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RESEARCH ARTICLE

The phyto-impact of fluazinam fungicide on cellular structure, agro-physiological, and yield traits of pepper and eggplant crops

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Abstract

Fluazinam is a widely used fungicide; most of the available information associated with its impact predominately on birds, invertebrates, mammals, and algae and scarce works studied its impact on crop plants. A two years-field experiments were conducted to study the response of pepper and eggplant to fluazinam at 0, 1, 2, and 3 times of the fluazinam-recommended dose (0, 0.5, 1, and 1.5 mL/L). The results revealed that fluazinam did not cause toxic effect on the tested plants except for temporary decline of shoot weights and lengths after 3 days of fluazinam application. However, fluazinam improved the physiological status of leaves via promoting metabolites, antioxidants, better membrane integrity, and adjustment of the redox status of fluazinam-sprayed plants. The ultrastructure changes of fluazinam-treated leaves associated with increment of chloroplasts' starch granules, giant nucleus, and elevated number of mitochondria. After 35 days of treatments, plant length of fungicide-treated plants was found to be higher than control and flowering time showed significant earliness. Furthermore, the yield traits were increased significantly in response to fluazinam. Our findings suggested that fluazinam-treated plants could initiate an early defense mechanism to mitigate the permanent growth retardation. This study could serve as a matrix for further studies to seek elucidation of plants' response to other doses of fluazinam.

Keywords Antioxidants · Cell ultrastructure · Eggplant · Fungicide fluazinam · Metabolites · Pepper

Introduction

In the field, crop plants are cultivated under conditions that predominately focused on the final yield. In this regard, the farmers' activity depends on both preferring plant growth through the fertilizer's supplementation and conserving crops against pathogen invasion and competition with weeds. Indeed, during their biological cycle, crops are normally invaded by many pathogens such as fungi, bacteria, viruses, insects, mites, and nematodes as well as weeds which create a competition with cultivated plants for uptake of the soil minerals. All these biotic stresses cause deteriorations for crops that will result in severe yield losses and/or a decline of yield quality (Pandey et al. 2017). The utilization of agrochemicals is the prime strategy for restraining these problems (Saladin and Clément 2005). Among the various classes of agrochemicals, fungicides, pesticides, bactericides, and nematicides are the most commonly used which correspond to 90% of chemicals used in agriculture. Besides their toxicity that related to their specific target, several fungicides trigger stress to crops which are associated with an alteration of nitrogen and/or carbon metabolism that lead to a lower nutrient availability for plant growth (Saladin and Clément 2005). Fluazinam is a type of protective fungicides that belongs to the chemical group of the 2,6-dinitroanilines (Schepers et al. 2018). It has a wide range of activities and an efficient product against many pathogens like *Alternaria*, *Botrytis*, *Phytophthora*, *Plasmopara*, *Sclerotinia*, *Venturia*, and others

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(Smith et al. 2008). After fluazinam fungicide absorption, it moves to plant tissues and penetrates beyond the cuticle and into the treated leaf tissue itself and it undergoes for a very little movement (translocation) within the leaves of plants. Its mechanism of action depends on breaking off the fungal energy production of the cell process (Schepers et al. 2018).

Many studies have been conducted on the risk of the environmental toxicity with fluazinam chemical groups on macroorganisms. Generally, toxic effects of fluazinam fungicide have been studied mainly on microorganisms, birds, and animals (NRAAVC 2011). The danger of fluazinam to herbivorous birds, insectivorous birds, earthworm-eating birds, fish-eating birds, aquatic invertebrates, algae, and mammals from ingestion is considered to be low at the recommended dose but is highly toxic to aquatic organisms (EFSA 2008). Studies on animal toxicity of the chemical group fluazinam indicate that it has low acute toxicity, leading to slight irritation in the skin and eye and it possesses dermal sensitization potential (Van Ginkel and Sabapathy 1995). The liver and eye have been found to be the most sensitive organs to the exposures to fluazinam groups in the short and long term. In addition, it has an effect on kidneys, pancreas, and testis and bone marrow in rats only when exposed to the long term (NRAAVC 2011). Fluazinam chemical groups give rise to reproductive and developmental toxicity, impairing fetal growth and survival. It increases the frequency of hernia, facial/palatal clefts, and skeletal anomalies; such embryo effects were noticed predominately at doses resulted in maternal toxicity (NRAAVC 2011). Draper et al. (2003) reported the toxicity of fungicides appeared in the form of asthma and dermatitis of the arms and the face on human.

For crops under continuous cropping, additional stress arises from continuous cropping obstacles, which include the frequent occurrence of pests, the gradual accumulation of serious pathogens, decline of soil physicochemical properties, and accumulation of certain poisonous root exudates in the soil (Chen et al. 2011). All of these stresses threaten plant growth and may cause the plant to produce reactive oxygen species and initiation of lipid peroxidation, membrane destruction, protein denaturation, and DNA mutation (Yin et al. 2015) which adversely affect the crop yield. So, the farmers apply fungicides, pesticides, and bactericides in uncontrolled manner postulating that they protect the plants from microorganisms' invasions.

Eggplant and pepper are main daily used vegetables (Younes et al. 2020). Pepper is the second world's important fruit vegetable, ranking after tomatoes. The fruit of pepper have nutritional, medicinal importance and is considered an important source of natural colors and antioxidant compounds (Howard et al. 1994). Eggplant is rich in some minerals and antioxidants. For example, 100 g fresh weight of eggplant can provide ~5% of the recommended daily amount of copper, potassium, and phosphorus as well as ~10% phenolic compounds. In addition, the peels of eggplant fruits are rich in anthocyanins which characterized with its free radical scavenging properties. Furthermore, eggplants are rich in alkaloids which induce "apoptosis in tumor cells" (Frary and Doganlar 2013).

Despite quite studies on the interactive effect of fungicides on crops, the available information about the phyto-impact of fluazinam fungicide on plant cellular structure and agro-physiological traits is scarce. Therefore, the study of the ultrastructure of leaves, and antioxidant response plus metabolites, may help to provide new reference information for highlighting roles of these agrochemicals on the whole plant's life cycle to recognize the stress and recovery periods in tissues, thereafter the influence on crop yield. For that, plant growth evaluations were performed paralleled with the characterization of the responses of the enzymatic and non-enzymatic antioxidant defense systems in pepper and eggplant, thereby the yield responses in relation to fluazinam application as a foliar spray. Thus, the aim of the current work was studying the metabolic profile and antioxidative changes jointed with cellular ultrastructure as well as yield traits of pepper and eggplant in response to different concentrations of fluazinam fungicide.

Materials and methods

Experimental site

The phyto-impact of the fungicide fluazinam as spraying on developmental, physiological, yield, and related traits was conducted for eggplant and pepper within two seasons (2016 and 2017), at the research farm of Faculty of Agriculture, Al-Azhar University, Assiut, Egypt (31° 11′ 1.25″ E and latitude 27° 10′ 51.46″ N).

Soil analysis

Soil samples from 0- to 25-cm depth were taken from each plot before transplanting the eggplant and pepper seedlings in 2016 and 2017. These samples were bulked and subsampled and the analysis was carried out according to Carter and Gregorich (2008). The tested soil texture was clay loam with sand:silt:clay by 25.5%:39.8%:34.7%, pH 7.6, ECe 1.2 dS/m, OM (organic matter) 12%, and $CaCO_3$ 2.7%. The soluble ions (mEq/L; milliequivalents per liter) of the tested soil were HCO_3^- 2.41, Cl^- 2.2, Mg^{+2} 1.9, Na^+ 6.2, and K^+ 0.21. Available nutrients (ppm) were NH_4^+ 48, N 62.4, P 9.2, K^+ 356, SO_4^{-2} 6.6, and Ca^{+2} 4.3.

Eggplant and pepper transplanting and agricultural practices

The commercial F1 hybrid blackberry of eggplant (importer Mecca TRADE Co. as Monarch Seed product, China) and F1 hybrid F-16 of pepper (importer Mecca TRADE Co. as Asia Seed co. Lid product, Korea) were used in this study. Hybrid cultivar seedlings (30 days) were transplanted on rows and planting space as recommended in the previous study (Younes et al. 2019). "Hybrid cultivar seedlings were transplanted in rows 50 cm apart in rows spaced 70 cm for eggplant plants and in rows 30 cm apart in rows spaced 70 cm for pepper plants. Each experimental unit consisted of six rows, 3m long." Experimental units were arranged as complete randomized block design (RCBD) with three replicates for each treatment.

Fungicide treatments

The fungicide fluazinam (fluazinam SC 50%, SC = suspension concentrate) was used as foliar spray after 7 days from transplanting eggplant and pepper seedlings with similar lengths and sizes (same developmental stage) to get uniform responses. Both crops were sprayed with the fungicide at the recommended dose (0.5 mL/L; 120 ml/240 L/ha), and 2-fold (1 mL/L; 240 mL/240 L/ha) and 3-folds of the recommended dose (1.5 mL/L; 360 mL/240 L/ha) as well as distilled water spraying as control plants. The stock solution was prepared by adding the calculated amount of the fungicide in distilled water.

Growth and yield data

The traits were recorded from ten randomly chosen plants per replication (three replicates/treatment) for some growth criteria viz., plant length after 3, 18, and 35 days from fungicide spraying (PL), and days to 50% flowering (50% F) calculated from transplanting date; also at harvesting time (nearly after 5 months), plant length was recorded at harvest (PL_h, cm), branches number per plant (NB), fruits number per plant (NF/P), and fruit yield ton/ha (FY, t/h).

Metabolic activity and antioxidants

All the physiological data were recorded in the second season 2017 after 18 days of fungicide spraying. Fresh shoots and dry weight of eggplants and peppers were recorded.

Photosynthetic pigments The leaf quantification of chlorophyll a, chlorophyll b, and carotenoids (mg/g FW) was performed using the method recommended by (Dawood and Azooz 2019).

Non-enzymatic antioxidants Reduced glutathione (GSH, μg/g FW) and ascorbic acid (ASA, μg/g FW) were determined using the methods adopted by Nahar et al. (2016) and Tahjib-UI-Arif et al. (2019), respectively.

The secondary metabolites Phenolic compounds (mg/g FW), flavonoid content (mg/g FW), and anthocyanins (μg/g FW) of leaves were estimated by the methods of Aery (2010), Zou et al. (2004), and Krizek et al. (1993), respectively.

Reactive oxygen species The oxidative stress was assessed by determination of superoxide anion (μg/g FW, $O_2^{\cdot-}$), hydrogen peroxide (μmol/g FW, H_2O_2), and hydroxyl radical (μmol/g FW, $^{\cdot}OH$) via published methods of Yang et al. (2011), Mukherjee and Choudhuri (1983), and Halliwell et al. (1987), respectively.

Leaf membrane damage–related traits Lipoxygenase activity (LOX, U/mg protein) and lipid peroxidation (μmol/g FW), as well as electrolyte leakage (EL), were determined following Minguez-Mosquera et al. (1993), Zhang and Huang (2013), and Silveira et al. (2009), respectively.

Enzymatic antioxidants The antioxidative properties of leaves were conducted by screening the specific activities of catalase (CAT, U/mg protein/g FW/min), superoxide dismutase (SOD, U/mg protein/g FW/min), ascorbate peroxidase (APX, μmol/mg protein/g FW/min), guaiacol peroxidase (POD, U/mg protein/min), which were performed utilizing the procedures of Noctor et al. (2016), Misra and Fridovich (1972), Silva et al. (2019), and Tatiana et al. (1999), respectively. Glutathione peroxidase (GPX, μmol/mg protein/g FW/min) as well as glutathione-S-transferase (GST, (U/mg protein/min) were done using the methods of Flohé and Günzler (1984) and Ghelfi et al. (2011), respectively. The activities of phenylalanine ammonia lyase (PAL, μmol/mg protein/min) and polyphenol oxidase (PPO, U/mg protein/min) were conducted based on the methods of Sykłowska-Baranek et al. (2012) and Lavid et al. (2001), respectively.

Nitrate reductase activity and nitric oxide content Nitrate reductase activity (NR) was quantified following the described method of Downs et al. (1993) and expressed as micromoles of NO_2 grams/hour. Nitric oxide content (NO) was estimated based on Ding et al. (1998) and Hu et al. (2003) and expressed as nanomoles/gram FW.

Primary metabolites Water extract of leaves was used for the estimation of carbohydrates as was described by Fales (1951) and Schlegel (1956). Soluble proteins were detected by Lowry et al. (1951), and free amino acids (mg/g DW) was performed via Lee and Takahashi (1966). Proline (mg/g DW) was estimated based on published work of Zhang and Huang (2013).

Ultrastructure of plant cells affected by the fungicide fluazinam

The leaves of control and fluazinam-treated leaves were cut, fixed, stained, and visualized with the electron microscope as recommended by Younes et al. (2019). Summarization of the periods and data collected throughout 2017 season from transplanting to yield harvesting is provided in Fig. 2.

Statistical analyses

The obtained results from the two studied seasons (2016 and 2017) were used for the analysis of variance (ANOVA) pertinent to randomized complete block design. Upon the error variances, combined analysis of variance was performed over the years (Gomez and Gomez 1984). The significance of different partitioned components of the total variation was presented and used to decide the meaningful mean comparisons at $p < 0.05$. The least significant difference test (LSD) at 0.05 probability level was employed to separate the means (three replicates per treatment). The statistical analyses were conducted using the MSTATC (software version–4).

Experimental results

Phenotypic and productivity of eggplant and pepper

The mean performance of eggplant and pepper seedlings that received different fluazinam doses is presented in Figs. 1 and 2 as well as Tables 1 and 2. Summarized significance was also given for the different components of the total variation over the 2 years using the combined analysis of variance.

Plant length (PL $_{3d, 18d, 35d, h}$) The effects of different fluazinam doses on plant length after 3, 18, and 35 days from fungicide application on eggplant and pepper plants are presented in Fig. 1 and Table 1, respectively. After 3 and 18 days from spraying, fluazinam negatively influenced and decreased the eggplant and pepper shoot lengths compared to the untreated control where the plants received 1.5 mL/L of fluazinam which was more affected compared to the other doses. After 35 days from spraying, the shoot length of plants treated with fluazinam recovered significantly and had been higher than those of plants derived from hydro-sprayed plants (Fig. 1). The plant length at the end of harvesting time (5 months from spraying the fungicide) of eggplant and pepper was significantly increased when plants derived from seedlings were sprayed with fluazinam which was maximally recorded at 1.5 mL/L. Such results were confident over the two season trials (2016 and 2017).

Number of branches per plant The analysis of variance for the mean number of branches per plant demonstrated a significant effect of the fungicide treatments (Table 1). The plants derived from seedlings sprayed with 1.5 mL/L of fluazinam unequivocally had the greatest number of branches in eggplant and pepper plants as compared to 0, 0.5, and 1 mL/L of fluazinam.

Days to flowering The days to 50% flowering recorded for eggplant and pepper crops differentially responded to the various doses of fluazinam (Table 1). There was no difference in days to 50% flowering for eggplant and pepper plants over the 2 studied years, but highly significant for the applied fungicide. Thus, pepper and eggplant received 1.5 of fluazinam significantly exhibited the shortest days to 50% flowering (in average, 25 and 32 days) as compared to control (36 and 46 days), respectively.

Number of fruits per plant Eggplant and pepper plants that received 1.5 and 1 mL/L of fluazinam, respectively, produced significantly higher number of fruits per plant. In contrast, the plant that received 0 and 0.5 mL/L of the fungicide fluazinam produced the minimum fruit number per plant. Such a result was documented over the 2-year trial (2016 and 2017) as represented in Table 1.

Total fruit yield (ton/h) The aerosol of fluazinam with doses 1.5 and 1 mL/L produced significantly higher total fruit yield on pepper and eggplant, respectively (Table 1), compared to control. The percent increment of the total fruit yield was 86% and 66% over the control for pepper and eggplant, respectively. This result was consistent over the 2 years (2016 and 2017). The concentration of 0.5 mL/L had non-significant effect on yield traits.

Leaf biochemistry

For all growth criteria (fresh and dry weight of shoot) represented in Table 2, hydro-sprayed plants exhibit the highest shoot biomass values followed by 0.5 mL/L of the fungicide fluazinam, and 1.5 mL/L of the fungicide spraying had the lowest values. A gradual reduction of fresh and dry weight of shoot was found in response to fluazinam spraying at 1-, 2-, and 3-folds of the recommended dose. The reduction of shoot weight by fluazinam was found to be not significant for pepper, while it was highly significant for eggplant plants.

Chlorophyll a, chlorophyll b, and carotenoids (Table 2) found to be stimulated by fluazinam application for both plants, but the increment of Chl. b and carotenoids was not significant for eggplants. The data presented in Table 2 denoted that spraying of fluazinam highly significantly influenced primary metabolites of both crop plants. Proteins, carbohydrates, amino acids, and proline were accumulated by the application of fluazinam where the dose of 1.5 mL/L recorded

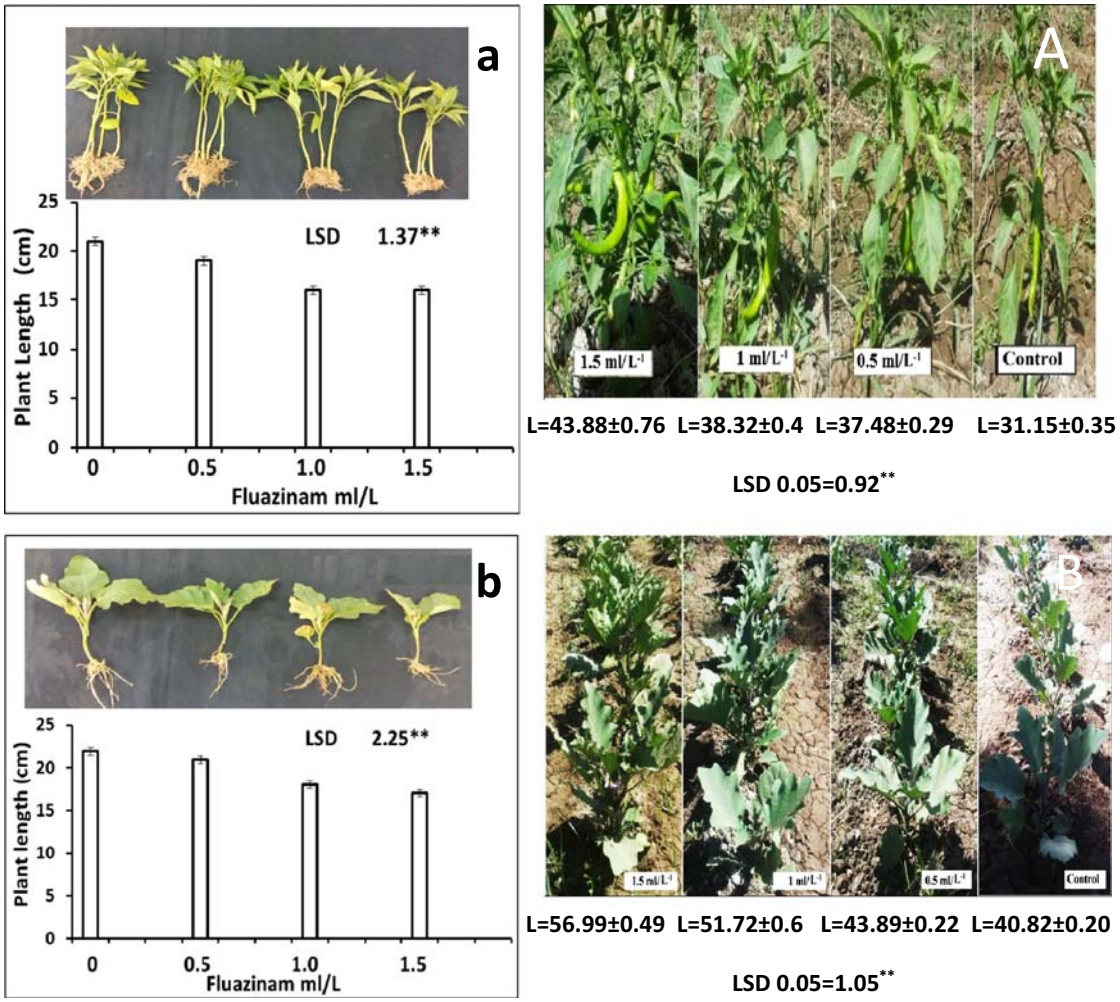

Fig. 1 Effect of spraying different concentrations of fluazinam (0, 0.5, 1, and 1.5 ml/L) on mean plant lengths ± SE of peppers (a, A) and eggplants (b, B) recorded after 3 and 35 days, respectively from spraying which compared using LSD at $p \leq 0.05$

the topmost values of these metabolites for both plants. In this regard, the content of proteins, carbohydrates, amino acids, and proline in pepper at the concentration of 1.5 mL/L fluazinam were 180, 125, 24, and 11 mg/g DW compared to 121, 95, 14, and 1.6 mg/g DW for the control and that of eggplant was 142, 122, 20, and 7 mg/g DW at the concentration of 1.5 mL/L fluazinam compared to 96, 87, 9, and 2 mg/g DW of control.

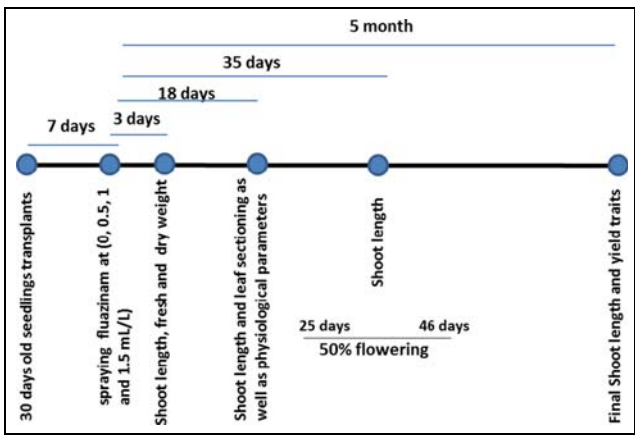

Fig. 2 Summarization of the period and data collected throughout 2017 season from transplanting to yield harvesting

Similarly, the activity of nitrate reductase enzyme highly significantly enhanced via fluazinam treatments to be maximally recorded at 1.5 mL/L by about 33% and 52% over the control for pepper and eggplant, respectively.

Fluazinam non-significantly affected superoxide radical whatever the concentration applied or crop used. On the other hand, hydrogen peroxide and hydroxyl radical were reduced significantly as the dose of fungicide fluazinam intensified on leaves. Furthermore, nitric oxide of the leaves of fungicide fluazinam promoted which found to be up mostly recorded at the dose 1.5 mL/L with percent increment 32% and 37% over the control for pepper and eggplant, respectively (Table 3).

The leaves of pepper and eggplant exposed to different doses of fungicide fluazinam exhibited retardation of lipoxygenase activity, while electrolyte leakage as well as

Table 1 Growth and yield traits as affected by different doses of fluazinam fungicide (0, 0.5, 1.0, and 1.5 mL/L) on pepper and eggplant crops. Each value represents a mean value of three replicates ± SE

Traits		Pepper						Eggplant					
Treatment		PL_{18d}	PL_h	NB	50%F	NF/P	FY	PL_{18d}	PL_h	NB	50%F	NF/P	FY
2016 Fluazinam, mL/L	0	28.95 ± 0.77	78.89 ± 1.38	6.77 ± 0.13	36.98 ± 0.85	20.88 ± 1.4	8.85 ± 0.26	34.09 ± 0.79	78.89 ± 0.98	3.50 ± 0.15	46.91 ± 0.29	13.83 ± 0.73	33.88 ± 1.27
	0.5	25.48 ± 0.44	77.15 ± 0.30	6.93 ± 0.15	37.31 ± 0.6	22.45 ± 0.57	8.42 ± 0.31	29.66 ± 0.38	77.15 ± 0.53	3.43 ± 0.12	45.07 ± 0.20	13.14 ± 0.56	32.27 ± 0.64
	1.0	22.15 ± 0.55	85.95 ± 0.37	8.33 ± 0.42	28.58 ± 0.28	25.90 ± 1.43	12.08 ± 0.75	24.75 ± 0.42	85.95 ± 0.31	4.80 ± 0.06	38.21 ± 0.93	19.59 ± 0.71	49.22 ± 0.62
	1.5	18.92 ± 0.85	91.33 ± 1.19	9.10 ± 0.55	25.63 ± 0.92	33.09 ± 1.65	15.43 ± 0.54	16.86 ± 1.21	91.33 ± 0.84	5.78 ± 0.28	32.58 ± 1.39	17.67 ± 0.88	53.66 ± 0.87
Mean over treatments		23.88	83.33	4.38	32.13	25.58	11.28	26.34	82.03	7.78	83.33	16.3	42.26
2017 Fluazinam, mL/L	0	29.34 ± 0.69	49.70 ± 0.43	6.78 ± 0.67	37.91 ± 0.79	20.4 ± 0.85	8.51 ± 0.30	34.39 ± 1.62	74.54 ± 1.14	3.17 ± 0.12	46.93 ± 0.53	11.99 ± 0.33	33.12 ± 1.95
	0.5	24.78 ± 0.99	50.83 ± 1.69	7.07 ± 0.45	38.95 ± 0.84	20.52 ± 0.33	8.93 ± 0.24	29.88 ± 0.73	74.86 ± 0.92	3.4 ± 0.15	44.66 ± 1.4	14.02 ± 0.33	32.27 ± 1.6
	1.0	22.67 ± 0.27	53.74 ± 0.93	8.08 ± 0.14	28.71 ± 0.82	29.27 ± 0.57	11.81 ± 0.46	26.48 ± 0.6	87.84 ± 0.29	5.0 ± 0.15	38.97 ± 0.85	17.77 ± 0.79	50.48 ± 1.14
	1.5	19.09 ± 0.12	61.06 ± 0.68	9.41 ± 0.9	26.17 ± 0.48	33.4 ± 1.69	15.58 ± 0.85	19.25 ± 1.56	90.87 ± 0.64	6.0 ± 0.17	33.46 ± 0.48	19.55 ± 0.81	55.60 ± 1.1
Mean over treatments		23.99	53.83	4.39	32.94	25.9	11.21	27.50	82.03	7.83	82.03	15.83	42.87
ANOVA													
Source of variance		**Mean square (MS)**						**Mean square (MS)**					
Y		0.053 ns	0.004 ns	0.001 ns	3.929 ns	0.592 ns	0.031 ns	7.924 ns	10.19 ns	0.015 ns	0.580 ns	3.176 ns	2.233 ns
T		110.423*	167.638**	9.138**	229.034**	207.688**	66.531**	284.04**	337.66**	7.952**	238.907**	70.916**	773.315**
Y × *T*		0.456 ns	1.783 ns	0.101 ns	0.618 ns	7.523 ns	0.234 ns	1.707 ns	10.613 *	0.082 ns	0.566 ns	2.706 ns	2.225 ns
LSD 0.05		*T* = 1.27	*T* = 2.29	*T* = 2.29	*T* = 1.57	*T* = 2.18	*T* = 1.02	*T* = 2.43	*T* = 1.45	*T* = 1.45	*T* = 2.02	*T* = 1.63	*T* = 2.74

PL_{18d} = plant length at 18 days from spraying, *PL_h* = plant length at the end of harvesting time, *NB* = number of branches/plant, *50%F* = days to 50% flowering (day), *NF* = number of fruit/plant, *FY* = fruits yield (ton/ha). *Ns*, *, ** = non-significant, significant, and highly significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

Table 2 Fresh and dry weight of shoot, chlorophyll a, chlorophyll b, carotenoids, carbohydrates, proteins, amino acids, and proline of pepper and eggplant as affected by different doses of fluazinam fungicide (0, 0.5, 1.0, and 1.5 mL/L). Each value represents a mean value of three replicates ± SE and means were compared using LSD at $p \leq 0.05$

	Pepper treated with fluazinam				LSD	Eggplant treated with fluazinam				LSD
	0 mL/L	0.5 mL/L	1.0 mL/L	1.5 mL/L		0 mL/L	0.5 mL/L	1.0 mL/L	1.5 mL/L	
Shoot fresh weight	3.45 ± 0.33	3.44 ± 0.17	3.36 ± 0.05	3.16 ± 0.06	NS	14.31 ± 0.16	11.41 ± 0.12	9.3 ± 0.25	8.77 ± 0.11	0.49**
Shoot dry weight	0.45 ± 0.02	0.45 ± 0.03	0.44 ± 0.02	0.42 ± 0.02	NS	1.66 ± 0.01	1.12 ± 0.02	1.31 ± 0.19	0.85 ± 0.01	0.26**
Chlorophyll a	0.64 ± 0.06	1.51 ± 0.07	0.95 ± 0	0.94 ± 0.02	0.14**	0.87 ± 0.01	0.88 ± 0.01	0.88 ± 0	0.97 ± 0.01	0.04**
Chlorophyll b	0.23 ± 0.02	0.55 ± 0.02	0.39 ± 0.04	0.38 ± 0.01	0.05**	0.31 ± 0.04	0.31 ± 0. 01	0.31 ± 0.01	0.30 ± 0. 02	NS
Carotenoids	0.47 ± 0.03	0.97 ± 0.08	0.81 ± 0.08	0.93 ± 0.08	0.14**	0.39 ± 0.04	0.46 ± 0.03	0.43 ± 0.01	0.43 ± 0.04	NS
Carbohydrates	95.62 ± 1.55	107.11 ± 1.97	116.45 ± 2.19	125.94 ± 2.5	4.47**	87.22 ± 0.16	94.55 ± 3.13	108.18 ± 1.02	122.72 ± 2.69	4.109**
Proteins	121.58 ± 0.92	140.9 ± 1.11	156.47 ± 1.29	180.22 ± 1.12	4.232**	96.47 ± 1.53	110.68 ± 0.86	124.16 ± 1.73	142.48 ± 3.08	2.043**
Amino acids	14.11 ± 0.95	15.33 ± 0.71	18.46 ± 1.32	24.76 ± 1.06	1.998**	8.94 ± 0.68	10.74 ± 0.75	10.89 ± 7.92	20.02 ± 1.35	2.361**
Proline	1.6 ± 0.06	3.41 ± 0.03	6.81 ± 0.62	11.56 ± 1.05	1.44**	2.00 ± 0.02	3.15 ± 0.03	5.10 ± 0.15	7.01 ± 0.24	0.34**

Ns, *, ** = non-significant, significant, and highly significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

Table 3 Physiological traits as affected by different doses of fluazinam fungicide (0, 0.5, 1.0, and 1.5 mL/L) on pepper and eggplant crops. Each value represents a mean value of three replicates ± SE and means were compared using LSD at $p \leq 0.05$

	Pepper treated with fluazinam				LSD	Eggplant treated with fluazinam				LSD
	0 mL/L	0.5 mL/L	1.0 mL/L	1.5 mL/L		0 mL/L	0.5 mL/L	1.0 mL/L	1.5 mL/L	
Hydrogen peroxide	217.91 ± 1.43	211.93 ± 2.01	205.33 ± 1.86	198.58 ± 3.99	5.22**	257.96 ± 2.6	250.02 ± 2.87	244.89 ± 3.49	234.01 ± 9.11	12.56**
Superoxide anion	22.4 ± 1.85	22.31 ± 0.31	21.8 ± 0.33	21.61 ± 0.18	NS	38.62 ± 4.06	37.75 ± 0.65	37.41 ± 1.58	37.05 ± 3.3	NS
Hydroxyl radical	8.28 ± 0.05	8.14 ± 0.07	6.51 ± 0.07	4.17 ± 0.17	0.32**	13.51 ± 0.15	13.37 ± 0.15	11.63 ± 0.2	9.07 ± 0.32	0.53**
Electrolyte leakage	26.9 ± 0.29	25.85 ± 1.16	26.91 ± 0.25	26.66 ± 1.27	0.425*	31.05 ± 0.36	33.81 ± 0.44	34.01 ± 0.45	34.57 ± 0.67	1.72*
Lipid peroxidation	91.47 ± 0.99	87.47 ± 2.31	77.36 ± 0.06	75.17 ± 5.68	8.78**	49.46 ± 0.48	46 ± 0.59	45.21 ± 2.13	41 ± 0.79	NS
Lipoxygenase activity	3.31 ± 0.04	3.08 ± 0.02	2.64 ± 0.02	2.87 ± 0.2	0.31**	8.43 ± 0.09	8.64 ± 0.11	7.66 ± 0.12	7.45 ± 0.26	0.39**
Ascorbic acid	29.34 ± 0.35	40.76 ± 0.59	49.31 ± 0.61	59.01 ± 57.1	5.26**	25.74 ± 0.28	27.3 ± 2.89	31.52 ± 1.9	38.28 ± 3.55	7.14**
Reduced glutathione	49.09 ± 4.21	66.76 ± 0.86	71.31 ± 8.02	85.61 ± 0.71	11.08**	34.07 ± 2.45	37.61 ± 2.3	44.77 ± 1.3	50.79 ± 3.41	5.66**
Phenolic compounds	400.86 ± 2.9	524.6 ± 5.13	529.67 ± 4.66	544.36 ± 5.53	9.71**	434.34 ± 4.83	444.06 ± 8.82	497.86 ± 8.47	527.98 ± 8.69	13.07**
Flavonoids	114.91 ± 1.25	130.42 ± 4.84	134.57 ± 0.15	141.12 ± 3.1	8.00**	92.44 ± 1.07	94.33 ± 1.22	118.31 ± 2.28	133.78 ± 2.59	5.06**
Anthocyanin	16.02 ± 0.11	20.93 ± 0.2	21.61 ± 0.19	29.08 ± 2.82	3.54**	11.11 ± 0.12	15.7 ± 1.66	19.39 ± 1.17	22.1 ± 2.05	4.15**
Nitric oxide	71.37 ± 1.06	77.38 ± 1.74	82.92 ± 3.65	94.29 ± 1.12	1.845**	65.49 ± 1.19	74.93 ± 4.69	80.86 ± 1.3	89.74 ± 2.85	3.024**
Nitrate reductase	60.71 ± 0.89	66.37 ± 1.79	76.99 ± 1.51	80.85 ± 1.52	2.460**	43.93 ± 1.77	55.02 ± 1.2	58.39 ± 0.59	66.81 ± 2.05	3.119**

Ns, *, ** = non-significant, significant, and highly significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

lipid peroxidation was non-significantly changed, whatever the dose of fungicide fluazinam except for the reduction of lipid peroxidation in pepper which was significant at the concentrations 1 and 1.5 mL/L (Table 3).

The foliar application of fluazinam caused a stronger increase in the concentration of ascorbic acid (201% and 48%) and reduced glutathione (72% and 49%) of pepper and eggplant, respectively, compared to control. In the same vein, secondary metabolites as phenolic compounds, anthocyanins, and flavonoids were vastly triggered by fluazinam application to be maximally recorded at 1.5 mL/L (Table 3).

The fungicide fluazinam significantly stimulated the antioxidant enzyme system (CAT, POD, APX, GST, and GPX), but the increase of APX was not significant for pepper plants. On the other hand, SOD activity was not significantly affected whatever the concentration of fluazinam applied and crop plant tested (Table 4).

Phenylalanine ammonia lyase (PAL) was at its lowest activity in water-sprayed plants, while enormous increase in PAL activity was recorded for the leaves of fluazinam-treated plants which was maximally recorded at the level of 1.5 mL/L (Table 4). Polyphenol oxidase was at its highest values in water-sprayed plants. A slight reduction in PPO was observed for pepper, while relatively higher reduction was attained for eggplants. The highest reduction values were demonstrated for the dose 1.5 mL/L by about 7% and 26% compared to water-sprayed plants (Table 4).

Leaf ultrastructure

The effects of spraying eggplant and pepper with fluazinam on leaf cell ultrastructure were examined at 18 days post fungicide treatment, which is presented in Figs. 3 and 4. Fluazinam treatment at 1.5 mL/L was selected to visualize the leaf cell ultrastructure by using a transmission electron microscope based on the best results of morphological and fruit yield in the first season (2016). The leaves submitted to fluazinam modified clearly compared to control plants where chloroplast displayed change in the size and the number of starch granules. Nucleus found to be giant and the cytoplasm of the cells was dense compared to the control. The number of mitochondria of fungicide-treated plants was higher than that of control plants. These modifications were vastly observed for eggplants compared to pepper (Figs. 3 and 4).

Discussion

The fungicide fluazinam was poorly inspected in relation to their effects on crop plants, though our data proved that the applied doses of fluazinam are nontoxic and healthful on leaves physiology. The data clearly revealed that fungicide-sprayed plants underwent a stress-sensitive period where

Table 4 Enzymatic antioxidants as affected by different doses of fluazinam fungicide (0, 0.5, 1.0, and 1.5 ml/L) on pepper and eggplant crops. Each value represents a mean value of three replicates ± SE and means were compared using LSD at $p \leq 0.05$

	Pepper treated with fluazinam				LSD	Eggplant treated with fluazinam				LSD
	0 mL/L	0.5 mL/L	1.0 mL/L	1.5 mL/L		0 mL/L	0.5 mL/L	1.0 mL/L	1.5 mL/L	
Catalase	77.51 ± 1.24	82.42 ± 0.8	79.62 ± 0.7	83.5 ± 4.14	4.79*	68.95 ± 0.76	69.58 ± 0.81	73.09 ± 2.27	78.9 ± 2.78	4.34**
Superoxide dismutase	46.82 ± 2.64	42.95 ± 2.35	41.25 ± 0.39	44.55 ± 5.02	NS	69.25 ± 2.88	70.38 ± 0.91	75.9 ± 3.5	75.88 ± 1.24	NS
Guaiacol peroxidase	122.45 ± 1.65	125.52 ± 1.79	168.25 ± 4.3	179.99 ± 1.44	7.37**	198.52 ± 5.06	215.33 ± 3.54	259.37 ± 2.94	291.66 ± 7.49	6.33**
Ascorbate peroxidase	174.84 ± 4.5	180.96 ± 1.6	175.66 ± 1.67	178.03 ± 2.73	NS	119.84 ± 2.94	130.53 ± 1.7	179.05 ± 4.68	190.9 ± 3.12	9.08**
Glutathione peroxidase	61.95 ± 3.08	74.09 ± 4.04	91.3 ± 0.87	98.59 ± 2.03	7.62**	58.25 ± 1.43	79.01 ± 1.02	102.05 ± 2.72	154.39 ± 2.52	5.47**
Polyphenol oxidase	48.35 ± 2.02	49.49 ± 0.48	47.21 ± 0.41	45.25 ± 0.6	NS	50.01 ± 0.55	42.1 ± 0.49	41.95 ± 1.3	37.05 ± 1.3	9.54**
Phenylalanine ammonia lyase	29.87 ± 0.4	73.04 ± 1.04	57.01 ± 5.19	51.9 ± 0.41	2.37**	24.03 ± 0.85	27.99 ± 0.46	29.03 ± 0.29	33.66 ± 0.61	2.30**
Glutathione-S-transferase	84.25 ± 1.34	97.58 ± 0.95	111.66 ± 0.98	142.94 ± 5.28	9.31**	74.33 ± 0.82	98.65 ± 1.15	142.59 ± 4.44	192.75 ± 6.81	0.73**

Ns,*, ** = non-significant, significant, and highly significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

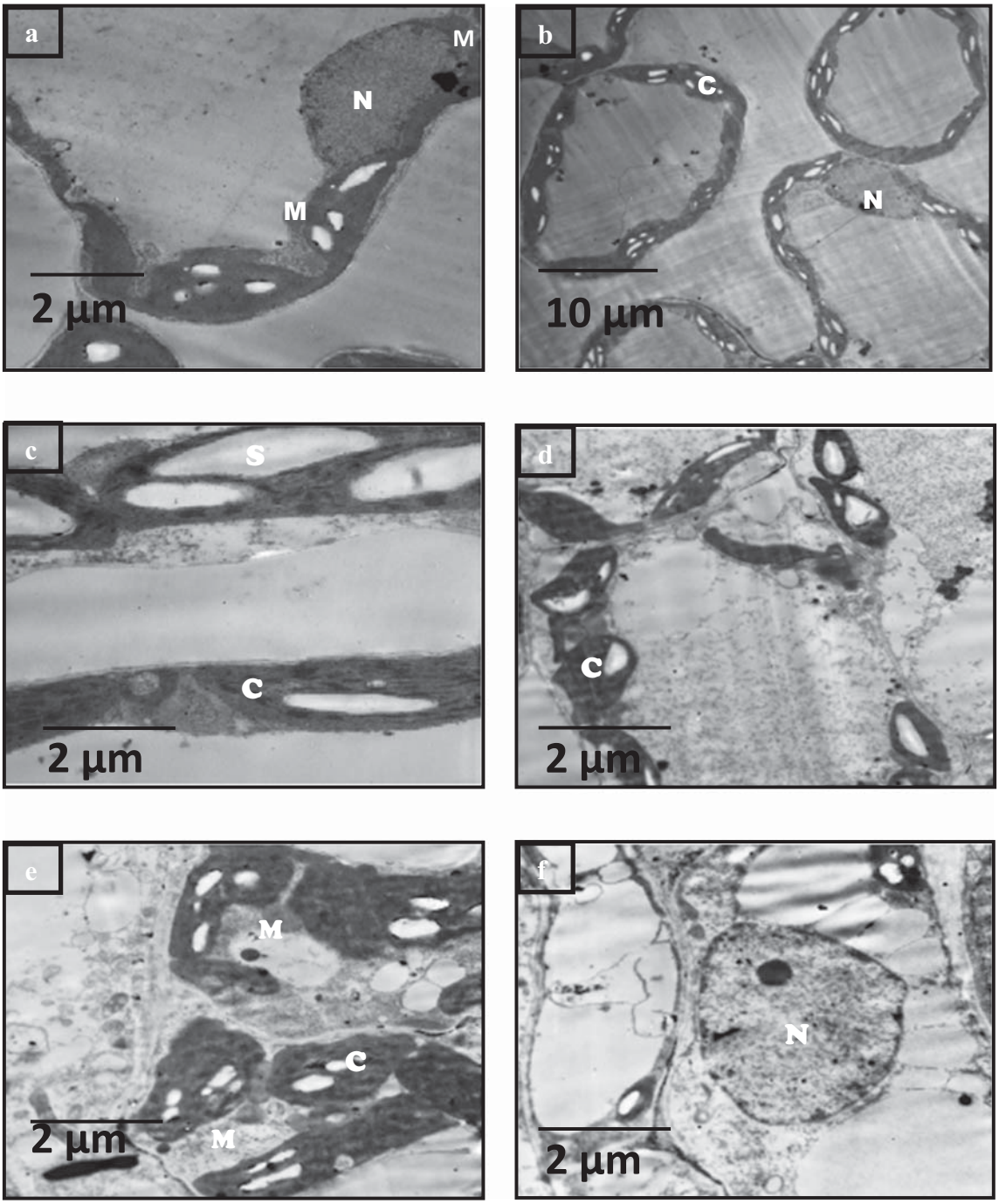

Fig. 3 The ultrastructure of eggplant leaves was studied using transmission electron microscope. (a) Cross-sections of palisade mesophyll cells in control eggplant. In (b–f), the leaves of eggplant exposed to 1.5 mL/L of the fungicide fluazinam. (C) Chloroplast, (M) mitochondria, (N) nucleus, and (S) starch grain. The leaves exposed to fluazinam showed large chloroplasts with large size and higher number of starch granules with well-developed grana (b, c, d). The number of mitochondria of fungicide-treated plants was higher than that of control plants (e). Nucleus found to be giant and the cytoplasm of the cells was dense compared to the control (f)

pepper and eggplants at the first 20 days after fluazinam application exhibited stunt growth. This could be due to the inhibition of both gibberellin and sterol biosynthesis as declared by Buchenauer and Röhner (1981) who found that the growth of barely was inhibited by two triazole fungicides. As the sprayed plants were at a stage of fast growth, the plant species may be more susceptible to any treatments as fungicide applications. During these stress, the plants may modulate nutrients and metabolites to evolve protection mechanisms on account of growth (Saladin and Clément 2005). After 1 month of fluazinam spraying, the plant growth turned over from a state of stunt growth to upregulation which

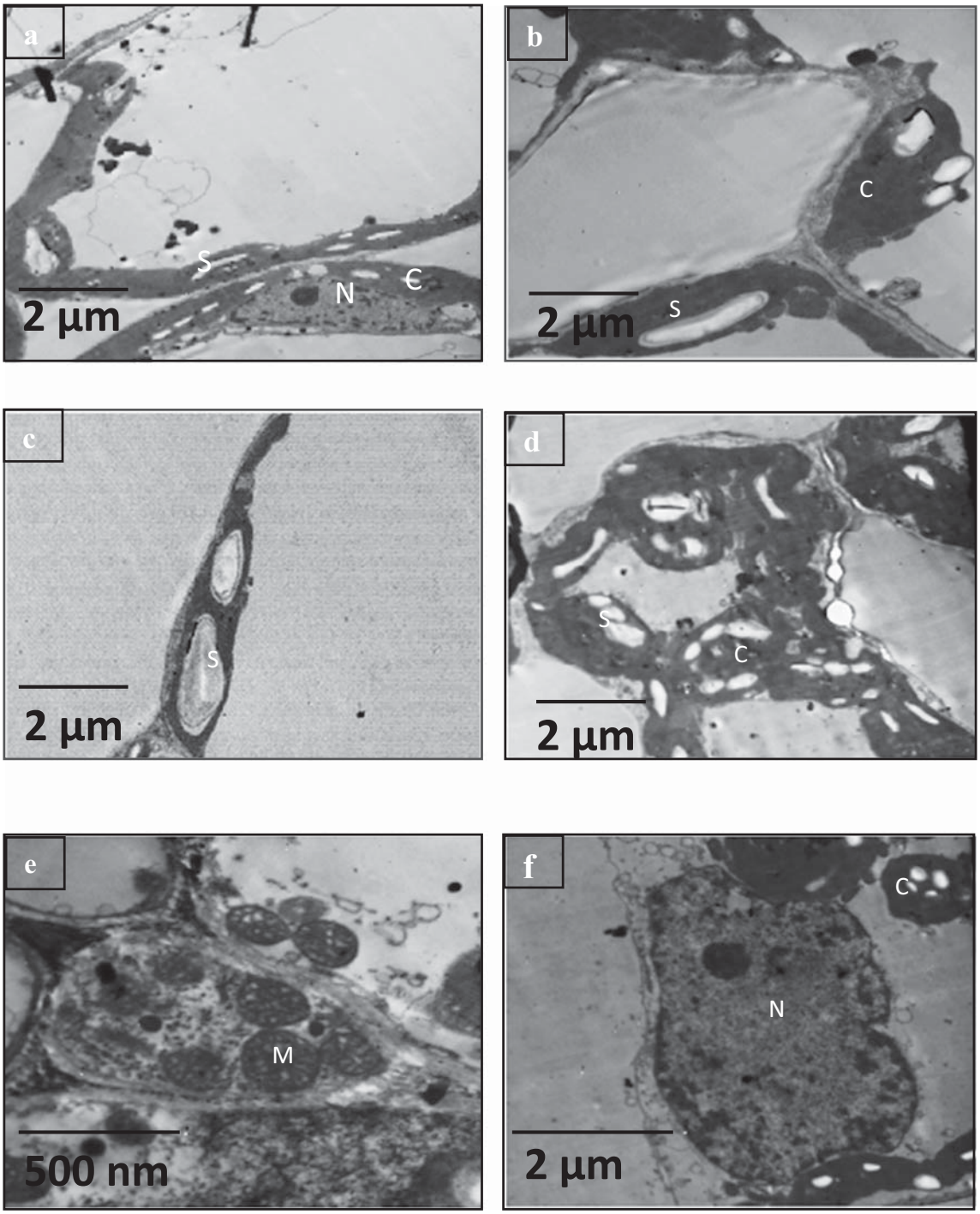

Fig. 4 Transmission-electron microscope of the leaves of pepper plant; (a) control plants and (b–f) cross-sections of pepper leaves exposed to 1.5 ml/L of the fungicide fluazinam. (C) Chloroplast, (M) mitochondria, (N) nucleus, and (S) starch grain. The leaves exposed to fluazinam showed large chloroplasts with large size and higher number of starch granules (b, c, and d) of the cells was dense compared to the control. The number of mitochondria of fungicide-treated plants was higher than that of control plants (e) and the nucleus found to be giant compared to control (f)

appeared from the higher shoot length of the fungicide-sprayed plants compared to hydro-sprayed plants. This implied that pepper and eggplant were able to withstand this temporary stress via eliciting different pathways of plant defenses enabling crops to recover better morphology.

Unlike morphological appearance, the biochemical responses of leaves to fungicide seemed to be a protective agent. In this respect, the fungicide-sprayed plants enhanced photosynthetic pigments (chlorophyll a and b content) higher than water-sprayed plants. Similarly, Saladin et al. (2003b)

reported that the application of pyrimethanil and fludioxonil stimulated the photosynthesis of vineyard. Thus, the carbon metabolism in terms of sugar content of fluazinam-treated leaves, herein, stimulated for pepper and eggplant. In the same context, the herbicide flumioxazin caused a strong accumulation of soluble carbohydrate and starch contents of grapevine grown in vitro (Saladin et al. 2003a). Thus, in the present study, the accumulation of sugars may be revealed that the leaves of fluazinam-treated plants were of peak accumulation of photo-assimilates that accelerated the switch from vegetative to the flowering stage; thereby, early flowering resulted. As flowering coincided with triggering photo-assimilate production in the green area which was the main source until the admittance of the vegetables into reproductive phase.

The earliness of flowering was underpinned by high upregulation of leaves' nitrogenous metabolism where the accumulation of amino acids and proteins by the fluazinam indicates well-constructed leaves with high metabolic efficacy. Similar stimulation of protein synthesis was reported by application of other fungicides as pyrimethanil on grapevines (Llorens et al. 2000) as well as azoxystrobin and epoxiconazole applied on barley leaves (Wu and Von Tiedman 2002). The results, herein, indicated that both plants responded to spraying of fluazinam by de novo nitrogenous components synthesis. This was connected to the invigoration of nitrate reductase activity which is an indispensable enzyme for nitrogen uptake by plants. Likewise to amino acids, proline promoted highly significantly by fluazinam treatment relative to control. This accumulation may act as a pivotal medium for respiration, thereby providing energy required for stress mitigation (Schröder 2001). Moreover, proline was speculated as a storage compound providing nitrogen and carbon for post-stress growth retrieval (Vartanian et al. 1992). This stimulation of carbon and nitrogen metabolites might accomplish the requirements of organic components needed for the formation of new branches where the highest increase of proteins, carbohydrates, and amino acids corresponded to more branches per plants.

Many studies denoted that NR is a fundamental participant for NO biosynthesis in plants (Chamizo-Ampudia et al. 2017). Thus, the activation of NR under fluazinam treatment was concomitant with the increment of NO. This enhancement of NO could be added to the regulatory role of the applied doses of fluazinam especially the dose of 1.5 mL/L. This response is affected by the NO status which plays an important role in plant immune signaling in addition to the enhancement of whole plant development. Furthermore, NO is soundly reported as an antioxidant itself, and concomitant with inducing the antioxidant enzyme production, it itself quenches the cellular reactive oxygen species (Dawood and Azooz 2019).

The productions of reactive oxygen species draw the susceptibility of plants to the test applicant. The abatements of H_2O_2 and $^{\cdot}OH$ and stabilization of $O_2^{\cdot-}$ production in the leaves sprayed with fluazinam reflected that this agent did not induce harmful impacts on plants. Thus, fluazinam spraying levels from 0.5 to 1.5 mL/L did not induce oxidative stress on the studied plants which could be ascribed to the participation of enzymatic and non-enzymatic antioxidants in eliminating these active oxygen species. These compounds control ROS biosynthesis and quenching molecules are pivotal mechanisms during abiotic stress, which display potential functions for yield permanence (Siebers et al. 2015). To attain a better understanding of the possible role of the fungicide fluazinam on enzymatic antioxidants, we screened the activities of SOD, POD, CAT, APX, GPX, and GST. SOD-specific activity, herein, was not affected by fluazinam; hence, $O_2^{\cdot-}$ content found to be kept around the control values. The decline of H_2O_2 content, a strong oxidant, is prevented in plant cells by the marked accumulation of CAT, APX, POD, and GPX as a reaction to fluazinam spraying. Similarly, the fungicides azoxystrobin and epoxiconazole sprayed on barley's leaves had boosted the activity of antioxidative enzymes as catalase, ascorbate peroxidase, and guaiacol peroxidase, after 4 days from the exposure (Wu and Von Tiedman 2002). The enhancement of GST in leaves exposed to fluazinam was compared to water-sprayed controls for both plants reflecting the involvement of GST as a defensive mechanism alarming the cell with the presence of xenobiotic agrochemical as fluazinam. This may trigger wide disciplines of antioxidants and metabolic pathways that collectively improved the physiological status of the leaves. Deavall et al. (2012) proposed that GSTs protect the plant cells against chemical-triggered toxicity and supply tolerance by inducing S-conjugation between GSH and electrophilic moiety in the hydrophobic and toxic molecules.

In this work, GSH was activated in fungicide-sprayed plants which could confer high detoxification capacity because GSH is the main contributor of ASA-GSH pool plus it is a substrate of GPX as well as GST, indicating that there was a balance in the utilization of elevated values of GSH by these antioxidant enzymes. Furthermore, GSH directly scavenges ROS and may protect enzyme thiol groups (Gill et al. 2013). Concomitantly, the stimulation of ascorbic acid or vitamin C by fluazinam could be a prominent indicator of plant healthiness. Furthermore, AsA accumulation went in parallelism with the accumulation of APX activity. Thus, AsA enhanced the defense mechanisms relevant to antioxidative properties to mediate oxidative stress in plants by controlling ROS detoxification alone or in combination with reduced glutathione and other ROS-metabolizing enzymes and compounds.

Another important defense pathway stimulated in pepper and eggplant as a response to fluazinam was the enhancement of secondary metabolism as phenolic compounds. A wide

range of phenolic compounds, such as flavonoids and anthocyanins, activates by phenylalanine ammonia lyase enzyme (Younes et al. 2019). These compounds have multiple functions related to antioxidant properties and the ability to remove free oxygen radicals (Dawood and Azooz 2019; Bagy et al. 2019). Bi et al. (2014) reported that anthocyanin participates more to H_2O_2 detoxification compared to other phenols. Similar response of flavonoids as a bioactive agents and a part of phenolic compounds positively reacted to fluazinam which conferred antioxidant properties to fungicide-submitted plants. These compounds stabilize membranes by decreasing their fluidity which in turn limits the diffusion of free radicals and reduces the peroxidation of membrane lipids. In addition, flavonoids specifically share in membrane stabilization due its ability to bind to some of integral membrane proteins and phospholipids (Kulbat 2016). This may be partially accounted for maintaining the lowering of the lipid peroxidation in plants treated by fluazinam, hence higher membrane integrity compared to control plants.

The cytotoxic compounds as malondialdehyde content, end product of lipid peroxidation, could also be detoxified by binding to GSH, via GSH-conjugate formation which is mediated by GST (Blair 2010). Thus, the fungicide-triggered defense mechanism prevented chain of reactions, thereby curtailing ROS formation and membrane deteriorations. Lipid peroxidation is detected also by the determination of lipoxygenase enzyme activity (LOX). It is worthy to mention that the increment of LOX activity is responsible for oxidation of polyunsaturated fatty acids, thus enhances lipid peroxidation under stress conditions (Sallam et al. 2019). Unlikely, the used fungicide highly significantly reduced LOX to be lower than the water-sprayed plants as was reported for lipid peroxidation and electrolyte leakage. Therefore, the decrease of LOX activity by fluazinam reduced the production of reactive oxygen species, which might protect lipids from oxidative damage, hence the enhancement of membrane stability. Because the leaves' senescence is generally associated with the burst of oxidative stress and membrane damages (Khanna-Chopra 2012), this maintenance of membrane integrity of fluazinam-treated leaves could delay leaves' senescence. The delay of senescing conferred the connection of the leaves to the growing plants for a prolonged time with a raised photosynthesis (Yu et al. 2004) and increased the translocation rate of photosynthates to the fruit production. In this sense, the fungicide fluazinam slowed down the process of senescence and improved pigment production as well as enhanced C metabolism; so that the fluazinam-treated plants carry greater number of fruits, consequently higher fruiting yield production.

Furthermore, PPO reduced significantly via fungicide fluazinam spraying. This could be due to the association of PPO activity to the accumulation of reactive oxygen species and overall redox potential values (Webb et al. 2014). The strict relationship of this enzyme with ROS which was clearly used as a stress marker explained its reduction by fluazinam-treated plants. Hence, these plants tried to keep the cellular conditions at the lowest level of ROS production and kept efficient the membrane criteria to delay the senescence of leaves.

Visualization of the cells response to fluazinam spraying of both crops was also included in the present investigation. The cells of the leaves exposed to fluazinam found to be likewise more densely packed with less intercellular spaces than the controls. On the same par, Benton and Cobb (1995) found that the fungicide epoxiconazole-treated leaflets of cleavers had more palisade and spongy cells per unit area with less air space. Moreover, Wetzstein et al. (2002) demonstrated that relatively few extracellular spaces and vacuolar areas of pecan leaves treated with propiconazole fungicide may be associated with the highest greenish of leaves. This could be similar to what was visualized, here, on peppers and eggplants treated with fluazinam. Obviously, chloroplast in leaves sprayed with fluazinam had thylakoid membranes with high stacking and internal membranes that were well defined compared to control plants. This effect represented vastly for eggplants compared to pepper. This behavior was supported higher membrane stability of fluazinam-treated plants compared to control plants. Interestingly, it was noticeable that the cells of fluazinam-treated plants were characterized with chloroplasts that filled the cells which were larger than those in non-treated leaves of eggplants. These ultrastructural changes of chloroplasts due to spraying eggplant leaves by 1.5 mL/L of fluazinam could interpret the positive effects on photosynthesis via increasing chlorophyll content. This could interpret that chloroplasts in leaf sections that were sprayed with fluazinam had more and larger starch grains stored in the chloroplasts than those in leaves of control plants of the same age. While the changes in pepper leaves sprayed with the fungicide included an increase in starch grain size, the number was slightly affected by fluazinam compared to the control plants. Starch granules in chloroplasts act as osmotically neutral storage of assimilatory starch, and starch hydrolysis maintains cell integrity under unfavorable conditions owing to the produced soluble carbohydrates that protect the thylakoid membranes and other cellular structures (Vecchia et al. 1998). Thus, the observed maintenance of chloroplast membranes in both plants under fluazinam fungicide may be in good correlation with the leaf sugars.

Furthermore, the morphometric analyses confirmed that fluazinam-treated plants increased the number and size of mitochondria as well as characterized by giant nucleus for both plants. The observed increase in the number of mitochondria per cell could balance the cell requirements

parallel to the enhanced chloroplasts and chlorophyll content. This may have provided the cells with an additional supply of energy, assuming that the increased number of mitochondria appears to be a compensatory mechanism involved in ATP synthesis. These observations to the cells with higher energy demands have larger numbers of mitochondria, because this organelle supplies the cells with the requirements of the ATP through oxidative phosphorylation (Kevin et al. 2001). The above structural modifications could have played a part in increasing the contact area between adjacent organelles and minimized the distance between cell compartments. This helped metabolite flow and eliminated energy expenditure associated with long-distance movement. This energy-saving mechanism could beneficial for plants growing in stress conditions (Hanson and Sattarzadeh 2011), as a temporary response to fluazinam.

Conclusion

Crops treated with agrochemicals undergo chemical stress as revealed by modifications in cell organelles and several physiological reactions that mimic the induction of defense mechanisms. Concomitantly, the energy cost necessary to induce new metabolic pathways or cellular changes can affect crop growth, resulting in temporary growth reduction. These defenses were found to be sufficient to overcome the temporal negative stress and recover growth retardation as was recommended for lengths from 35 days after spraying to harvest time. Thus, the increment of the yield and number of fruits was the mere benefit from metabolic and antioxidative upregulations. On the same par, because of the energetic cost of resistance and growth, plants have first to stimulate defense strategies for their survival under stress conditions.

Summarizing, visible temporal stunting of plant length did not affect the crop yield since the upregulation of plant physiology reflected on a yield development of both crops which occurred even if visible symptoms were monitored. The present study recommended the use of fluazinam fungicide to improve the crop growth and physiology, but further research should be conducted to study fungicide accumulation in fruits and its effect on human health.

Author contributions Nabil A. Younes, Mona F.A. Dawood, and Ahmed A. Wardany: all authors shared equally in validation, formal analysis, data curation, writing-original draft, writing-review, and editing the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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