many metabolites with the parent, Azoxystrobin, being the major component of the residue [10]. A single application of Azoxystrobin (Amistar 25% SC) on tomato plants at the concentration of 0.05% (v/v) in water showed that the fungicide concentration was lower in tomato leaves than in fruits. The corresponding half life time periods were 5.90 and 4.07 days [11].

This research work aims to determine the potential effects of foliar application of Azoxystrobin on increasing drought tolerance in tomato plants growing under open greenhouse conditions. To achieve this aim, physiological attributes, photosynthetic pigments, antioxidant defense system (enzymatic and phenolic activities), growth characteristics and anatomical features of leaflet blade and main stem of tomato plants were evaluated under well-watered and drought conditions.

2. Material and Methods

2.1. Plant material, transplanting and growth conditions

At Zagazig University, Faculty of Agriculture, a pot experiment, repeated 3 times, was carried out during the late summer season beginning of August 2016 to achieve the purpose of the current study. Plastic pots (20 cm in diameter), each one was filled with 5 kg sandy clay soil (1 sand: 1 clay, v/v). All pots were well-watered before transplanting. Thirty-day-old tomato transplants (cv. 765) were gently removed from their trays and were then transplanted, immediately, at a rate of one healthy seedling per pot.

Before transplanting, 0.8g phosphorus fertilizer (calcium super phosphate, 15.5% P_2O_2) was added per pot, and then all pots were well watered. After transplanting, 0.8g nitrogen (ammonium nitrate, 33% N) and 0.8g potassium (potassium sulfate, 48% K_2O) fertilizers were added per pot, each in two split doses. The first dose was added after 2 weeks from transplanting and the second was added 2 weeks later. Seedlings were provided with all other necessary agricultural practices as recommended for tomato production.

After transplanting, pots were left 2 weeks as an adaptation period before the beginning of the experimental treatments.

2.2. Treatments and Azoxystrobin application

In the 2nd week of adaptation, pots were distributed randomly into two groups; a control group in which plants were sprayed with water, and a comparable group in which plants were sprayed with Azoxystrobin. Each group was represented by 40 pots. Water and Azoxystrobin were sprayed, 3 times in 3 consecutive weeks, and then samples from the two groups were taken as a test to evaluate the potential promotion effects of Azoxystrobin under normal condition (Table 1). Thereafter, each group was divided into two sub-groups to specify treatments; spraying with water + full irrigation (a control), spraying with water + deficit irrigation (65% of ETc), spraying with Azoxystrobin + full irrigation, and spraying with Azoxystrobin + deficit irrigation. Full irrigation treatment means plants were irrigated at full recovering of crop evapotranspiration; ETc, 3 times weekly, while deficit irrigation (drought condition) treatment means plants were irrigated at 65% of crop ETc, 3 times weekly. Irrigation treatments were applied for three weeks (from the beginning of the 6th week up to the end of the 8th week). These basic treatments were selected based on our preliminary studies (data not shown). Irrigation of tomato plants with levels under 65% of ETc was led to the death of plants even with Azoxystrobin due to the aridity of the experimental region.

Table 1. Effect of azoxystrobin (AZOX) treatment on some physiological traits [cell membrane stability; CMS, relative water content; RWC, excised leaf water retension; ELWR, relative water loss; RWL, and contents of chlorophyll–a; Chl–a, chlorophyll–b; Chl–b, chlorophylls–(a+b); Chl–(a+b), total carotenoids; Carot (mg g^{-1} FW) and free proline (µmol g^{-1} FW) of well-watered tomato plants (at the end of the 5th week after transplanting)

Parameters	Treatments		
	Control#	AZOX	Significance
CMS (%)	10.8b	17.3a	**
RWC (%)	48.2	48.2	NS
ELWR (%)	25.2	24.0	NS
RWL (%)	18.4a	15.8b	*
Chl–a content	0.60b	1.03a	**
Chl–b content	0.29b	0.35a	*
Chl–(a+b) content	0.89b	1.38a	**
Carot content	0.53b	0.95a	**
Proline content	9.34b	11.02a	*

Means marked with the same letter in each row are not significant different ($P < 0.05$) by Duncan's test. #Control means well-watered plants that not sprayed with azoxystrobin but sprayed with water, NS means not significant, (*) means significant at $P < 0.05$, and (**) means significant at $P < 0.01$.

Spray treatments of water and Azoxystrobin were applied in another 3 consecutive (the 6th, 7th and 8th) weeks, and then samples from the four sub-groups were taken to evaluate the potential promotion effects of Azoxystrobin under drought stress condition (Tables 2–5). The applied concentration was 125 mg a.i. l^{-1} of water, which represents recommended rate of Amistar 25% SC (5 cm^3 10 l^{-1} of water). Water or fungicide solution was sprayed on tomato shoots to run-off by using backpack sprayer with affirmation on washing the sprayer tank three times before each usage.

Samples were harvested at the end of the 5th and at the termination of the experiment; 8th week after transplanting for conducting the physiological, antioxidant defense systems (enzymatic activities and phenolic contents) and anatomical determinations.

Table 2. Effect of irrigation condition and azoxystrobin (AZOX) treatments on some physiological traits [cell membrane stability; CMS, relative water content; RWC, excised leaf water retension; ELWR, relative water loss; RWL, and contents of chlorophyll–a; Chl–a, chlorophyll–b; Chl–b, chlorophylls–(a+b); Chl–(a+b), total carotenoids; Carot (mg g^{-1} FW) and free proline (µmol g^{-1} FW) of well-watered and drought-stressed tomato plants (at the end of the 8th week after transplanting)

Parameters	Treatments				
	Control#	AZOX	Drought (D)	D+AZOX	Significance
CMS (%)	14.0b	11.7c	9.0d	17.7a	**
RWC (%)	71.7b	79.8a	23.3c	74.3ab	*
ELWR (%)	85.8ab	85.1b	88.0ab	91.2a	*
RWL (%)	51.7a	46.9b	26.5c	25.2c	**
Chl–a content	1.16ab	1.24a	1.11b	1.09b	*
Chl–b content	0.77	0.80	0.73	0.78	NS
Chl–(a+b) content	1.94b	2.04a	1.83c	1.87bc	*
Carot content	1.29ab	1.32a	1.21b	1.21b	*
Proline content	20.6b	20.8b	23.1a	24.1a	*

Means marked with the same letter in each row are not significant different ($P < 0.05$) by Duncan's test. #Control means well-watered plants that not sprayed with azoxystrobin but sprayed with water, NS means not significant, (*) means significant at $P < 0.05$, and (**) means significant at $P < 0.01$.

Table 3. Effect of irrigation condition and azoxystrobin (AZOX) treatments on enzymatic [catalase; CAT (μmol H_2O_2 mg^{-1} FW min^{-1}), peroxidase; POD and polyphenol oxidase; PPO (change in absorbance g^{-1} min^{-1})] activities, and phenolic compounds [free, bound and total phenols (mg g^{-1} FW)] contents in well-watered and drought-stressed tomato plants (at the end of the 8th week after transplanting)

Parameters	Treatments				
	Control#	AZOX	Drought (D)	D+AZOX	Significance
CAT activity	317b	331b	380a	383a	**
POD activity	0.58c	0.63b	0.78a	0.77a	*
PPO activity	0.29b	0.38a	0.40a	0.41a	*
Free phenols content	1.05b	1.10b	1.12b	1.23a	*
Bound phenols content	0.59	0.63	0.63	0.64	NS
Total phenols content	1.64c	1.73bc	1.78ab	1.87a	*

Means marked with the same letter in each row are not significant different ($P < 0.05$) by Duncan's test. #Control means well-watered plants that not sprayed with azoxystrobin but sprayed with water, NS means not significant, (*) means significant at $P < 0.05$, and (**) means significant at $P < 0.01$.

Table 4. Effect of irrigation condition and azoxystrobin (AZOX) treatments on growth characteristics of well-watered and drought-stressed tomato plants (at the end of the 8th week after transplanting)

Parameters	Treatments				
	Control#	AZOX	Drought (D)	D+AZOX	Significance
Root length (cm)	35.8a	29.8b	26.4c	29.0b	*
Shoot length (cm)	36.4a	28.0c	29.3bc	30.7b	*
Plant length (cm)	72.2a	57.8b	55.7b	59.7b	**
Root fresh weight (g)	15.0b	15.2b	19.0a	15.1b	*
Shoot fresh weight (g)	74.8a	63.1b	57.1c	52.1d	**
Plant fresh weight (g)	89.8a	78.3b	76.1b	67.2c	*
Root dry weight (g)	2.06d	2.80c	4.18a	3.26b	**
Shoot dry weight (g)	11.9a	11.5a	10.5b	10.5b	*
Plant dry weight (g)	14.0ab	13.9b	14.7a	13.8b	*
Number of fruits $plant^{-1}$	1.5b	2.0a	1.3c	2.0a	*

Means marked with the same letter in each row are not significant different ($P < 0.05$) by Duncan's test. #Control means well-watered plants that not sprayed with azoxystrobin but sprayed with water, (*) means significant at $P < 0.05$, and (**) means significant at $P < 0.01$.

Table 5. Responses of histological features in transverse section of leaflet blade and main stem of well-watered and drought-stressed tomato plants (at the end of the 8th week after transplanting) to treatment with azoxystrobin (AZOX)

Parameters (μm)	Treatments				
	Control#	AZOX	Drought (D)	D+AZOX	Significance
Leaflet anatomy					
Upper epidermis thickness	39.7a	41.0a	30.0c	36.6b	*
Lower epidermis thickness	38.0ab	39.0a	31.0c	35.6b	*
Blade thickness	330b	400a	300c	310bc	*
Palisade tissue thickness	110b	120a	100c	102bc	*
Spongy tissue thickness	120c	200a	140b	141b	**
Diameter of vessel average	43.3b	46.6a	30.0d	33.3c	*
Phloem tissue thickness	121a	130a	90c	100b	*
Xylem tissue thickness	160a	170a	109b	160a	*
Midvein thickness	1009b	1109a	749c	949b	**
Midvein width	1309a	1349a	849c	1099b	**
Main stem anatomy					
Epidermis thickness	39.0	40.0	37.6	38.1	NS
Cortex thickness	220b	310a	110d	160c	**
Phloem tissue thickness	240b	300a	160d	220c	**
Xylem tissue thickness	550a	500b	400c	490b	*
Diameter of vessel average	73.3a	76.6a	56.6c	66.6b	*
Stem diameter	3439b	4616a	3077c	3276bc	*
Hollow pith diameter	1539b	2534a	1358c	1484bc	*

Means marked with the same letter in each row are not significant different ($P < 0.05$) by Duncan's test. #Control means well-watered plants that not sprayed with azoxystrobin but sprayed with water, NS means not significant, (*) means significant at $P < 0.05$, and (**) means significant at $P < 0.01$.

2.3. Cell membrane stability (CMS)

The CMS was determined according to the method described by [12] using the upper full expanded leaves from each treatment. Twenty leaf discs (1 cm in diameter) were taken from leaves and washed with deionized water. For desiccation treatment, ten leaf discs were flooded in 10 ml of 30% polyethylene glycol (PEG6000) in 15 ml test tubes for 24 h at 10 °C. For control treatment, ten leaf discs were flooded in distilled water. Thereafter, the treatment and control leaf discs were washed with deionized water and 10 ml of deionized water was added to each tube. The tubes were maintained for 24 h at 10 °C, and then conductivity of the solutions was determined by electrical conductivity meter. Finally, the tubes were boiled in a water bath for 60 min, cooled to room temperature, and the conductivity of the solutions was recorded again. Percent of CMS value for leaf tissues was calculated using the following equation:

$$\mathbf{CMS}\ (\%) = \left(\mathbf{1} - \frac{(\mathbf{1} - \boldsymbol{T1/T2})}{(\mathbf{1} - \boldsymbol{C1/C2})}\right) \times \mathbf{100}$$

where: T_1 and T_2 are the first and second (pre and after boiling, respectively) measurements of the conductivity of solutions, and C_1 and C_2 are the respective values for the controls.

2.4. Relative water content (RWC)

Four upper full expanded leaves were collected from each treatment and immediately weighted to record fresh weight (FW). Then, were placed in distilled water for 4 hours at 25 ± 2 °C and weighed again to record turgid weight (TW). Leaves were then subjected to drying oven at 70 °C until constant weight and the dry weight (DW) values were recorded. The RWC was calculated using the following equation suggested by [13]

$$\mathbf{RWC}\ (\%) = \frac{\mathrm{FW}\ -\ \mathrm{DW}}{\mathrm{TW}\ -\mathrm{DW}} \times \mathbf{100}$$

2.5. Excised leaf water retention (ELWR)

Four upper full expanded leaves were collected and weighed to record fresh weight (FW), and then left for 4 h to wilt at 25 °C and reweighed (WW4h). ELWR was calculated using the following formula according to [14]

$$\mathbf{ELWR}\ (\%) = \left[\mathbf{1} - \frac{\mathrm{FW} - \mathrm{WW4h}}{\mathrm{FW}}\right] \times \mathbf{100}$$

2.6. Relative water loss (RWL)

Four upper full expanded leaves were sampled for each treatment and samples were weighed to record fresh weight (FW), wilted for 4 hours at 35°C in incubator and reweighed (WW4h), and then oven dried at 72 °C until constant weight and weighed (DW). The RWL was calculated using the following formula according to [15]

$$\mathbf{RWL}\ (\%) = \frac{(\mathrm{FW} -\ \mathrm{WW4h})}{(\mathrm{FW} -\ \mathrm{DW})} \times \mathbf{100}$$

2.7. Chlorophyll and carotenoids contents determinations

Leaf photosynthetic pigments; chlorophyll–a (Chl–a), chlorophyll–b (Chl–b) and carotenoids (Carot) were measured by the method described by [16]. Pigments were extracted from upper full expanded leaf samples by pure acetone and extracts were filtered. The optical density (E) of the filtrate was determined Spectrophotometrically at 662, 644, 440.5 nm for pigments that were calculated as mg g^{-1} FW using the formulae adopted by [17] as follows:

Chl–a = $(9.784 \times E_{662}) - (0.99 \times E_{644})$
Chl–b = $(21.426 \times E_{644}) - (4.65 \times E_{662})$
Carot = $(4.695 \times E_{440.5}) - (0.268 \times$ Chl–a + Chl–b)

2.8. Proline content (PC) determination

Using upper full expanded leaves, proline was determined according to the method of [18]. Samples (0.5 g of each) were grinded with 10 ml of 3% sulfosalicylic acid, and the homogenates were filtered. Two ml of glacial acetic acid and 2.0 ml acid ninhydrin reagent were added to a 2.0 ml of the filtrate, and the mixture was then shaken by hand and incubated in boiling water bath for 1 h. Thereafter, it was transferred to ice bath and cooled to room temperature. Four ml of Toluene was added to the mixture and the absorbance of the upper toluene layer after separation was measured at 520 nm using spectronic 20-D spectrophotometer.

2.9. Determination of catalase (EC 1.11.1.6) activity

Measurement of catalase activity was made according to the perborate method of [19]. Leaf tissue (0.5 g) was crushed in a mortar with 5 ml of pH 6.8 phosphate buffer, and the macerate was then squeezed through muslin. A series of flasks containing 5 ml of 1.5% sodium perborate + 1.5 ml phosphate buffer (pH 6.8) were prepared. At zero time, 1 ml of the macerate was pipetted into each flask. The reaction was stopped by rapidly adding 10 ml 2N sulfuric acid. One drop of manganese chloride 1% was added for each flask and titration was carried out by means of potassium permanganate (0.05 N) using an appropriate blank for each series.

2.10. Determination of peroxidase (EC 1.11.1.7) activity

One g of sample (upper full expanded leaves) was crushed well in 2 ml of 0.1 M sodium phosphate buffer (pH 7.1). The homogenate was filtered through Whatman No.1 filter paper. The suspension was centrifuged at 6000 rpm at 4 ºC for 20 min and stored at –18 ºC until use. Peroxidase activity was analyzed spectrophotometrically [20]. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 100 µl of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2 °C). Color density was measured in a spectrophotometer at 425 nm every 30 sec [21]. The enzyme activity was expressed as changes in the absorbance min^{-1} g^{-1} of fresh leaf tissue.

2.11. Determination of polyphenol oxidase (EC 1.14.18.1) activity

Polyphenol oxidase activity was determined by the procedure described by [22] using upper full expanded leaves. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 100 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} g^{-1} of fresh leaf tissue.

2.12. Determination of phenolic compounds contents

Phenolic contents were colorimetrically determined using the folin reagent according to [23]. Two g from upper fully expanded leaves were crashed and immersed in 25 ml of 70% ethanol in brown bottles, and were then stored in dark at room temperature. After a month, ethanolic extraction was dried at room temperature, and then transported quantitatively into 5 ml of 50% isopropanol and stored in vials at 1 °C. For total phenols content, a reaction mixture consisted of 1 ml extraction and 0.25 ml HCl was boiled in a water bath for 10 min, and then cooled. One ml of Folin-Denis reagent and 6 ml of 20% Na_2CO_3 were added. The mixture was completed to 10 ml with distilled water and the color density was measured at 520 nm. Tannic acid was used as a standard compound. For free phenols content, a reaction mixture consisted of 1 ml of extracted sample, 1 ml of distilled water, 1 of ml Folin-Denis reagent and 3 ml of 20% Na_2CO_3, was adjusted to a final volume of 10 ml with distilled water. Free phenols content was then measured as mentioned before for total phenols content. For bound phenols content, the difference between total and free phenols contents gives the content of bound phenols.

2.13. Growth and fruit yield parameters

At the endpoint (end of 8 weeks after transplanting), 10 plants from each treatment were selected randomly to measure plant height, shoot and root lengths, fruit number per plant, fresh and dry (70 °C for 48 h or until a constant weight) weights of shoots and roots. Fruits number was taken at the end of the experiments (at the 56^{th} day; the 8^{th} week after transplanting, with the beginning of the fruiting process and before its completion).

2.14. Anatomical Responses

Specimens from a blade of the selected leaflet and main stem were obtained from various treatments. The specimens were killed and fixed in FAA (10 ml of formalin, 5 ml of glacial acetic acid, and 85 ml of ethyl alcohol 70%), washed in 50% ethyl alcohol, dehydrated in butyl alcohol series and cleared by xylene and absolute alcohol. Finally, specimens were embedded in liquefied pure paraffin wax. Sections at thickness of 14µm were cut. Paraffin ribbons were mounted on slides, stained with Safranin and Light green, then mounting in Canada balsam [24]. The prepared slides were examined with a light microscope equipped with a digital camera (Canon Power Shot S80) connected to a computer. Sections were photographed and examined to detect histological manifestations of the chosen treatments and micrograph. All parameters measured using calibrated micrometer slide under light microscope.

2.15. Experimental layout and statistical analysis of data

Experimental treatments were arranged in a completely randomized blocks design. The experimental data were analyzed statistically by one-way ANOVA. The mean values of treatments

were separated by Duncan's multiple range test ($P < 0.05$ and 0.01) according to [25] using CoStat 6.4 software package.

3. Results

3.1. Using 35-day-old tomato plants after transplanting to test the potential improving effects of Azoxystrobin under normal (well-watered) condition

Data in Table 1 show that spraying tomato plants with Azoxystrobin significantly increased some physiological attributes; cell membrane stability and contents of chlorophyll–a (Chl–a), chlorophyll–b (Chl–b), chlorophylls–(a+b) [Chl–(a+b)], total carotenoids (Carot) and free proline. These increases were 60.2, 71.7, 20.7, 55.1, 79.2 and 18.0%, respectively compared to those in plants sprayed with water under normal (well-watered) conditions. Per contra, relative water loss (RWL) was significantly decreased (by 14.1%), while relative water content (RWC) and excised leaf water retension (ELWR) were not affected by spraying plants with Azoxystrobin.

3.2. Using 56-day-old tomato plants after transplanting to test the potential improving effects of Azoxystrobin under drought (deficit irrigation) condition

Under normal (well-watered) condition, foliar spray with Azoxystrobin to tomato plants significantly increased RWC by 11.3% and Chl–(a+b) content by 5.2% (Table 2), peroxidase (POD) activity by 8.6% and polyphenol oxidase (PPO) activity by 31.0% (Table 3), root dry weight (DW) by 35.9% and number of fruits per plant by 33.3% (Table 4), and many features of leaflet (blade thickness by 21.2%, palisade tissue thickness by 9.1%, spongy tissue thickness by 66.7%, average diameter of vessels by 7.6% and midvein thickness by 9.9%) and main stem (cortex thickness by 40.9%, phloem tissue thickness by 25.0%, stem diameter by 34.2% and hollow pith diameter by 64.7%) (Table 5) compared to the control plants sprayed with water. Contrariwise, Azoxystrobin treatment significantly decreased CMS by 16.4% and RWL by 9.3% (Table 2), root length by 16.8%, shoot length by 23.1%, plant length by 19.9%, shoot fresh weight (FW) by 15.6% and plant FW by 12.8% (Table 4), and the stem anatomy feature of xylem tissue thickness by 9.1% (Table 5) compared to the control plants. However, the following parameters were not affected by Azoxystrobin treatment: ELWR, Chl–a content, Chl–b content and total carotenoids content (Table 2), catalase (CAT) activity, and free, bound and total phenols contents (Table 3), root FW, shoot DW and plant DW (Table 4), and some features of leaflet (upper epidermis thickness, lower epidermis thickness, phloem tissue thickness, xylem tissue thickness and midvein width) and main stem (epidermis thickness and average diameter of vessels) (Table 5).

Deficit irrigation (drought)-stressed tomato plants showed significant reductions in most tested parameters of physiology, growth and leaflet and main stem anatomical features (Tables 2–5). These reductions were 35.7% for CMS, 67.5% for RWC, 48.7% for RWL, 5.7% for Chl–(a+b), 26.3% for root length, 19.5% for shoot length, 22.9% for plant length, 23.7% for shoot FW, 15.3% for plant FW, 11.8% for shoot DW, 13.3% for number of fruits per plant, 24.4% for upper epidermis thickness of leaflet, 18.4% for lower epidermis thickness of leaflet, 9.1% for leaflet blade thickness, 9.1% for leaflet palisade tissue thickness, 30.7% for leaflet diameter of vessels, 25.6% for leaflet phloem tissue thickness, 31.9% for leaflet xylem tissue thickness, 25.8% for leaflet midvein thickness, 35.1% for leaflet midvein width, 50.0% for stem cortex thickness, 33.3% for stem phloem tissue thickness, 27.3% for stem xylem tissue thickness, 22.8% for stem diameter of vessels, 10.5% for stem diameter, and 11.8% for stem hollow pith diameter compared to the well-watered control plants. In contrast, drought-stressed tomato plants showed significant increases in proline content by 12.1%, CAT activity by 19.9%, POD activity by 34.5%, PPO activity by 37.9%, total phenols content by 8.5%, root FW by 26.7%, root DW by 102.9%, and leaflet spongy tissue thickness by 16.7%. On the other hand, there are some parameter that were not affected by drought stress, e.g., ELWR, contents of Chl–a, Chl–b, total carotenoids, and free and bound phenols, plant DW and stem epidermis thickness.

However under drought stress, spraying tomato plants with Azoxystrobin significantly increased some of the physiological attributes (CMS by 26.4% and free proline content by 17.0%),

antioxidative defense systems (CAT activity by 20.8%, POD activity by 32.8%, PPO activity by 41.4%, free phenols content by 17.1% and total phenols content by 14.0%), and growth traits (root DW by 58.3% and number of fruits per plant by 33.3%), in addition to spongy tissue thickness of leaflet by 17.5% compared to the control plants sprayed with water. In contrast, Azoxystrobin foliar treatment significantly decreased another some of the physiological attributes (RWL by 51.3%), growth characteristics (root length by 19.0%, shoot length by 15.7%, plant length by 17.3%, shoot FW by 30.3%, plant FW by 25.2% and shoot DW by 11.8%), and anatomical features of leaflet (upper epidermis thickness by 7.8%, average diameter of vessels by 23.1%, phloem tissue thickness by 17.4% and midvein width by 16.0%) and main stem (cortex thickness by 27.3%, phloem tissue thickness by 8.3%, xylem tissue thickness by 10.9% and average diameter of vessels by 9.1%) compared to water foliar treatment. While others of the physiological parameters (RWC, ELWR, and the contents of Chl–a, Chl–b, total Chl–(a+b) and total carotenoids), growth traits (root FW and plant DW) and anatomical features of leaflet (lower epidermis thickness, blade thickness, palisade tissue thickness, xylem tissue thickness and midvein thickness) and main stem (epidermis thickness, stem diameter and hollow pith diameter), in addition to the content of bound phenols were not affected by Azoxystrobin treatment.

4. Discussion

Impairing crop plant growth and limiting its production, water deficit, particularly in arid and semi-arid regions, considers as one of the major constraints that threats the agricultural productions nowadays and may cause more severe in future due to continuous climate changes. In the present study, a deficit of irrigation water caused a reduction in tomato growth (Table 4), disruptions in many physio-biochemical attributes, the most important of which were plant/cell water relations and leaf photosynthetic pigments (Table 2), and negative modifications in leaf and stem anatomy (Table 5).

These negative effects of water deficit stress are attributed to a decrease of cell division, cell elongation, and differentiation affected due to impaired enzyme activities, loss of cell turgor, and decreased energy supply. In addition to that plants tend to partially close their stomata under drought stress causing reductions in photosynthesis rate and membrane damage, and disturbances in activity of various enzymes, especially enzymes involved in ATP synthesis [26, 27, 28]. Plant water relations attributes such as relative water content (RWC), leaf water, osmotic and pressure potentials, and transpiration rate are negatively affected under water deficit due to a shortage in water supply [29, 30]. Impaired activities of essential photosynthetic enzymes, the most important one is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is responsible for the decrease of photosynthesis rate under drought [27, 31]. Progressive downregulation and metabolic processes inhibition decrease ribulose-1,5-bisphosphate (RuBP) contents, of which conversion to 3-phosphoglyceric acid (3-PGA) will decrease with reducing leaf RWC under drought [32]. At moderate water deficit stress, Rubisco acts as oxygenase due to higher internal O_2 than CO_2 contents due to stomatal closure, and increase photorespiration at the expense of carbon-fixation [33]. Photorespiration may be beneficial under drought stress due to its involving in energy dissipation and thus it reduces [32]. In addition, it produces glycine (amino acid) to use for synthesis of glutathione as an important component of antioxidant defense [34], and it also enhances the RuBP supply to Calvin cycle [35]. As reported, low CO_2 influx under drought stress deteriorated RuBP contents, Rubisco activities or ATP synthesis which downregulate carbon fixation, consequently, oxidation of reduced NADPH in the Calvin cycle is decreased, and consequently, $NADP^+$ (primary electron acceptor) is not sufficiently available [32]. Plants exposed to drought stress leads to increased oxidative stress with ROS (e.g., $O_2^{\bullet-}$, $^1O_2$, H_2O_2 and OH^-) overproduction that are highly reactive, toxic and deteriorate normal plant metabolism through oxidative causing damages to proteins, lipids, carbohydrates, and DNA [36, 37]. The major sites of ROS generation are found in both chloroplasts (e.g., PSI and PSII) and mitochondria (e.g., complex I, ubiquinone and complex III of electron transport chain; ETC) [38]. Consequently, diminished activities of essential enzymes and ATP synthesis due to oxidative damage lead to minimize photosynthetic and respiratory activities.

Plants adopt a lot of mechanisms to tolerate drought stress, some of them are found in our results such as reduced water loss (Table 2) by increased diffusive resistance, increased water uptake with prolific and deep root systems (Table 4), increased low-molecular-weight osmolytes such as proline (Table 2) sustaining cellular functions under water deficit stress. Higher cell membrane stability (CMS; Table 2), elevated phenolic compound contents and enzymatic antioxidants activities (Table 3) and increased spongy tissue thickness (Table 5) modulate plant responses to drought stress. In addition to reported earlier, plants exhibit several morphological and physio-biochemical adaptations at subcellular, cellular and organ levels to survive under drought stress. However, drought stress tolerance is a complex phenomenon associated with stomatal regulation, hormonal balances, antioxidant defense system, osmotic adjustment, root system and maintenance of tissue water contents, in addition to cuticle thickness etc. Drought escape (the ability of a plant to complete its life cycle before the beginning of drought) and avoidance (the ability of a plant to sustain high plant water status or cellular hydration under drought; [39]) are also of important mechanisms for drought tolerance.

The most important plant challenges with drought undergo adaptive mechanisms at molecular levels such as up- and downregulation of many gene transcripts and accumulation of stress proteins [40]. Underwater deficit stress, a considerable rise in CDSP 32 (chloroplastic drought-induced stress protein) mRNA and protein protects chloroplast from drought-induced oxidative damage [41]. In addition, aquaporins (an important group of intrinsic membrane proteins) are able to regulate hydraulic conductivity of membranes [42]. Many dehydration-responsive element-binding genes are also involved in signaling pathways in response to water deficit stress [43]. The dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element and its DNA-binding protein are a major transcription system that modulates ABA-independent gene expression in response to drought stress and includes dehydration-responsive element binding proteins (DREB)/C-repeat binding factors (CBF) family of proteins. DREB2 subclass of DREB/CBF family proteins are expressed under drought stress to articulate genes involved in stress tolerance [44]. Signal transduction pathways are also induced in plants under water deficit stress to regulate plant growth. An early-warning response mechanism exists in plant roots to activate the hydrogen pump ATPase protein (H^+-ATPase) on plasma membrane of root hairs before a substantial decline in tissue RWC of plant. The activation of root hair cell plasma membrane H^+-ATPase stimulates magnified biosynthesis of key osmolytes such as leaf proline and glycine betaine to maintain the water content in plants. Moreover, interspecific and intraspecific differences in the timing of inducing early responses may exist to initiate warming responses to drought stress [45]. It has been concluded that, polyamines (PAs) associate with the response of plants to drought stress by signaling [46]. Ornithine decarboxylase (TcODC), arginine decarboxylase (TcADC), S-adenosylmethionine decarboxylase (TcSAMDC), spermidine synthase (TcSPDS), and spermine synthase (TcSPMS) are the expression patterns of genes encoding enzymes involved in PAs in plant leaves. Expression of TcODC, TcADC, and TcSAMDC is induced at the start of drought which modulates stomatal conductance, photosynthesis, photosystem II efficiency, and leaf water potential. Induction of TcSAMDC in leaves is most closely correlated with changes in water potential. The earliest measured responses to drought are enhanced expression of TcADC and TcSAMDC in plant roots along with decreases in stomatal conductance, photosynthesis, and PS II efficiency due to elevated levels of PAs; putrescine, spermidine and spermine [46].

Although plants have many endogenous mechanisms to survive under abiotic stress conditions including water deficit stress, in most cases they need exogenous help to support their mechanisms. In the present study, a fungicide Azoxystrobin was used as foliar spray to potentially increase drought stress tolerance in tomato plants. As shown in our data, proline content was significantly increased by application of Azoxystrobin supporting plant osmoprotectants concentrations to tolerate drought conditions. However, plants might be already surrendered to chemical stress resulted from azoxystrobin degradation in the plant as a xenobiotic. In this concern, [47]. have concluded that at higher concentrations, phytotoxicity of Azoxystrobin varied with host genotype. Among the tested plants, Catjang (*Vigna unguiculata* subsp. *cylindrical*) was most sensitive to the fungicide. The fungicide at different concentrations significantly decreased community respiration and gross primary

productivity. However, the net primary productivity was significantly increased by Azoxystrobin treatment up to 0.0073 μg a.i. ml^{-1}. They have added that increase in fungicide concentration and incubation period of treated leaf tissue resulted in increased electrolyte leakage measured by increased electrical conductivity. In addition, [48] have noticed that using Strobilurins to improve water status of wheat grown under drought conditions by the decrease of the conductance of water through the epidermis and lower rate of transpiration, lower intercellular carbon dioxide concentration, and lower net rate of photosynthesis. They have also mentioned that the probable mechanism for the photosynthetic effects may be caused either by Strobilurin fungicides acting directly on ATP production in guard cell of mitochondria or by stomata responding to Strobilurin-induced changes in mesophyll photosynthesis. In addition, application of Azoxystrobin improved tomato water status, especially under water stress conditions in terms of osmotic potential, relative water content and crop water stress index as a result of improving the formation of ABA [8]. All inspected reviews did not refer to probable action of water stress tolerance but it proved a phytotoxic effect invisible on plant parts, however, it detected on physiological level. Variation in plant tolerance comes from the diversity of the fungicide detoxification mechanism, varying metabolites and their rate which called physiological selectivity. The main metabolic pathway for Azoxystrobin in plant ended with the compound 2-hydroxybenzonitrile which has an herbicidal effect. Information on Azoxystrobin metabolism is studied [49, 50, 51].

In the present study, CMS did not record a significant difference between irrigation treatments or Azoxystrobin spraying. Also, Azoxystrobin application caused a significant increase of RWC and excised leaf water retention (ELWR) in tomato leaves, while caused a significant reduction of relative water loss (RWL) under water deficit stress. These measures represent beneficial mechanisms for tomato plants to tolerate water deficit stress. In contrast, 6 Azoxystrobin sprays may represent a toxic effect on some resulted metabolites, but the plant may be developed a detoxification mechanism leading to stability of parameters values and to vanish the deductions claiming the potential of Azoxystrobin to raise the drought tolerance. In this connection, [7] have noticed that tomato plants sprayed with Azoxystrobin look more vigorous than plants without Azoxystrobin, and the effect on yields was positive, particularly when fungal infection had caused stress. Several studies [52, 53, 54] have reported that the physiological effects of Pyraclostrobin (a Strobilurin like Azoxystrobin) on plants as enhancement of nitrate-reductase activity, change in hormonal balance, water conservation and delayed senescence were reflected in improved plant yield.

[47] have observed that Azoxystrobin causes no visible phytotoxic when applied with a recommended dose. On bean leaves, [55] have stated that Azoxystrobin exhibits no effect on chlorophylls and nitrogen contents. In addition, [56] have reported that foliar application of Azoxystrobin under drought conditions had positive influences on plant water status, water use efficiency and yields in tomato. But, anti-water stress should be possessed a steady physiological response which did not fluctuate with a continuing application, as shown in our results, and did not interfere with physiological function for increasing plant production and yield, as well as, it had no effect on photosynthesis and respiration. In contrast, [57] have indicated that Azoxystrobin was shown to be phytotoxic in some situations during controlling Aphanomyces root rot caused by *Aphanomyces euteiches*, which consider a serious disease in certain green bean (*Phaseolus vulgaris*).

Our results also show that Azoxystrobin treatment, drought condition and integrative Azoxystrobin + drought treatment significantly increased catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO) activities exhibiting vigor enzymes response to drought condition comparing with well-irrigated treatment, and the same trend was observed with free and total phenols contents. These enzymatic and non-enzymatic antioxidants have been shown to support the role for detoxification enzymes, increasing phenols that the main part of Azoxystrobin structure and preparing for the second phase in Azoxystrobin metabolism which called conjugation phase that represented in bound phenols, this assumption ascertained by interaction between irrigation and Azoxystrobin factors. Degradation rate of Azoxystrobin may be increased under drought condition, otherwise, it is unlikely that Azoxystrobin has a role in increased drought tolerance or induced plant defense

mechanism directly, but, its metabolites may cause fluctuation in different phenols depending on plant and its detoxification enzymes.

It has been obviously shown (Table 4) that Azoxystrobin treatment reduced tomato plant size to reduce water loss from drought-stressed plant, and this was gathered with increasing total root volume by Azoxystrobin treatment to increase the root capacity to absorb more water under water deficit stress. This was reflected in increased root dry weight and maintained plant dry weight to increase tomato fruit number under the studied stress. These two mechanisms occurred by Azoxystrobin treatment are very important for tomato plants to survive under drought stress.

Shoot system represents the contact surface area for foliar application with agrochemicals due to their huge surface area, and consequently, more uptake and permeability for a chemical substance to occur their action. After systemic chemical application substance entering in the plant to take place and exhibit their effect, plant deal with it as a xenobiotic and should be eliminate by metabolizing depending on chemical structure of xenobiotic and detoxification enzymes available inside plant, therefore, differentiation of metabolites according to plant. Some metabolites may have a hormonal or herbicidal effect, although, the parent compound don't have this effect as shown in Azoxystrobin fungicide which has no effect on sprayed plants while metabolites has a potential hormonal effect on tested plant which shown clearly on histological level. In this connection, our results showed improvements in most features measured in tomato leaflet and main stem with Azoxystrobin treatment under drought stress condition (Table 5, and Figs 1 and 2). It has been obviously exhibited that leaflet spongy and palisade tissues thickness correlated positively with Azoxystrobin treatments. Increased spongy and palisade tissues contain more chloroplasts that lead to a rise in photosynthesis and production of carbohydrates required to different plant activities. Photosynthesis activity requires more transpiration subsequently, more well water condition is crucial for hormonal activity of Azoxystrobin metabolites.

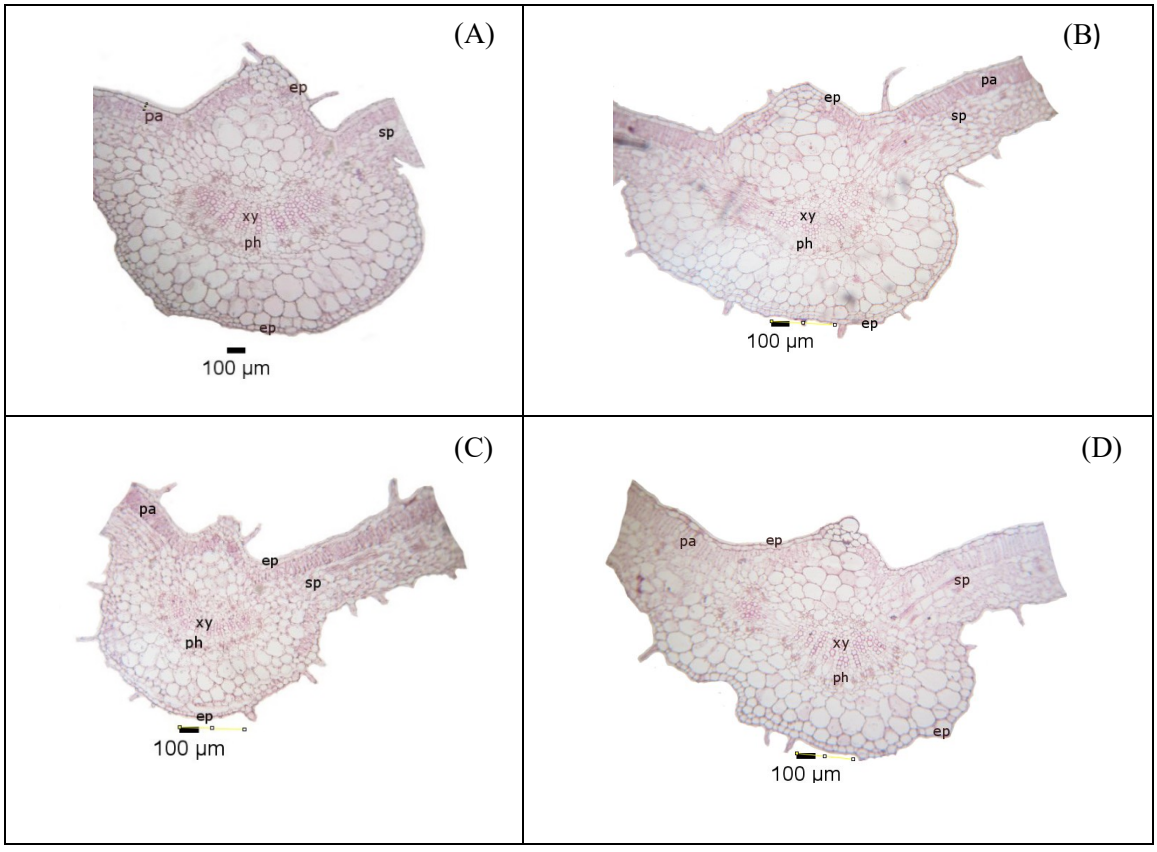

Figure 1. Cross section of tomato leaflet blade from: (A) well watered, (B) well watered + azoxystrobin, (C) drought condition, and (D) drought condition + azoxystrobin
(ep means epidermis, pa means palisade tissue, sp means spongy tissue, ph means phloem tissue, and xy means xylem tissue)

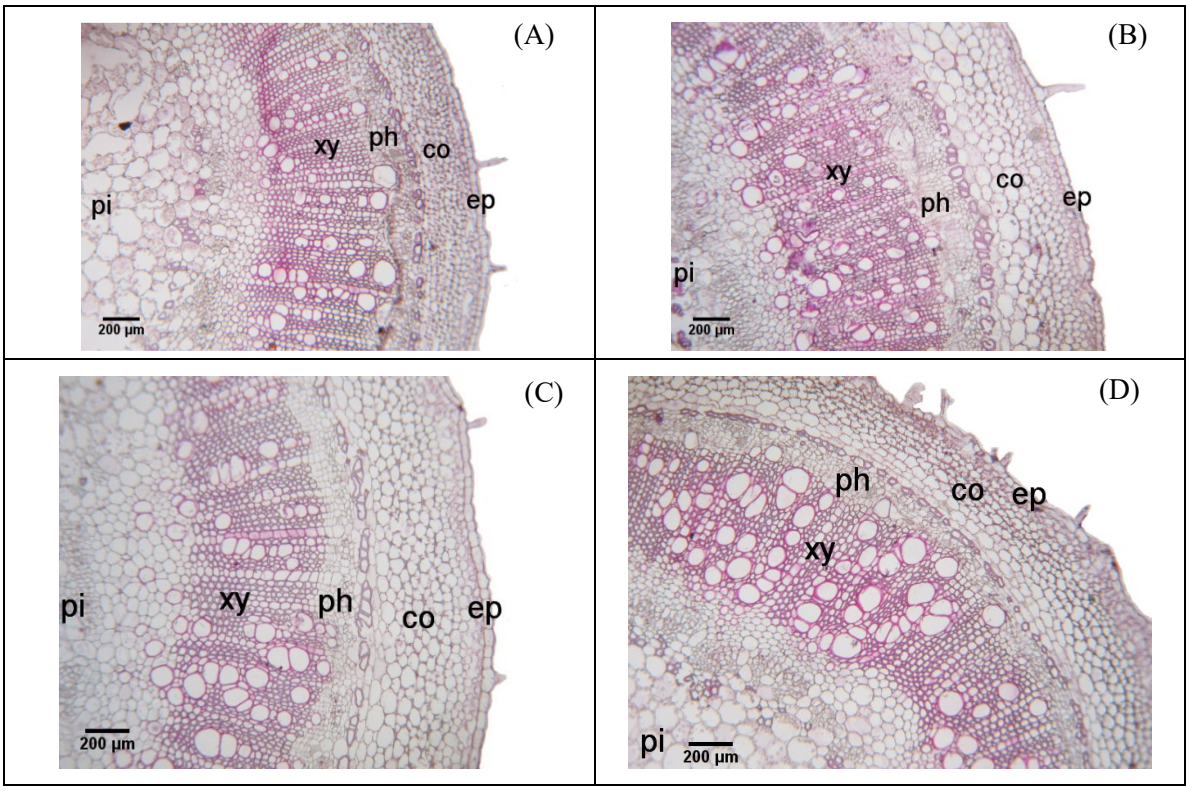

Figure 2. Cross section of tomato main stem from: (A) well watered, (B) well watered + azoxystrobin, (C) drought condition, and (D) drought condition + azoxystrobin
(ep means epidermis, co means cortex, ph means phloem tissue, xy means xylem tissue, and pi means pith)

Conclusion

Azoxystrobin fungicide application was found, in this study, to be pivotal important anti-stress ameliorating drought effects. This was shown through increasing osmoprotectants, antioxidants and photosynthetic efficiency and reducing tomato plant size to reduce water loss from drought stressed-tomato plants, and this was gathered with increasing total root volume by Azoxystrobin application to increase the root capacity to absorb more water under drought stress. These results were reflected in increased root dry weight and maintained plant dry weight to increase the final fruit yield of tomato plant under the studied stress.

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