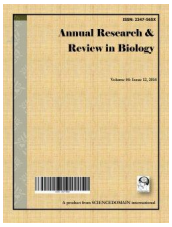

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Impact of Glyphosate Herbicide and Salicylic Acid on Seed Germination, Cell Structure and Physiological Activities of Faba Bean (*Vicia faba* L.) Plant

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Authors' contributions

This work was carried out in collaboration between both authors. Author KAF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author EFA managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The effects of salicylic acid (SA), glyphosate (Gly) and interaction of SA + Gly on seed germination and seedling growth, leaf morphology, cell ultrastructure and physiological alterations of *Vicia faba* plants were evaluated. SA (50 and 100 µM) reduced root seedling growth. Exposure to 250 and 500 µM Gly with or without SA caused an inhibition in seed germination and seedling growth of *V. faba* plants. Chlorosis, necrosis, welting, and growth reduction of *V. faba* was noticed in response to Gly and SA + Gly treatments. The photosynthetic pigment (Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*) and carotenoid (Car) contents of glyphosate and SA + Gly-treated plants were declined compared to the control. Electron microscopic observations of glyphosate-treated plants revealed disorganization in the internal structure of chloroplasts. Formation of vesicles within chloroplasts was observed in

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glyphosate and interaction of SA + Gly-treated plants. The lamellae and stroma thylakoids of chloroplasts were degenerated. Contents of soluble proteins and total phenolic compounds were decreased in glyphosate and SA + Gly-treated plants compared to the control. Peroxidase (POX) activity significantly increased with application of SA and glyphosate as well as with the interaction of SA + Gly compared to the control. Malondialdehyde (MDA) content in glyphosate and interaction of SA + Gly-treated plants increased compared to untreated one. The obtained results indicate that the root seedling growth of *V. faba* is sensitive to SA and Gly, while shoot was only sensitive to Gly herbicide treatments. The vegetative growth, cell organelles and physiological functions was negatively affected by Gly. Application doses of SA appear to be did not alleviate the toxicity of Gly herbicide on *V. faba*.

Keywords: Glyphosate; metabolites; oxidative stress; salicylic acid; Vicia faba.

1. INTRODUCTION

Glyphosate (Gly) is a broad-spectrum, nonselective, postemergence herbicide (with low risk to humans and the environment, lack of residual soil activity, and rare occurrence of resistant weeds [1,2]. Gly is mainly absorbed by leaves and stems, although some root absorption has also been reported [3]. Glyphosate (N-(phosphomethyl) glycine) directly affects the synthesis of secondary compounds, competing with the substrate phosphoenolpyruvate (PEP) for a binding site on the 5-enolpyruvylshikimic-3-phosphate synthase enzyme (EPSPS; E.C. 2.5.1.19), reduces the biosynthesis of aromatic amino acids and cause a general metabolic disruption in the plant [1,4-7]. EPSPS is present in plants, bacteria and fungi. Only one substitution in the amino acid sequence of EPSPS can contribute significantly to the reduction of sensitivity to glyphosate [8]. The inhibition of EPSPS also affects the biosynthesis of proteins, auxins, phytoalexins, folic acid, cinnamic acids, lignin precursors, flavonoids, plastoquinone and other phenolic compounds and alkaloids [9].

About 20% of the carbon fixed by green plants is routed through the shikimate pathway [10]. Toxic effects on leaf photosynthetic carbon fixation, disorganization of grana and inter-grana, allocation of newly fixed and stored carbon can interfere with exportation of both the assimilated carbon and the herbicide [11,12]. Gly applications reduced aromatic amino acid pools, photosynthesis, water and nutrient uptake, growth, yield and symbiotic N_2 fixation [13-15]. Gly immobilize essential micronutrients such as Fe [16] and Mn [17] that are necessary for physiological functions. Zobiole et al. [18] confirmed chelation of Ni by Gly and reduced biological nitrogen fixation in soybean.

Herbicides like many other biotic and abiotic stresses caused growth retardation, disordered cell organelles, initiate development of reactive oxygen species (ROS) and alternate physiological activities in plants [19-28]. The endogenous defense system combats such oxidative stress in living organisms [29,30]. ROS like superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) are predictable consequences in all living aerobes due to disturbance in electron transport chain (ETC). However, these undesirable reactions are efficiently controlled within living cells [24]. Gly herbicide caused oxidative stress and exhibited negative effects on photosynthesis and gas exchange of peanut [31]. Gly water pollution was a source of indirect phytotoxicity for *Bolboschoenus maritimus* [32].

Plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ enzymatic and non-enzymatic antioxidant defense systems protection against oxidative stress. The antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (POX, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) which minimizes the oxidation stress in plant cells through a limit generation of $^{\bullet}OH$, $O_2^{\bullet-}$ and H_2O_2 [33-35].

Salicylic acid (SA) is an important signal molecule involved in the activation of plant defence responses against abiotic and biotic stress and involved in various physiological responses such as thermogenesis, stomatal closure, flowering, photosynthetic rate, and transpiration [36,37]. Exogenous SA could regulate the activities of antioxidant enzymes and increase plant tolerance to the abiotic stress [38,39]. Salicylic acid (SA) plays an important role in the regulation of plant growth and development, seed germination and fruit yield

[40,41]. Moreover, it has been shown that SA provides protection against herbicides and oxidative stress [42-45]. On contrast, increase SA application decreases chlorophyll content in wheat and moong seedlings [46], and cause death of tomato plants [47].

Vicia faba (Fabaceae) is a crop well known by Egyptians, Greeks and Romans. Faba bean seeds are rich in protein and energy [48]. Faba bean production has a long history of numerous and valuable uses in feed and food. Nevertheless, faba bean seeds contain different constituents that may exert anti-nutritional effects. *V. faba* is among the most common food crops and a primary dietary legume for humans and animals. It contributes about 33% of the dietary protein nitrogen needs of humans [49]. Germination and/or seedling - an early stage of growth and a complex physiological process in plants are commonly used for evaluation of environmental-contaminants-phytotoxicity [50, 51]. To our knowledge, study the interaction of Gly + SA on *V. faba* have not been examined, therefore, the present work aims to study the response of *V. faba* to SA, Gly and interaction of Gly + SA treatments. Seed germination, seedling growth, plant morphology, photosynthetic pigments, leaf cell ultrastructure and physiological activities parameters were used for assessment the sensitivity of *V. faba*.

2. MATERIALS AND METHODS

2.1 Seed Surface Sterilization, Seed Germination and Seedling Treatment Process

Seeds of faba bean (*Vicia faba* L.) were first immersed in 70% ethanol for 3 min and then in 0.1% $HgCl_2$ for 2 min. The seeds were then washed five times with distilled water to remove the sterilized materials. The concentration of treatments solution was comprised of control (distilled water), two SA (50 and 100 µM), two Gly (250 and 500 µM) and four interactions (250 µM Gly + 50 µM SA, 500 µM Gly + 50 µM SA, 250 µM Gly + 100 µM SA, 500 µM Gly + 100 µM SA). Treatment solutions (10 mL) were applied to each Petri-dish. The treatment solutions were renewed every two days. At the day 6, the germinated seedlings were transferred to another set of sterilized Petri-dishes with double-layered filter papers containing 10 mL of the treatment solutions. The Petri-dishes were sealed with parafilm tape and placed in dark for 4 days followed by 6 days exposure to a 14 h photoperiod under suitable of white fluorescent light with 25 ± 2°C culture temperature. On day 10, three independent parallel experiments for germination assay were measured. Seedling growth of various treatments was photographed. Germination index of the different treatments were calculated using the formula: G.I. = (number of seeds germinated/total number of seeds) ×100.

2.2 Plant–soil Experiment

In the second experiment, faba bean seeds were surface sterilized as mentioned above. Seeds were sown in appropriate clean plastic pots containing 500g soil mixture composed of sand and soil at:1:1 ratio. Based on the seed germination results, the concentration of glyphosate (Gly) and salicylic acid (SA) was chosen for treatments. After two weeks from the seed emergence, faba bean plants had the same growth were distributed into six groups. The plants were treated for three weeks in open field. The leaves were sprayed once with treatment concentrations until saturation (run-off). The identification of six plant group treatments was as follows.

Group 1; plants sprayed with water (Control H_2O).
Group 2; plants sprayed with 100 µM SA (Control SA).
Group 3; plants sprayed with 250 µM Gly.
Group 4; plants sprayed with 500 µM Gly.
Group 5; plants sprayed with 250 µM Gly + 100 µM SA.
Group 6; plants sprayed with 500 µM Gly + 100 µM SA.

At vegetative stage, leaf samples of the control and treated plants were collected separately in three replicates for each group. Leaf morphology, mesophyll cell ultrastructure, photosynthetic pigment contents, protein contents, phenolic compounds, guaiacol peroxidase activity and malondialdehyde contents of the control, SA, Gly and interaction of Gly + SA -treated *V. faba* were estimated.

2.3 Morphology and Cell Ultrastructure of Faba Bean Leaves

The morphological changes of faba bean caused by SA and Gly treatments were examined. For symptom observations, the leaves of control and treated plants were photographed. For ultrastructure studies, fresh leaf samples (1-2 mm) were fixed in 3% glutaraldehyde prepared in 0.05 M phosphate buffer (pH 7), for 3 h.

Samples were rinsed several times in 0.05 M phosphate buffer and then were post fixed with 1% OsO_4 in 0.05 M phosphate buffer for 2 h. Samples were rinsed several times with 0.05 phosphate buffer and then dehydrated in a gradient ethanol series, and embedded in epon 812 [52]. Ultrathin sections (60-70 nm thick) were stained with uranyl acetate and lead citrate. Specimens were viewed with a Jeol-1011 transmission electron microscope at 100 kV (Unit of Electron Microscopy at Taif University, Saudi Arabia).

2.4 Physiological and Biochemical Measurements

2.4.1 Photosynthetic pigments content

Contents of Chlorophyll a (*Chl a*), chlorophyll b (*Chl b*) and total carotenoids were spectrophotometrically determined according to Metzner et al. [53]:

Chlorophyll *a* = $10.3 \times E_{663} - 0.918 \times E_{644}$ = µg/mL

Chlorophyll *b* = $19.7 \times E_{644} - 3.87 \times E_{663}$ = µg/mL

Total carotenoid = $4.2 \times E_{452}$ – {(0.0264 × Chl *a*) + (0.426 × Chl *b*)} = µg/mL. Finally, the pigment fractions were calculated as mg g^{-1} FW.

2.4.2 Determination of soluble proteins content

Soluble proteins content of leaves determined according to Lowry et al. [54] using Bovine serum albumin as a standard. Leaf samples (0.1 g fresh weight) were extracted in 10 mL distilled water for 2 h at 90°C. The extracts were centrifuged and the supernatants were collected. One mL of extract was added to 5 mL of alkaline reagent (50 mL 2% Na_2CO_3 prepared in 0.1 N NaOH and 1 mL 0.5% $CuSO_4.5H_2O$ prepared in 1% sodium potassium tartarate) and mixed thoroughly then allowed to stand for 10 min. A total of 0.5 mL of diluted Folin-Ciocalteu reagent was then added and mixed immediately. After 30 min, the extinction against appropriate blank was measured at 700 nm. Protein contents were expressed as mg g^{-1} FW.

2.4.3 Total phenolics content

Total phenolic content was determined with the Folin-Ciocalteu reagent according to a procedure described by Singleton and Rossi [55]. Gallic acid standard solution (2.0 mg/mL) was prepared by accurately weighing 0.01 g and dissolving 50 ml of distilled water. After standing 60 min at room temperature, absorbance was measured at 765 nm. Results are expressed as mg/g gallic acid equivalents (GAE).

2.4.4 Guaiacol peroxidase activity

Peroxidase (EC 1. 11. 1. 7) activity was carried out by grinding a known weight of the fresh leaves material at 4°C in a mortar in extraction buffer of 50 mM phosphate buffer pH 7.0 (1:1 w/v). The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. Supernatants were collected for measuring POD activity. Increase in the absorbance due to oxidation of guaiacol (extinction factor = 26.2 mM cm^{-1}) was measured at 470 nm. Enzyme activity was calculated in terms of µmol of guaiacol oxidized min^{-1} g^{-1} fresh weight at 25 ± 2ºC [56].

2.4.5 Determination of malondialdehyde

Malondialdehyde (MDA) content was determined as an indication of leaf lipid peroxidation according to Hernández and Almansa [57]. Fresh leaf samples (500 mg) were homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000*g* for 20 min at 4°C. One mL aliquot of the supernatant was mixed with 3 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and incubated at 90°C for 20 min. After stopping the reaction in an ice bath, samples were centrifuged at 10,000*g* for 5 min. The supernatant absorbance at 532 nm was then measured. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 mM^{-1} cm^{-1}.

2.5 Statistical Analysis

The results were statistically analyzed with SPSS 9.0 statistical software for Windows. Mean values were statistically compared by least significant differences (LSD). Differences were considered to be significant at levels $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Seed Germination and Seedling Growth

SA application (50 and 100 µM) slightly affected seed germination while the Gly as well as the interaction of SA + Gly was significantly (P ≤

0.001) inhibited seed germination of *V. faba.* The inhibition of seed germination in response to Gly and interaction of Gly + SA was clearly noticeable with increasing dose treatments (Table 1). In response to 250 and 500 µM Gly, the germination of *V. faba* seed was 61 and 23.6% of the control, respectively. Interaction of Gly + SA increased germination inhibition of *V. faba* seeds. Due to addition of 50 µM SA to 250 and 500 µM glyphosate, the % of seed germination was 47.3 and 32.0, respectively, compared to the control. While application of 100 µM SA to 250 and 500 µM glyphosate-treated plants, the seed germination was 40.0 and 21.3 %, respectively, compared to the control (100%). May be, this inhibition in seed germination resulted from the toxicity of Gly on seed embryo and seedling cells. Sharp [58] reported that herbicide affects some metabolic processes during initial seed germination and further development such as protein synthesis. Herbicides and toxic heavy metal ions inhibited seed germination and affect seedlings development [59-61].

The reduction in root growth of *V. faba* seedling was shown in response to SA, Gly and interaction of SA + Gly (Fig.1A-C). With increasing SA dose application, the root growth exhibited much more reduction compared with the control. Shoot seedling growth appear to be unaffected by SA. The Gly dose treatments (250 and 500 µM) caused inhibition in seedling growth with more noticeable inhibition in shoot growth compared with the control (Fig. 1A). In response to interaction of SA + Gly, both shoot and root growth was inhibited. Our results indicate that the SA caused inhibition of root growth, while Gly caused inhibition of shoot and root of *V. faba* seedlings. The inhibition of root growth with SA, probably, resulted from the sensitivity of root than shoot to hormone action. On the other side, the inhibition of seedling growth in response to Gly treatments suggested that the herbicide penetrated the seedling cells and disturbed its physiological activities. Herbicides of chlorsulfuron, norflurazon and triallate reduced root growth and injured root cap of pea and bean plants [62].

3.2 Leaf Morphology, Photosynthetic Pigments and Cell Ultrastructure

Untreated leaves and SA-treated *V. faba* plants appear to be healthy and did not show any symptoms such as chlorosis or necrosis and they were dark green (Fig. 2A and B). In response to Gly concentration treatments (250 and 500 µM), the reduction in growth was clearly shown. At 500 µM Gly, chlorosis and welting of leaves were noticed (Fig. 2A), reflecting weakness status and development retardation. Due to interaction of Gly + SA effect, the chloroses and welting of leaves was increased compared to that only treated with Gly (Fig. 2B).

Salicylic acid (SA) significantly reduced Chl *a* (≤ 0.01), Chl *b* (P ≤ 0.05) and Car (P ≤ 0.05). Glyphosate (Gly) and interaction of Gly + SA treatments caused highly significant (P ≤ 0.001) reduction in the contents of Chl *a*, Chl *b* and Car of *V. faba* leaves compared to the control (Table 2). Application of 100 µM SA reduced Chl *a*, Chl *b* and Car contents by 14.8, 11.5 and 11.7%, respectively compared to the control. In response to 250 and 500 µM Gly, the Chl *a* contents were 48.6 and 44.8 % of the control, respectively. With the interaction treatments of Gly + SA, the Chl *a* contents were significantly (P ≤ 0.001) reduced compared to those of the control and SA treatments. For example, Due to 250 µM Gly + 100 µM SA-treated plants, the content of Chl *a,* was 56.3% of the control. In response to 500 µM Gly + 100 µM SA-treated plants, the content of Chl *a,* was 44.3% of the control. Similarly, due to Gly and/or Gly + SA treatments, the decrease in Chl *b* and Car contents approximately like that of Chl *a*. Chl *a*/*b* ratio and total pigment contents were also tended to decrease with Gly and with interaction of Gly + SA treatments (Table 2). The most decrease in Chl *b* and Car contents were resulted with 500 µM Gly + 100 µM SA treatment (29 and 47% of the control, respectively). The value of Chl *a/b* ratios tended to be decrease with increasing Gly dose and with SA applications. With Gly and SA treatments, the decrease in Chl *a/b* mostly resulted from the reduction in Chl *a* content. The reduction in total pigment contents was more 50% in response to 500 µM Gly and to 500 µM Gly + 100 µM SA treatments. These findings are consistent with that reported in Gly-treated soybean varieties [14]. Other study showed that the chlorotic symptoms may be related to decrease photosynthetic rates as a result of direct damage of Gly to chlorophyll [63]. Muñoz-Rueda *et al.* [64] reported that the decreased in chlorophyll content caused by carotenoid loss which induced by sub-lethal doses of Gly. Generally, decrease chlorophyll contents are characteristic in plants exposed to sub lethal concentrations of Gly [65] and other herbicides [20,66-69]. Therefore, the decrease in photosynthetic pigments content might be a good indicator for monitoring injury of plant growth and metabolites affected herbicides.

Table 1. Effect of 10-day SA, Gly and interaction SA+ Gly on seed germination (%) of *V. faba* compared to the control

Treatments	Germination (%)			%
	M	±	SD	
Control	96.0	±	1.00	100
50 µM SA	95.0	±	1.00	98.09
100 µM SA	93.3	±	2.51	97.2
250 µM Glyphosate	61.0***	±	4.00	63.5
500 µM Glyphosate	23.6***	±	4.51	24.9
250 µM Gly + 50 µM SA	47.3***	±	5.86	49.3
500 µM Gly + 50 µM SA	32.0***	±	6.00	33.3
250 µM Gly + 100 µM SA	40.0***	±	5.29	41.6
500 µM Gly + 100 µM SA	21.3***	±	5.03	22.2

*The values are means (M) of three replicates ± SD. ***, significant at P ≤ 0.001*

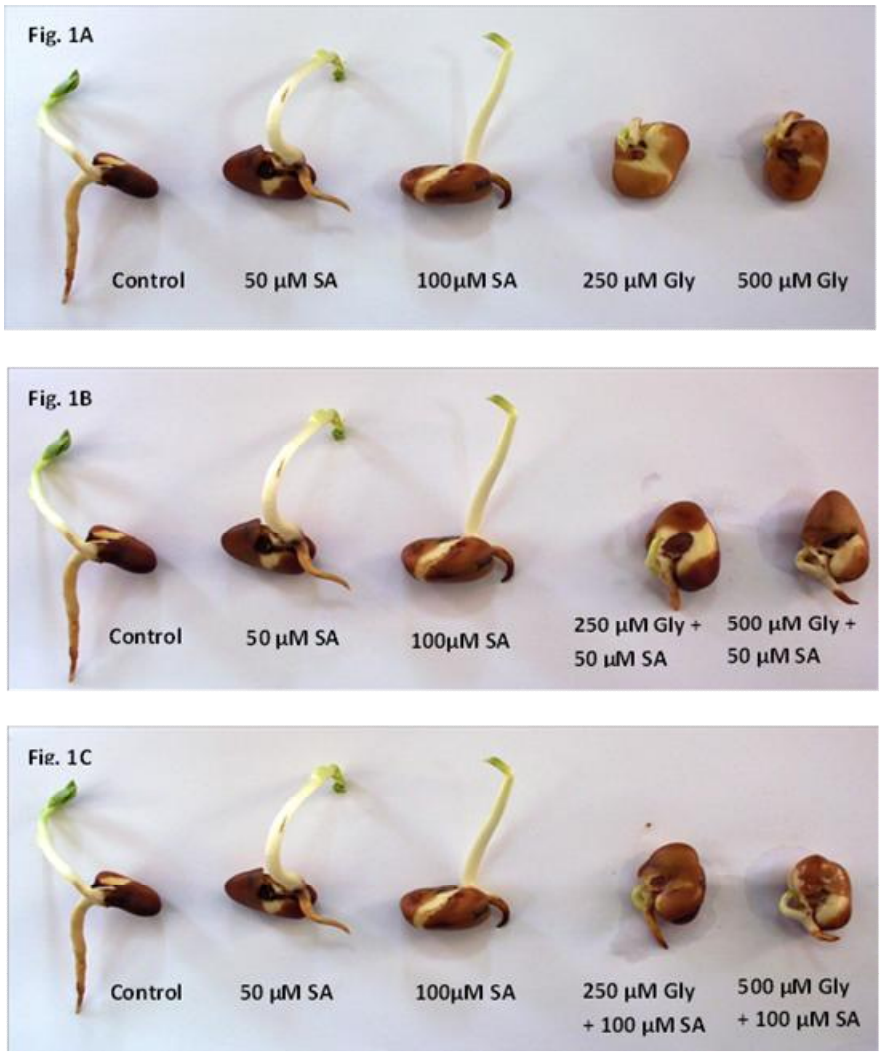

Fig. 1A-C. Effect of SA, Gly and interactions of SA + Gly on seedling growth of *V.faba* compared to the control

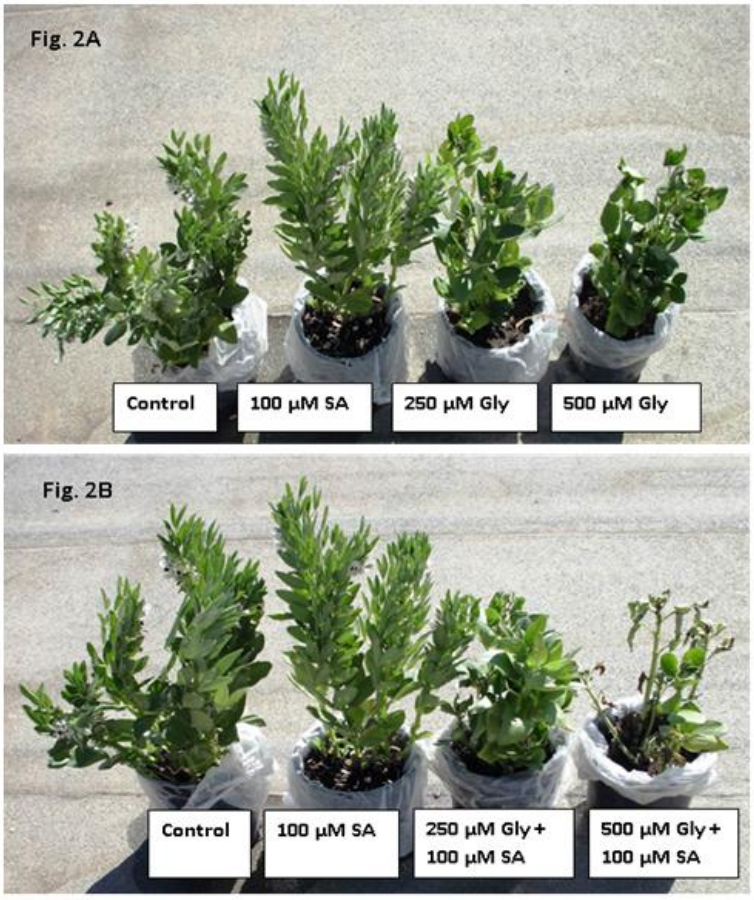

Fig. 2A and B. Effect of SA, Gly and interaction of SA+ Gly on vegetative growth of *V.faba* compared to the control

Electron microscopic observations of Gly (Fig. 3A) and SA + Gly (Fig. 3B), and treated *V. faba* showed considerable subcellular disorder. Chloroplasts of Gly and SA + Gly-treated plants were strongly injured (Fig. 3A and 3B). Disappear of grana thylakoids and stroma lamellae and accumulation of starch grains were resulted in response to Gly treatments. Chloroplasts of Gly + SA-treated plants showed many vesicles containing electron dense materials. Gly induced alterations in lupine photosynthetic apparatus and nodule ultrastructure [11]. In the present study, Gly treated plant ruptured the chloroplast envelope (Fig. 3A and B).

3.3 Soluble Protein and Total Phenolic Compounds

Soluble proteins of *V. faba* leaves were declined due to SA, Gly and Gly + SA treatments (Table 3). Leaf soluble proteins content of 100 µM SA significantly decreased by 18.9% ($P \leq 0.05$) compared to the control. A significant ($P \leq 0.001$) decrease in soluble proteins of *V. faba* leaves was detected in response to 250 and 500 µM Gly treated plants, the soluble proteins content decreased by 28.4 and 44.4%, respectively, compared to the control. Interaction of Gly + SA effect exhibited more reduction in soluble protein content of *V. faba* leaves compared to the control and to those only treated with SA or Gly. Due to 500 µM Gly + 100 µM SA effect, the soluble proteins content decreased by 45.6% compared to the control. Changes of plant protein with various abiotic stresses were reported [28,61,66].

Total phenolic compound of *V. faba* leaves significantly reduced due to SA ($P \leq 0.05$), Gly and interaction of Gly + SA ($P \leq 0.001$) treatments (Table 3). With 100 µM SA, phenolic compounds decreased by 15.8% compared to the control. Leaf total phenolic content of 250

and 500 µM Gly-treated plants decreased by 30.4 and 34.2%, respectively, compared to the control. In response to the interactions, total phenolic of the plant leaves showed much decrease than that only treated with SA or with Gly. They decreased by 42.1 and 38.9% in response to 250 µM Gly + 100 µM SA and 500 µM Gly + 100 µM SA, respectively, compared to the control. Phenolic compounds are widely distributed in the plant kingdom. Several of these compounds play important physiological and ecological roles being involved in resistance to different types of stresses [70,71].

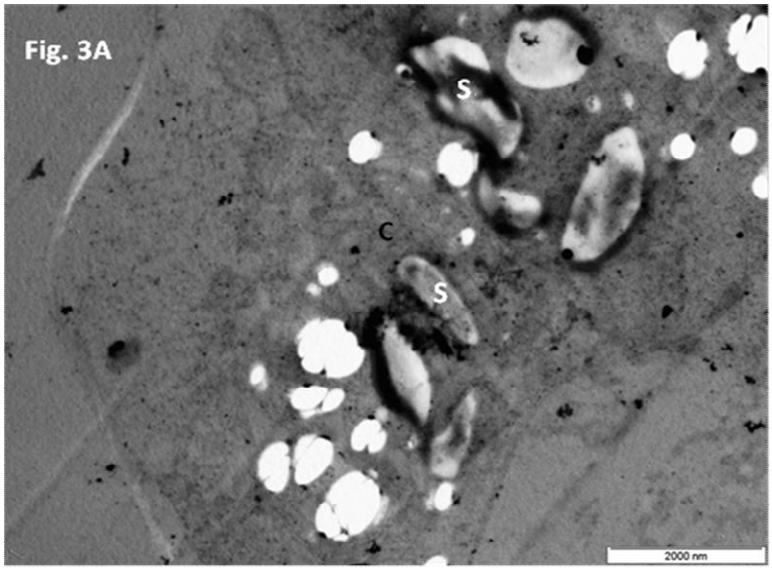

Fig. 3A. Electron micrograph of 500 µM Gly-treated plants for three weeks showed residual chloroplast of *V. faba* leaves

C, chloroplast; S, starch. Scale bar: 2000 nm

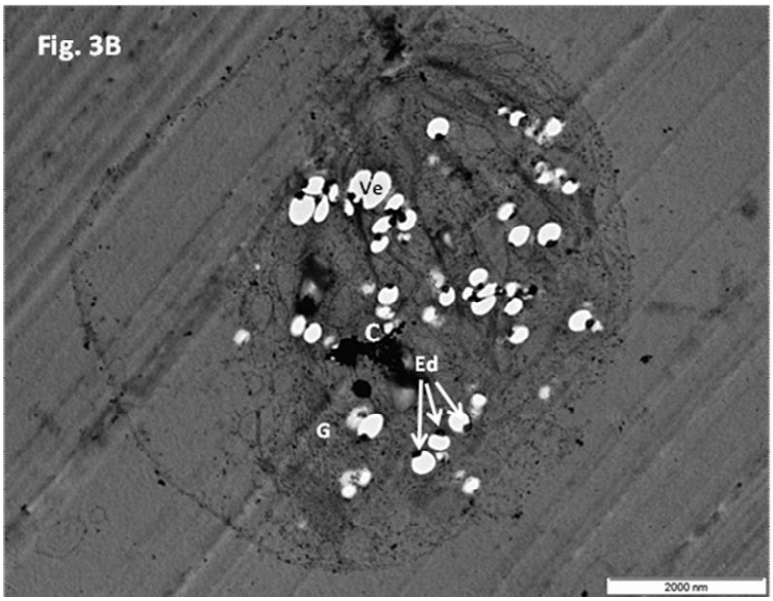

Fig. 3B. Electron micrograph of 500 µM Gly +100 µM SA-treated plants for three weeks showed residual chloroplast of *V. faba* leaves.

C, chloroplast; Ed, electron dense material; G, degenerated granum; Ve, vesicle. Scale bar: 2000 nm

Table 2. The effect of three-week application of SA, glyphosate, and interaction of SA + Gly on photosynthetic pigments (mg g^{-1} FW) of *V. faba* leaves compared to the control

Treatments	Chl *A*				Chl *B*				Carotenoids				*A/B* ratio	Total	%
	M	±	SD	%	M	±	SD	%	M	±	SD	%			
Control	1.83	±	0.16	100	0.61	±	0.04	100	0.94	±	0.07	100	3.00	3.38	100
100 µM SA	1.56**	±	0.08	85.2	0.54*	±	0.02	88.5	0.83*	±	0.05	88.3	2.88	2.93	86.7
250 µM Glyphosate	0.89***	±	0.08	48.6	0.30***	±	0.04	49.2	0.53***	±	0.02	56.4	2.97	1.72	50.9
500 µM Glyphosate	0.82***	±	0.07	44.8	0.28***	±	0.03	45.9	0.49***	±	0.02	52.1	2.93	1.59	47.0
250 µM Gly + 100 µM SA	1.03***	±	0.07	56.3	0.35***	±	0.04	57.4	0.58***	±	0.04	61.7	2.94	1.96	58.0
500 µM Gly + 100 µM SA	0.81***	±	0.09	44.3	0.29***	±	0.04	47.5	0.47***	±	0.03	50.0	2.79	157	46.4

The values are mean (M) of three replicates ± standard deviation (SD)., significant at P ≤ 0.05, ** significant at P ≤ 0.01, *** significant at P ≤ 0.001*

3.4 Peroxidase Activity

The POX activity significantly increased with application of SA and Gly as well as with the interaction of SA + Gly compared to the control (Table 4). The increase in peroxidase activity was higher with increasing dose of Gly treatment ($P \leq 0.05$). Leaf POX activity increased by 22.1% in response to 100 µM SA, while POX increased by 18.5% in response to 250 µM Gly treatment. With 500 µM Gly, the POX increased by 33.3% compared with the control. Due to interaction of 100 µM SA with 250 and 500 µM Gly, the activity of POX enhanced by 31.2 and 47.1%, respectively, compared with the control. The increase in POX activity under effect of SA and Gly treatments may be an attempt of plant to overcome the excess of ROS. Enhance POX activity was reported as one scavenger agent for ROS and survival plant tissues under stress toxic substances [31,72,73].

3.5 Lipid Peroxidation

SA (100 µM) slightly decreased MDA content (about 4 %) of *V. faba* leaves than that of the control (Fig. 4). MDA contents of *V. faba* leaves increased with the increasing concentration of Gly. In detailed, the MDA contents significantly increased by 37.2 ($P \leq 0.05$) and 146.3% ($P \leq 0.001$) in 250 and 500 µM Gly-treated leaves, respectively, compared with the control. In response to interaction of 100 µM SA with 250 and 500 µM Gly, the MDA content increased by 29.7 and 67.4%, respectively, compared with the control. The results of exhibited a decrease in the content of MDA in response to Gly + SA than that only treated with Gly. Excessive amount of herbicides are able to induce intracellular overproduction of ROS. So, damage of plant cells occurred [69].

So, symptoms appeared on the *V. faba* leaves due to the Gly treatments resulted from enhanced lipid peroxidation and photosynthetic pigment degradation and damage of cell membranes. Lipid peroxidation occurs as a result of attack by free radicals such as reactive oxygen species (ROS) in biological systems [74]. Peroxidation of lipid leads to the destruction of membranes of cell organelles and dysfunction of proteins, DNA and RNA [75,76]. The results in the present study illustrated the phytotoxicity of glyphosate relates with the reduction of pigments, damage of photosynthetic system and other cell organelles [20,27,28,60,61,67]. According to our finding in the present study and that reported by Iqbal [77], *V. faba* is useful for toxicity monitoring of all types of environments.

Table 3. Effect of three-week application of SA, Gly and interactions of SA+ Gly on soluble proteins and total phenolic contents of *V. faba* leaves compared to the controls

Treatments	Soluble proteins (mg g^{-1} FW) (FW)				Total phenolic (µg g^{-1} FW)			
	M	±	SD	%	M	±	SD	%
Control	14.91	±	1.37	100	21.14	±	1.94	100
100 µM SA	12.09*	±	2.42	81.1	17.80*	±	1.54	84.2
250 µM Glyphosate	10.67**	±	0.99	71.6	14.73***	±	1.45	69.6
500 µM Glyphosate	8.29***	±	1.32	55.6	13.91***	±	1.98	65.8
250 µM Gly + 100 µM SA	9.54***	±	0.95	64.0	13.26***	±	0.97	57.9
500 µM Gly + 100 µM SA	8.11***	±	1.54	54.4	12.96***	±	1.00	61,1

The values are means (M) of three replicates ± standard deviation (SD)., significant at P ≤ 0.05; **, significant at P ≤ 0.01;***, significant at P ≤ 0.001*

Table 4. Effect of three-week application of SA, Gly and interaction of SA+ Gly on peroxidase activity (µM guaiacol oxidized min^{-1} g^{-1} FM) of *V. faba* leaves compared to the control

Treatments	POX [µM (guaiacol oxidized) min^{-1} g^{-1}(FM)]			
	M	±	SD	%
Control	69.25	±	6.13	100
100 µM SA	84.56*	±	6.72	122.1
250 µM Glyphosate	82.09**	±	2.72	118.5
500 µM Glyphosate	92.33***	±	3.05	133.3
250 µM Gly + 100 µM SA	90.89***	±	7.28	131.2
500 µM Gly + 100 µM SA	101.88***	±	5.29	147.1

*The values are means (M) of three replicates ± standard deviation (SD). *, significant at P ≤ 0.05; **, significant at P ≤ 0.01;***, significant at P ≤ 0.001*

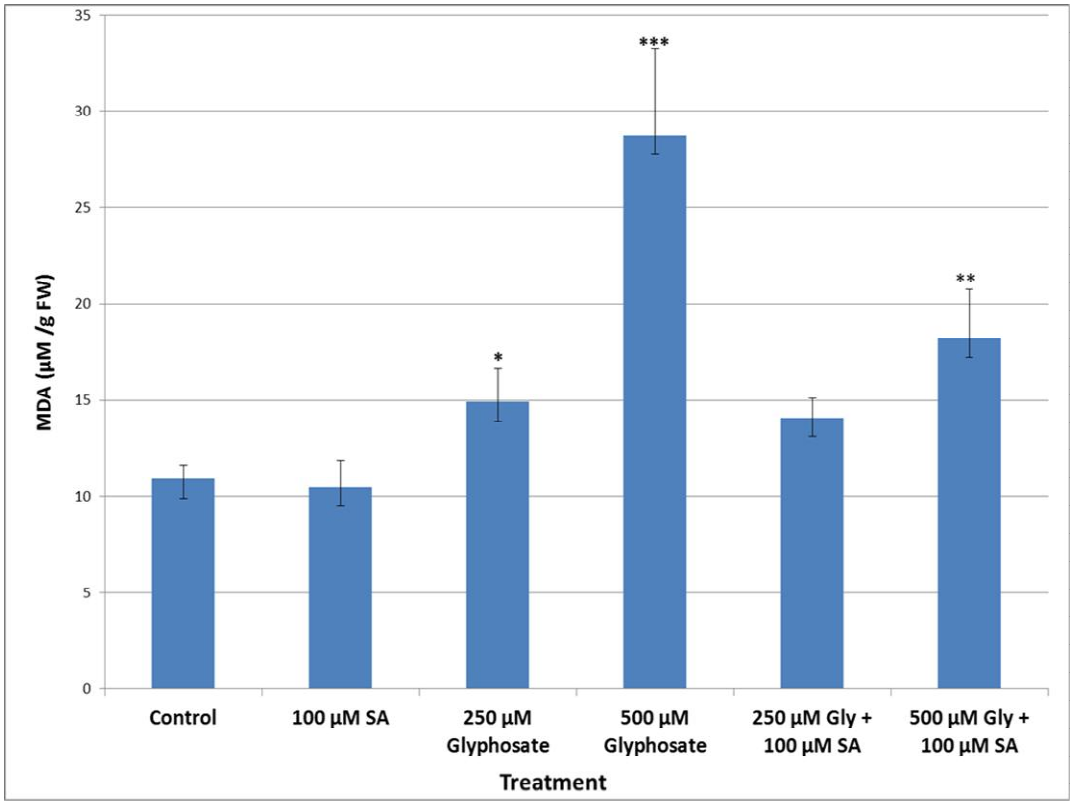

Fig. 4. Effect of three-week application SA, Gly and interaction of SA+ Gly on malondialdehyde (MDA) content (µmol g^{-1} FM) of *V. faba* leaves compared to the control

*The values are means (M) of three replicates ± SD. *, significant at P ≤ 0.05; **, significant at P ≤ 0.01;***, significant at P ≤ 0.001*

5. CONCLUSION

The study concludes that *V. faba* showed sensitivity to SA and Gly herbicide and can be used as a test for toxicity monitoring. The results showed that the SA strongly reduced root seedling growth of *V. faba* while shoot was unaffected suggested a sensitively of root to hormone action. Gly has a strong inhibition on both root and shoot seedling growth of *V. faba* indicated the Gly toxicity effect. The inhibition cases in seedling growth of *V. faba* increased with increasing SA and Gly-treated doses. Gly herbicide induced oxidative stress in a concentration dependent manner that was monitored through seed germination, seedling growth, leaf morphology, cell organelles and physiological activity. Decrease photosynthetic pigments, degeneration of chloroplast ultrastructure and increase lipid peroxidation represent good indicators for arising oxidative stress in Gly-treated *V. faba.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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