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Aromatic Amino Acids Partially Alleviated Glyphosate Induced Effects on Metabolites and Growth in Faba Bean with a More Phytotoxicity in *Orobanche crenata* Parasite

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Abstract

The holoparasite *Orobanche crenata* is considered the most damaging pathogens on the faba bean grown in the Mediterranean region. Sub-lethal dose of glyphosate was used for controlling *O. crenata*, but it caused injury effects on faba bean growth. Glyphosate inhibits the shikimic pathway of the synthesis aromatic amino acids (AAAs), phenylalanine, tyrosine and tryptophan. Greenhouse experiment was conducted using infested pots with *O. crenata* to explore the possibility of co-exposure AAAs with glyphosate to alleviate glyphosate effects on faba bean and determine their effects on the parasite. Moreover, changes in the metabolites related to shikimic pathway were examined in faba bean leaves, 3 and 7 days after treatments. Application of twice sprays with glyphosate at 170 or 340 g ai ha^{-1} on faba bean was effective in controlling *O. crenata* infestation. Co-exposure AAAs (0.5 and 1 mM) with glyphosate allowed to partially mitigating the detrimental effects of the herbicide on faba bean growth. Combined treatments produced more sever effects on *O. crenata* parasite and completely inhibited *O. crenata* parasite by 120 days from sowing. Glyphosate induced differential effects on amino acids composition, endogenous free AAAs and protein content in faba bean leaves. Moreover, it increased shikimic acid content accompanied by decreases in phenolic contents and antioxidant activity. Level of these compounds was partially mitigate by co-applied glyphosate with AAAs, proposing a potential role of AAAs in increasing tolerance of the host plant by ameliorating the glyphosate induced effects on metabolites related to shikimic acid pathway.

Key words: Aromatic amino acids; Vicia faba L.; Holoparasite; Orobanche; Glyphosate; Shikimicacid.

1. Introduction

Faba bean (*Vicia faba* L.) is one of the most important legumes in Egypt, which constitute the major part of the diet of Egyptian people. The obligate holoparasite *Orobanche crenata*is considered the most damaging pathogens on the faba bean grown in the Mediterranean region [1]. Grain yield losses can reach more than 90% in highly infested fields [2]. *Orobanche* parasite attaches to the host root by a haustorium in that it absorbs water, minerals and organic compounds producing tubercle from which a spike arises and emerges from the soil to flower and set seeds [3]. Sequential application of sub-lethal doses of glyphosate on faba bean at the beginning of the flowering stage and after 21 days from first application was effective in controlling *O. crenata* infestation [4, 5]. Glyphosate absorbed through foliage of host plants with rapid translocation to the attached parasite, and exerted its toxic effect, thus inhibiting parasite growth prior to its shoot emergence [6]. Eid et al. [5] found that spraying glyphosate twice at the rate 85 g ai ha^{-1} produced more than 70% decreases in number and dry weight of *O. crenata* spikes and more than 80% increases in faba bean seeds, compared with untreated check. However, in a field trial, Zeid and Hemeid [7] found that application of this glyphosate treatment on four faba bean cultivars decreased emerged spikes, only

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between 18-49% without any improvements on faba bean yield.

Glyphosate exerts its herbicide activity by inhibiting 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS), an enzyme in the shikimate pathway. Inhibition of this enzyme leads to excessive accumulation of shikimate, preventing the biosynthesis of the three aromatic amino acids (AAAs) i.e. Phenylalanine (Phe), Tyrosine (Tyr) and Tryptophan (Trp) [8]. Since the shikimate pathway metabolizes about 30% of the assimilated carbon in photosynthetic plants [9], it is reasonable to assume that related metabolic pathways may be affected within hours after the inhibition of EPSPS, due to the impairment of carbon metabolism [10]. Reduction in the levels of AAAs by glyphosate treatments was previously observed by Shaner and Lydon [11], they found that the endogenous levels of Tyr and Phe decreased by 50% 6 h after the beginning of the glyphosate treatment while the levels of other amino acids increased. Also, levels of AAAs in holoparasite *Phelipanche aegyptiaca* were significantly decreased within 48 h after application of glyphosate on tomato host [12].Besides to their function in protein synthesis, AAAs serve as precursors of a wide variety of natural products that play crucial roles in plant signaling, growth and development, including responses to biotic and abiotic stresses [13].Thus, it can be hypothesized that the exogenous application of AAAs could revert the changes induced in the shikimic acid pathway by glyphosate and alleviate glyphosate-induced phytotoxicity.The reversion of glyphosate effects on growth with exogenous AAAs has been previously shown in fungi and bacteria [14,15], while in higher plants, the reversion of the effect on growth effect has been detected in Arabidopsis [16]. Recently, many investigators found that exogenous application of some metabolites related to AAAs such as salicylic acid alleviated the glyphosate toxicity [17,18]. The regulatory mechanisms underlying the response of the shikimic acid pathway and the specific role of final products (AAAs) have not been thoroughly investigated [8].

In Egypt, for control *O. crenata* infestation, it is recommended to spray glyphosate at 85 g ai ha^{-1} on faba bean leaves at the beginning of the flowering and after 21 days from first application [5]. However, this glyphosate treatment caused injury effects on faba bean growth [19]. Understanding the effects of AAAs impacts on the survival of host and parasites-treated plants is a matter of particular importance that needs an accurate answer. In order to maximize the efficiency of glyphosate for controlling *O. crenata* in faba bean, this study was conducted to i) examine the effect of application glyphosate at two and four times the recommended dose on faba bean growth and *O. crenata* infestation, ii) assess the potential of exogenous AAAs to alleviate the detrimental effects of glyphosate in faba bean plants, with a focus on the metabolites related to shikimic acid pathway.

2. Experimental

An experiment was conducted on November, 21, 2019 at the greenhouse of National Research Centre, Giza, Egypt. Seeds of faba bean (*Vicia faba*) cultivar Nubaria 3 were provided by Agricultural Research Centre, Egypt. Ten uniform faba bean seeds were sown in each 30-cm diameter plastic pots containing about 5 kg sandy loam soil (48.4% sand, 41.1% silt, and 10.5% clay; pH 8.0). About 200 mg of *O. crenata* seeds were mixed with the soil of infested pot. At sowing, commercial rhizobia and 8 g of superphosphate (15%, P_2O_5) were incorporated into the top 30-mm of the soil. The pots were placed in a greenhouse (25 ± 3 °C, 12 h photoperiod). After 20 days from sowing, seedlings were thinned to three uniform seedlings per pot. Plants were supplied with nitrogen (ammonium sulfate, 21% N, 6 g/pot) three times at 4, 6, 8 weeks after sowing. Pots were divided into eight groups with five replications; seven *O. crenata* infested groups and one non-infested group (control healthy) and arranged in a randomized complete block design. As for the seven infested groups, one group served as infested control and the other six groups were treated by two levels of glyphosate (Roundup, 360 g ai l^{-1}, Monsanto, USA) at 170 and 340 g ai $ha^{-1}$with three levels of AAAs solutions (0, 0.5 and 1 mM). Solutions of AAAs were prepared by mixing the same concentration (0.5 or 1 mM) of each of Phe, Tyr and Trp ((Sigma Aldrich, Germany). At flowering stage (January, 13), glyphosate treatments were conducted and AAAs solutions used as recovery treatments after three days from glyphosate treatments (January, 16). Glyphosate and AAAs treatments were repeated after three weeks (on 3rd and 6th January, respectively). The solutions were sprayed using an Epoca sprayer (Italy) evenly over the entire surface of the plant, including the

adaxial and abaxial surface of leaves. At 3 and 7 days from glyphosate treatments, vegetative plant samples were collected, fast cleaned with distilled water to remove the glyphosate or AAAs residues, oven-dried at 50 °C, and ground in a mortar. Dried 4th leaf of faba bean plants used to determine metabolites related to shikimic acid pathway. Samples from healthy and infected plants were collected twice at February 18 and March 17, 2020 (90 and 120 days after sowing, respectively) and growth parameters of faba bean and *Orobanche* were determined. The plant growth parameters were evaluated in terms of plant height (cm), branches number, leaves number, fresh weight (g), dry weights of plant (g), flowers number, pods number and weight (g). Also, total number and weight (g) of attached *Orobanche*, tubercles, and emerged spikes per pot were determined.

2.1 Extraction and determination of free Phe, Tyr and Trp

The extraction of endogenous levels of the three AAAs, Phe, Tyr and Trp was performed according to Matallo et al. [20]. One hundred milligrams of 4th dried powder of faba bean plant was placed in a centrifuge tube, and 10 mL of acidified water (pH 2.5) was added and subjected to an ultra-sonication bath with an ultrasonic frequency of 50/60 Hz for 30 min. Samples were subjected to centrifugation at 4000 g for 10 min at 20 °C. The supernatant was collected and filtered through a nylon filter 45 µm. Quantification of AAA was performed by High Performance Liquid Chromatography (HPLC), LC-10 AD, Shimadzu, Japan. The AAAs was analysed using a Luna RP-C18 (2) column (250 × 4.6mm i.d, 5 µm, Phenomenx). The mobile phase of phosphate buffer (pH 6.5) in methanol (90:10, v/v) was used at a flow rate 0.9 ml/min. The detecting wavelength was 220 nm. For each compound, the calibration curve was constructed with the concentrations of the standards (0.01–0.1 mg/mL) that covered the range of levels of the compounds found in the plant tissues. The retention time for Tyr, Phe andTrp are 6.08, 9.32 and 11.59 min, respectively.

2.2 Determination of shikimic acid by HPLC

Due to the polarity of shikimic acid, the previously mentioned mobile phase was not suitable to identify and determine this polar compound with HPLC. Shikimic acid in acidified water extract of leaves was determined by using HPLC under all previously mentioned conditions, except the mobile phase. Depending on Bueno-Solano et al [21], determination was conducted by using 5 mM ammonium acetate in methanol (72: 28) at 0.8 ml/min as a mobile phase and shikimic acid purchased from Sigma-Aldrich (Sweden) used as a standard (retention time, 3.6 min).

2.3 Determination of total protein content and amino acids composition

Total protein content in faba bean leaves was determined by Kjeldahl method. Moreover, the amino acids composition was determined using HPLC-Pico-Tag method according to White et al. [22].Dried sample was hydrolysed with 6 N hydrochloric acid with 0.1% phenol in a sealed tube at 110 °C for 24 hours. The Pico-Tag method, was developed commercially by Waters Associates, was an integrated technique for amino-acid analysis. Phenyl isothiocyanate (PITC, or Edman's reagent) was used for pre-column derivatization. The chromatographic analysis by HPLC was carried out using the following gradient of Pico-Tag two eluents solvents purchased from Waters Associates. Eluent A comprises 940 ml of 0.14 M sodium acetate, pH 6.40, containing 0.05% triethylamine, mixed with 60 ml acetonitrile. Eluent B is 60% acetonitrile. A standard gradient elution programmer, recommended by Waters with eluent B increasing, was employed for the work reported here. Both eluents purchased from Waters Associates. Sample was injected and loaded on Pico-Tag amino acids column (150 x 3.9 mm) stainless steel. The PTC derivatives were detected by using ultraviolet absorption measurements (Waters detector) at 254 nm. Before injecting, calibration was constructed with the amino acids standards.

2.4 Determination of total phenolics

Total phenolics in faba bean leaves was extracted with 70% acetone, and determined according the method of Singleton and Rossi [23] using the Folin-Ciocalteu reagent. In brief, 0.1 ml of extract was added to 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent, 1.5 ml of sodium carbonate solution (200 g/L) and then mixed vigorously. The mixture was allowed to stand for 1 h at the room temperature and then the absorbency was measured at

a wavelength of 765 nm. Gallic acid was used as a standard and the results were expressed as mg gallic acid g^{-1}dry sample.

2.5 Determination of antioxidant capacity

Antioxidant capacity or free radical scavenging activity was determined according to Brand-Williams et al. [24] using 1,1-diphenyl-2-picryl-hydrazil (DPPH) reagent. In brief, 1.5 ml of freshly prepared methanolic DPPH solution (0.02 mg/ml) was added to 0.75 ml of 70 % acetone extract and then stirred. The de-colorizing process was recorded after 5 min of reaction at a wavelength of 517 nm and compared with a blank control. The DPPH radical scavenging activity of the extracts was measured using the Trolox standard curve. Results were expressed as μmol Trolox g^{-1} sample.

2.6 Statistical analysis

Analyses were performed in triplicate. The data were subjected to analysis of variance according Gomez and Gomez [25], and comparison of means (LSD, 5% level) was performed using Stat graphics Plus Version 5.1.

3. **Results and discussion**

This study aimed to examine whether the toxicity and biochemical effects of glyphosate on faba bean plants and O. crenata parasite are affected by its combination with AAAs. Furthermore, this study explores the regulation of glyphosate and AAAs treatments on some metabolites related to shikimic acid pathway.

3.1 Effect of glyphosate and AAAs on faba bean growth

Effect of spraying glyphosate (at 170 and340 g aiha^{-1}) and AAAs (at 0.5 and 1 mM) twice, through 21 days, on growth parameters of faba bean plants was measured after 90 and 120 days from sowing and the tabulated data was statistically analysed against infected and healthy non-infected plants (Table 1).

After 90 days from sowing, *O. crenata* infestation and foliar spray with glyphosate alone or in their combination with AAAs treatments did not induce any significant effects on number of branches and height of faba bean plants, when compared with healthy non-infected plants. *O. crenata* infestation reduced number of leaves, fresh weight, dry weight and flowers number of faba bean with 24.2, 37.8, 54.7 and 63.7%, respectively, relative to healthy non-infected plants. Meanwhile, plants sprayed with glyphosate alone at 170 or 340 g ai ha^{-1}achieved great decreases in their number of leaves (13.6 and 30.6%, respectively), fresh weight (24.9 and 26.8%, respectively), dry weight (25.0 and 28.8%, respectively) and flowers number (33.1 and 28.7%, respectively). It can be observed that co-exposure of glyphosate with AAAs partially alleviated the depression effects induced by independently glyphosate treatments. The most enhancement effect was observed when co-applied low glyphosate dose (170 g ai ha^{-1}) with high dose of AAAs (1mM). This combined treatment increased number of leaves (9.9%), fresh weight (16%), dry weight (16.7%) and number of flowers (7.6%), relative to those of low glyphosate alone. Although, values of these growth parameters exhibited slight changes due to the co-application AAAs with high glyphosate dose, when compared with those of independently high glyphosate dose.

At 120 days from sowing, more sever effects in faba bean growth was observed as affected with *Orobanche* infestation and glyphosate treatments (Table 2). Obtained data indicated that *O. crenate* infestation caused great decreases in faba bean growth. Biomass accumulation by infected plant did not reach 14% of biomass accumulation in non-infected plants. Also, great decrease in tested growth parameters was observed due to independently glyphosate treatments, compared with healthy plants. The most sever effects was obtained by the highest glyphosate dose (340 g ai ha^{-1}), it reduced plant height and number of leaves with about 23% as well as fresh and dry weight of plant with about 57%, accompanied by a complete inhibition on pod production. Based on the obtained results, co-mixture of AAAs at 0.5 or 1 mM with glyphosate at 170 g ai ha^{-1} partially alleviated the phytotoxicity induced by glyphosate alone. Whereas, the toxicity induced by high glyphosate dose was reversed only by application high dose of AAAs (1mM). This combined treatment tended to produce significant increases in most faba bean tested parameters, compared with independently high glyphosate dose.

Sever effects on growth and productivity of faba bean due to *O. crenata* infestation was previously

observed by many investigators [4,5]. Due to their achlorophyllous nature, *Orobanche* uptake water and nutritional resources from the host, and its sink strength derived from accumulation of high concentrations from osmotic active compounds such as cations, sugars, amino acids, and polyols [26].

Table 1 Effect of glyphosate (Gly) and aromatic amino acids (AAAs) on growth of faba bean plants, 90 days after sowing.

Treatment Gly + AAAs (g ai ha^{-1}) (mM)	Branches number	Plant height (cm)	Leaves number	Plant fresh weight (g)	Plant dry weight (g)	Flowers number
170 + 0.0	1.5^{a}	48.0^{a}	20.3^{b}	24.4^{c}	4.8^{b}	10.5^{b}
170 + 0.5	1.5^{a}	49.3^{a}	19.3^{b}	26.5^{bc}	5.2^{b}	11.0^{b}
170 + 1.0	1.6^{a}	49.0^{a}	22.3^{a}	28.3^{b}	5.6^{ab}	11.3^{b}
340 + 0.0	1.5^{a}	48.3^{a}	16.3^{c}	23.8^{c}	4.7^{b}	11.2^{b}
340 + 0.5	1.4^{a}	48.5^{a}	18.0^{bc}	25.6^{c}	5.0^{b}	10.0^{b}
340 + 1.0	1.6^{a}	47.5^{a}	18.8^{b}	24.0^{c}	4.7^{b}	11.0^{b}
Infected plants	1.4^{a}	46.2^{a}	17.8^{bc}	20.2^{d}	2.9^{c}	5.7^{c}
Healthy plants	1.6^{a}	48.5^{a}	23.5^{a}	32.5^{a}	6.4^{a}	15.7^{a}

Values are given as means of three replicates. Means with the same letters in a column are not significantly different at $P<0.05$.

Table 2 Effect of glyphosate (Gly) and aromatic amino acids (AAAs) on growth of faba bean plants, 120 days after sowing

Treatment Gly + AAAs (g ai ha^{-1}) (mM)	Plant height (cm)	Leaves number	Plant fresh weight (g)	Plant dry weight (g)	Pods number	Pods weight (g)
170 + 0.0	55.0^{d}	40.6^{b}	40.0^{c}	7.5^{c}	1.7^{d}	4.0^{d}
170 + 0.5	61.0^{c}	41.1^{b}	57.7^{b}	10.8^{b}	2.3^{c}	5.7^{c}
170 + 1.0	66.0^{b}	42.0^{b}	58.3^{b}	10.9^{b}	3.0^{b}	11.0^{b}
340 + 0.0	54.0^{d}	35.6^{c}	31.5^{de}	5.9^{d}	0.0^{f}	0.0^{e}
340 + 0.5	47.3^{e}	36.0^{c}	27.0^{e}	5.1^{d}	0.0^{f}	0.0^{e}
340 + 1.0	51.3^{de}	37.6^{c}	34.2^{d}	6.4^{cd}	0.3^{e}	0.7^{e}
Infected alone	35.7^{f}	23.0^{d}	10.0^{f}	1.9^{e}	0.0^{f}	0.0^{e}
Non-infected	71.0^{a}	46.3^{a}	73.7^{a}	13.8^{a}	3.7^{a}	15.7^{a}

Values are given as means of three replicates. Means with the same letters in a column are not significantly different at $P<0.05$.

Orobanche growth and faba bean reproduction compete directly for resources within faba bean plant and the increase in number of attached *Orobanche* per plant lead to faba bean death before maturity [27]. Whereas, the phytotoxic effect on faba bean exposed to low dose of glyphosate in order to control *O. crenata* parasitism was previously observed by Mesa-Garcia et al. [19]. Glyphosate exerts its herbicide activity by inhibiting EPSPS, an enzyme in the shikimate pathway that catalyses a key step in the biosynthesis of natural aromatic amino acids [10]. Also, several secondary effects of glyphosate action have been described, including: (1) a decrease in the rate of photosynthesis and in concentrations of photosynthates in plant tissues [28]; (2) inhibition of the biosynthesis of phenolic compounds, resulting in impaired defence mechanisms [29]. The relative potential of AAAs in increasing tolerance of faba bean to glyphosate herbicide is consistent with alleviation effect of AAAs on the growth of higher plants exposed to glyphosate herbicide [30]. Reversal effect of AAAs treatments may be due to the potential role of AAAs in decreasing the inhibition of the shikimate pathway, specifically of EPSPS enzyme by glyphosate [31].

3.2 Effect of glyphosate and AAAs on O. crenata parasite.

The effect of spraying faba bean plants with glyphosate alone or in combination with AAAs on growth and development of *O. crenata* parasite was determined twice at 90 and 120 days after sowing. As shown in Table 3, all glyphosate and AAAs treatments achieved great effects on various growth parameters of the parasite. After 90 days from sowing, glyphosate and AAAs treatments prevented the development of the *Orobanche* parasite from the first growth stage (tubercles stage) to sever advanced stages. Moreover, tubercles growth achieved significant decreases as affected with application glyphosate alone either at 170 or 340 g ai ha^{-1} when compared with infected non-sprayed plants. These two glyphosate doses produced depression in tubercles number with 29.4% and 82.4%, respectively at 90 days after sowing, corresponded with 68.4 and 95.6%, respectively in tubercles weight. As shown in Table 3, application of AAAs at 0.5 or 1 mM with low glyphosate dose produced further decrease in number and weight of tubercles compared those of independently low glyphosate treatments. On the contrary, co-exposure of high glyphosate dose with one of AAAs doses produced remarkable increases in number and weight of tubercles at 90 days after sowing, as compared with independently high glyphosate treatment. It can be observed that, application of glyphosate alone or in combination prevented the ability of *O. crenata* grown in faba bean plants for producing emerged spikes after 90 days after sowing. A further phytotoxic effect on growth and developments of *O. crenata* parasite was observed after 120 days after sowing (Table 3). Except the insignificant effect of low glyphosate dose alone on tubercles growth, all glyphosate and AAAs treatments tended to produce a complete inhibition on *Orobanche* growth and development by inhibiting tubercles and spike production.

The sever effects of glyphosate on *Orobanche* growth and development are in accordance with findings obtained by Eid et al. [5]. Glyphosate treatments completely prevented emerged spikes; this parameter was considered the best index for estimation the *Orobanche* control [32].This herbicide trans located from the host tissues to the attached parasite causing growth disruption [6]. Moreover, Hassanein and Kholosy [33] suggested that the effects of glyphosate on *Orobanche* tubercle was attributable to its selective accumulation in the young parasite up to a level of three times as high as that in faba bean host root, three days after spraying. Despite the importance of glyphosate as a mean of controlling *Orobanche*, the mechanism of action of the herbicide in this parasite is not clearly understood [10]. Recently, Shilo et al. [12] found that EPSPS enzyme was present in the tissues of *O. aegyptiaca* and the mechanism of glyphosate's control of *Orobanche* is due to EPSPS inhibition of parasite. Moreover, we found that spraying AAAs at 0.5 or 1 mM after three days from glyphosate treatments did not alleviate glyphosate-induced phytotoxicity on growth and development of *Orobanche* parasite. It can be expected that a large amount of glyphosate which accumulated in parasite tissues during the first three days could exert its phytotoxic effects on *Orobanche* before exposing to AAAs treatments [33].

3.3 Effect of glyphosate on amino acids composition and endogenous free AAAs.

Effect of twice sprays with glyphosate at 170 or 340 g ai ha^{-1}on amino acids composition in faba bean leaves, 3 days after treatment was illustrated in Table 4. Depending on HPLC analysis, major amino acids presented in faba bean leaves of control and glyphosate treated plants are Ala followed by Asp, Glu and Phe. It can be observed that amino acids composition achieved various responses to glyphosate treatments. A decrease in the levels of aromatic amino acids, Phe and Tyrdue to glyphosate treatments was observed. Following the 1st and 2nd sprays with glyphosate at high dose (340 g ai ha^{-1}) decreased Phe with 52.8 and 47.0%, respectively as well as Tyr with 13.7 and 78.8%, respectively. Moreover, remarkable decrease in the level of acidic amino acids e.g. Asp and Glu was observed after the 1st and 2nd glyphosate sprays. High dose of glyphosate decreased Asp with 17.7 and 41.8%, respectively and Glu with 7.5 and 19.9%, respectively. On contrast, application of glyphosate at170 and 340 g ai ha^{-1} tended to produce noticeable increases in levels some amino acids as Gly, Arg and Thr. Meanwhile, the other detected amino acids did not achieve any clear effects with glyphosate treatments. Confirming with the obtained results, differential effects on the amino acids composition of

tomato leaves due to glyphosate application was previously observed by Shilo et al [12].

Table 3 Effect of glyphosate (Gly) and aromatic amino acids (AAAs) on *O. crenata* grown in faba bean plants.

90 Days after sowing						
Treatments **Gly + AAAs** (g ai ha^{-1}) (mM)	***Orobanche* number**	***Orobanche* weight** (g)	**Tubercles number**	**Tubercles weight** (g)	**Spikes number**	**Spikes weight** (g)
170 + 0.0	12^{b}	1.42^{b}	12^{b}	1.42^{b}	0^{b}	0^{b}
170 + 0.5	6^{d}	0.50^{b}	6^{d}	0.50^{e}	0^{b}	0^{b}
170 + 1.0	11^{b}	1.20^{b}	11^{b}	1.20^{c}	0^{b}	0^{b}
340 + 0.0	3^{e}	0.20^{b}	3^{e}	0.20^{f}	0^{b}	0^{b}
340 + 0.5	5^{dc}	0.31^{b}	5^{d}	0.31^{f}	0	0^{b}
340 + 1.0	9^{c}	1.00^{b}	9^{c}	1.0^{d}	0^{b}	0^{b}
00 + 0.0	35^{a}	39.9^{a}	17^{a}	4.5^{a}	18^{a}	35^{a}
120 Days after sowing						
170 + 0.0	6^{b}	14^{b}	6^{b}	14^{a}	0^{b}	0^{b}
170 + 0.5	0^{c}	0^{c}	0^{c}	0^{b}	0^{b}	0^{b}
170 + 1.0	0^{c}	0^{c}	0^{c}	0^{b}	0^{b}	0^{b}
340 + 0.0	0^{c}	0^{c}	0^{c}	0^{b}	0^{b}	0^{b}
340 + 0.5	0^{c}	0^{c}	0^{c}	0^{b}	0^{b}	0^{b}
340 + 1.0	0^{c}	0^{c}	0^{c}	0^{b}	0^{b}	0^{b}
00 + 0.0	35^{a}	150^{a}	11^{a}	14^{a}	24^{a}	136^{a}

Values are given as means of three replicates. Means with the same letters in a column are not significantly different at P<0.05.

Table 4 Amino acids composition (%) in faba bean leaves, 3days after glyphosate treatments

Treatments **Amino acids**	**Control**	**Glyphosate 170 g ai ha^{-1}**	**Glyphosate 340 g ai ha^{-1}**	**Control**	**Glyphosate 170 g ai ha^{-1}**	**Glyphosate 340 g ai ha^{-1}**
	1st Spray			**2nd Spray**		
Aspartic (Asp)	2. 90	2.56	2.39	4.89	4.85	2.85
Glutamic (Glu)	2.03	2.04	1.88	2.87	2.74	2.30
Serine (Ser)	0.35	0.60	0.41	0.47	0.48	0.45
Glycine (Gly)	0.46	0.90	0.64	0.66	0.67	0.74
Histidine (His)	0.46	0.41	0.45	0.48	0.48	0.48
Arginine (Arg)	0.55	1.23	0.90	1.11	1.31	1.05
Threonine (Thr)	0.93	1.14	1.13	0.82	0.88	1.13
Alanine (Ala)	8.90	6.30	8.39	7.84	7.91	6.68
Proline (Pro)	0.49	0.10	0.77	0.38	0.44	0.32
Tyrosine (His)	0.63	0.74	0.55	0.62	0.79	0.13
Valine (Val)	0.77	0.70	0.68	0.44	0.36	0.71
Methionine (Met)	0.36	0.06	0.49	0.03	0.09	0.25
Cysteine (Cys)	0.03	0.02	0.02	0.02	0.02	0.02
Isoleucine (Ile)	0.48	0.64	0.41	0.66	0.54	0.56
Leucine (Leu)	0.53	1.01	0.59	0.89	0.95	0.86
Phenylalanine (Phe)	1.96	1.94	0.92	2.57	2.33	1.36
Lysine (Lys)	0.65	1.17	0.70	1.08	0.98	0.78

Also, Vivancos et al. [34] reported that glyphosate decreased the levels of Phe, Glu and Asp in soy bean leaves, while Thr, Lys, Val and Leu were increased. Decline in the AAAs, Phe and Tyr is consistent with the inhibition of the shikimate pathway by glyphosate [8]. While, the decline in Glu and Asp by glyphosate likely reflect a shift towards the conversion of these amino acids to the synthesis of the corresponded nitrogen-rich amino acids, glutamine and asparagine as suggested by Vivancos et al. [34].

Concentration of free AAAs, Phe and Trp in leaves of faba bean was determined by HPLC after 3 days or 7 from each glyphosate spray. More than 12% decreases in Phe concentration was observed after 3 or 7 days from the 1st spray with glyphosate either at 170 or 340 g ai ha^{-1} (Table 5).But, after 3 days from the 2nd spray, applied glyphosate doses did not induce any significant effect on protein content. A remarkable increase in protein content was observed after 7 days from the second spray with glyphosate treatments. Different effect of glyphosate on free Phe contents was previously observed by Shilo et al. [12]. Such diversity effects depending on applied dose, measuring time and application technique. Although, with one exception, two sprays with glyphosate at 170 or 340 g ai ha^{-1} did not induce any significant effects on Trp content when measured after 3 or 7 days from each spray, as compared with non-treated plants. In line of these results, Zulet-Gonzalez et al. [8] reported that Trp level did not differ between glyphosate treated and untreated plants. Indeed, glyphosate is a specific inhibitor of the EPSPS enzyme, but the effects of this inhibition on the shikimic pathway itself and the regulation of the AAAs pathway remain poorly understood in plants [9].

3.4 Effect of glyphosate and AAAs treatments on metabolites related to shikimic pathway

In this study, the effect of glyphosate alone and in combination with AAAs on some metabolites related to shikimic acid pathway in faba bean leaves was determined. In the combined treatments, AAAs was applied 3 days after each glyphosate spray and the treated leaves were collected after 7 days from glyphosate treatments (4 days from AAAs treatments) and then subjected to analysis.

3.4.1 Shikimic acid content

Application of glyphosate alone either at 170 or 340 g ai ha^{-1}produced increases in shikimic acid content with 52.1 and 67.3%, respectively after 1st spray and with 44.4 and 35.4%, respectively after the second one (Table 6). It can be observed that the presence of AAAs significantly decreased the levels of shikimic acid in glyphosate treated plants, compared with independently glyphosate treatments. Spraying AAAs at 0.5 and 1 mM on plants exposed to low glyphosate dose reduced shikimic acid content with 15.3 and 5%, respectively after the 1st spray and with 30 and 31.9%, respectively after the second one, when compared with glyphosate alone treatment. Also, when AAAs was simultaneously applied with high glyphosate dose, level of shikimic acid was significantly diminished in relation to plants exposed to high glyphosate dose alone.

Table 5 Effect of glyphosate (Gly) on free AAAs in faba bean leaves, 3 and 7 days after treatments

Gly (g ai ha^{-1})	**Free Phe** (µg g^{-1})				**Free Trp** (µg g^{-1})			
	1st Spray		2nd Spray		1st Spray		2nd Spray	
	Days after treatment							
	3	7	3	7	3	7	3	7
170	230^{b}	230^{b}	240^{a}	320^{a}	83.2^{a}	87.9^{a}	85.6^{a}	60.5^{a}
340	228^{b}	231^{b}	239^{a}	294^{a}	86.0^{a}	83.3^{a}	70.0^{b}	59.6^{a}
0.0	262^{a}	266^{a}	225^{a}	277^{b}	84.0^{a}	84.5^{a}	80.0^{a}	60.2^{a}

Values are given as means of three replicates. Means with the same letters in a column are not significantly different at $P<0.05$.

Table 6 Effect of glyphosate (Gly) and aromatic amino acids (AAAs) on some metabolites related to shikimic acid pathways, 7 days after glyphosate treatments

Gly + **AAAs** (g ai ha^{-1})(mM)	Shikimic acid (mg g^{-1})		Total protein (%)		Phenolics (mg g^{-1})		Antioxidant activity(µ mol trolox g^{-1})	
	1st Spray	2nd Spray	1st Spray	2nd Spray	1st Spray	2nd Spray	1st Spray	2nd Spray
170 + 0.0	2.42^{ab}	2.57^{a}	23.7^{a}	25.7^{a}	38.7^{c}	29.8^{c}	597^{d}	371^{d}
170 + 0.5	2.05^{c}	1.81^{b}	23.6^{a}	25.5^{a}	36.7^{c}	40.0^{b}	474^{e}	517^{b}
170 + 1.0	2.30^{b}	1.75^{b}	23.8^{a}	25.3^{a}	64.5^{a}	41.2^{b}	1139^{a}	533^{b}
340 + 0.0	2.66^{a}	2.41a	23.7^{a}	23.5^{b}	31.2^{d}	33.4^{c}	405^{f}	320^{e}
340 + 0.5	2.45^{ab}	1.69^{b}	23.7^{a}	23.3^{b}	47.8^{b}	32.2^{c}	699^{c}	328^{e}
340 + 1.0	1.75^{d}	1.28^{c}	23.8^{a}	23.3^{b}	62.4^{a}	37.7^{b}	1029^{b}	440^{c}
00 + 0.0	1.59^{d}	1.78^{b}	23.8^{a}	25.6^{a}	38.3^{c}	51.2^{a}	607^{d}	735^{a}

Values are given as means of three replicates. Means with the same letters in a column are not significantly different at $P<0.05$.

The greatest reduction effect on shikimic acid contents was obtained by combined treatments after the 2nd spray. In this time, application of AAAs at 0.5 or 1 mM completely ameliorated the glyphosate induced effects on shikimic acid contents and even this compound was found in lower level than its level in non-treated plants. Accumulation of shikimic acid has been described as a characteristic effect of glyphosate, due to EPSPS inhibition. Inhibition of this enzyme deregulates the carbon flow through shikimic acid pathway by decreasing chorismate synthesis and all its by-products, resulting in an increase in shikmate-3-phosphate [8]. In this study, accumulation of shikimate was similar in the two glyphosate treatments, suggesting that shikimate accumulation would be directly related to EPSPS inhibition and not to other physiological changes caused by the effect of the herbicide [8]. Therefore, presence of AAAs partially elevated levels of shikimate in glyphosate treated plants, proposing a potential role of AAAs in decreasing the inhibition of the shikimate pathway, specifically of EPSPS enzyme by glyphosate [31].These results disagree with recently obtained results by Zulet-Gonzalez et al. [8]. They found that incubation leaf desks of *Amaranths palmeri* for 24 h with AAAs in combination with glyphosate, the shikimate content was similar that accumulated by the glyphosate-alone treatment. This discrepancy between their findings and our results may be explained by the difference in treatment technique.

3.4.2 Protein content

The effect of two glyphosate doses (170 or 340 g ai ha^{-1}) independently or co-exposure with AAAs (0.5 or 1mM) on protein content in faba bean leaves was determined. As shown in Table 6, the 1st spray with independently glyphosate treatments did not produce any significant effects on protein content compared with non-sprayed plants. Moreover, un-changed in the level of protein was observed after the 2nd spray with independently low glyphosate dose alone. But, a significant decrease in protein was observed after the 2nd spray with high dose of glyphosate. Unchanged in protein with low glyphosate dose may be related, in part, to the low dose applied of glyphosate. In line of these results, Bellaloui et al [35] found that application of low glyphosate dose (0.11 kg of ai ha^{-1}) on soybean did not show significant differences in protein content. Reduction in protein content by increasing glyphosate dose was recorded previously by Salman et al. [36]. On the other side, the obtained results indicated that the protein content did not change in glyphosate-treated plants upon co-exposure with exogenous AAAs (Table 6). Hence, it can be suggested that the different effects on protein content by applied glyphosate doses did not attribute to the availability of the AAAs to maintain necessary protein synthesis [35].

3.4.3 Total phenolics content and antioxidant activity

Based on the obtained results, total phenolic contents and antioxidant activity in faba bean leaves nearly had the same trend in their response to the glyphosate

(at 170 and 340 gai ha^{-1}) and AAAs (at 0.5 and 1 mM) treatments (Table 6).Except the non-significant effect of low glyphosate dose after the 1st spray, glyphosate treatments caused great decreases in the levels on phenolic contents and antioxidant activity, compared with non-sprayed plants. Independently high dose of glyphosate decreased phenolic contents and antioxidant activity with 18.3 and 33.3%, respectively after the 1stspray and with 34.8 and 56.5%, respectively, after the 2nd one. Meanwhile, co-mixture of glyphosate and AAAs partially alleviated the decrease effects induced by independently glyphosate treatments. The great enhancement effect was obtained by combined glyphosate treatments with high AAAs dose. Co-exposure low glyphosate dose with 1 mM AAAs showed increases in phenolic contents constituted 66.7 and 38.3% after the 1st and 2nd spray, respectively corresponded increases in antioxidant activity constituted 90.8 and 43.7%, respectively, compared with low glyphosate alone. Also combination between high glyphosate dose and high dose of AAAs increased phenolic contents with 100 and 11.7% after the 1st and 2nd spray, respectively accompanied by 154.1% and 37.5%, respectively increases in antioxidant activity, when compared with those of high dose of glyphosate dose.

In accordance with obtained results, positive correlation between phenolic content and antioxidant activity was previously reported by Panzella et al., [37]. Phenolic compounds were considered natural antioxidants that have the character of quenching oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical [38]. Confirming with our results, the oxidative stress and decrease in production of phenolic and antioxidants following exposure to sub-lethal glyphosate doses was previously observed by Spormann et al. [17]. These effects may be due to the secondary effect of the blocked shikimic acid pathway by glyphosate [8] or due to the enhancement effect of glyphosate on production of reactive oxygen species (ROS) and oxidation of essential plant molecules. On the other hand, our findings about the capability AAAs treatments to reverse the decrease in phenolic contents and antioxidant activity induced by glyphosate alone are in line with the results obtained by Spormann et al [17]. They found that salicylic acid, a derivative compound produced from AAAs, was found to alleviate glyphosate-induced effects on phenolic content and oxidative stress on maize and *Hordeum vulgar*.

4. Conclusion

We studied the enhancement of glyphosate efficacy on controlling *O. crenata* grown in faba bean by co-applied aromatic amino acids. The obtained results revealed that application twice sprays with glyphosate at170 or 340 g ai ha^{-1} on faba bean plants at flowering stage caused injury effects on faba bean plants with a great depression effects on growth and development of *Orobanche* parasite. Co-exposure of AAAs (0.5 or 1 mM, each) with glyphosate treatments partially alleviated glyphosate-induced effects on the host plants with a more phytotoxicity on the parasite. Combined treatments completely inhibited the *Orobanche* growth by 120 days after sowing. Application of glyphosate alone induced differential effects on amino acids composition and free aromatic amino acids contents. Following glyphosate treatments an increase in shikimic acid was observed accompanied with decreases in phenolic content and antioxidant activity. Presence of AAAs partially alleviated the glyphosate effects on shikimic acid, total phenolics, antioxidant activity, without achieving any changes on protein content. In this study we applied two and four times recommended dose of glyphosate which used for control *Orobanche*, further studies must be conducted to evaluate the effect of co-applied of AAAs with glyphosate at recommended dose on faba bean and *O. crenata* parasite.

5. Conflicts of interest

The authors declare that they have no competing interests.

6. Acknowledgments

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