The metabolic profiles of phenolic acids and aromatic amino acids in the Orobanche crenata parasite and its host faba bean at different infestation stages

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Abstract

Orobanche crenata is a root holoparasite that depends on its host for nutritional requirements. The shikimate pathway that metabolizes about 30% of the assimilated carbon in photosynthetic plants plays a role in host-parasite relationships. Aromatic amino acids (AAAs) and phenolic acids are derived from the shikimate pathway and serve as precursors of a wide number of primary and secondary metabolites. The effects of the *O. crenata* parasite on the phenolic acid and AAA profiles of two faba bean varieties and the differences of these compounds between the host organs and attached parasite at infestation stages were studied. Hosts and attached parasites were collected and divided, depending on the stage of parasite development, into four stages. The results revealed that phenolic acids and free AAAs tended to increase in parasitized roots compared to those of healthy roots, and the greatest increase in phenolic contents occurred at the first infestation stage. Syringic acid was observed to be unique to the parasite. Profiles of phenolic acids and AAAs were changed during the developmental stages of the parasite and differed from those of its host. Caffeic in the parasite reached more than 100 times that in Nubaria 4 host roots at the fourth infestation stage. Free phenylalanine in the parasite ranged between 2.2 and 5.5 times its level in host roots at all infestation stages. This study provides much evidence that indicates *O. crenata* is able to self-regulate its phenolic and AAA metabolites during its developmental stages, which differ from those of its host.

Introduction

Many species in the family Orobanchaceae are considered destructive weeds, especially members of the genera Phelipanche, Orobanche, and Striga. *Orobanche crenata* Forsk is a root holoparasite widely distributed in the Mediterranean region, and it can cause up to 100% loss of crop yield (Fernandez-Aparicio et al. 2016). The holoparasitic plants are devoid of chlorophyll and entirely depend on their host plants for their carbon and other nutritional requirements (Abbes et al. 2020). *O. crenata* produces thousands of seeds that can remain viable in the soil for a long time and germinate in response to chemical molecules secreted by host roots in the rhizosphere. After seed germination, the parasite attaches to the host root by a haustorium, through which it absorbs water, minerals, and organic compounds, producing tubercles from which shoots arise and emerge from the soil to flower and set seeds (Fernandez-Aparicio et al. 2016).

Metabolic profiling, or metabolomics, has proven to be a powerful method for identifying metabolites whose levels are altered during various growth conditions and stresses. Such an analysis could also be proposed for putative biochemical pathways and provide more data on metabolism and physiological processes (Nakabayashi and Saito 2015). Our knowledge about holoparasite metabolic profiling and its effects on the metabolic profiling of the infected host's plants is mostly unknown (Flore-Sanchez and Garza-Ortiz 2019). There is discussion in the scientific literature about the nature of parasite's metabolites. The first option is that the parasite depends mainly on the metabolites of associated hosts, while the second option is that the parasite can self-regulate its own metabolites depending on the host metabolites (Kumar et al. 2022; Kumar and Amir 2021). Few studies have compared the metabolite

profiles of the host root to those of Orobanchaceae holoparasites (Clermont et al. 2019; Hacham et al. 2016). The shikimate pathway and their related metabolites may play a role in host-parasite relationships because: (I) a key enzyme of the shikimate pathway (5-enolpyruvylshikimate-3-phosphate synthase, EPSPS) is the target of the widely used herbicide glyphosate for killing Orobanch*e* parasite (Shilo et al. 2016); (II) plant phenolics are secondary metabolites biosynthesized mainly from the shikimate pathway and involved in the defense mechanisms of plants against parasitic weeds (Briache et al. 2020); and (III) this pathway metabolizes about 30% of the assimilated carbon in photosynthetic plants (Maeda and Dudareva 2012). However, the dependence of the holoparasites on their own production of shikimate pathway metabolites is still under debate in the scientific literature (Kumar et al. 2022). These important properties account for the major motivation to elucidate the role of the metabolites related to the shikimate pathway in host-parasite relationships.

The shikimate pathway includes seven different enzymes catalyzing the conversion of erythrose4-phosphate and phosphoenolpyruvate to chorismate, which is considered the initial branch point metabolite in the synthesis of the three aromatic amino acids (AAAs) and the wide range of aromatic secondary metabolites derived from it (Vogt 2010). Phenolic acids are generated mainly from AAAs or dehydroshikimic acid produced via the shikimate pathway (Kumar and Goel 2019). AAAs and phenolic acids are the early products of the shikimic acid pathway and are considered the source of building blocks for a very wide number of primary and secondary products in the plant kingdom (Vogt 2010). The AAAs, phenylalanine, tyrosine, and tryptophan, are central molecules in plant metabolism. Besides their function as building blocks of proteins, they serve as precursors for a very wide range of aromatic secondary metabolites with multiple biological functions (Vogt 2010). Phenolic acids are a diverse class of plant polyphenols that contain one or more hydroxyl groups attached directly to the aromatic ring. Phenolic acids are divided into two sub-groups: benzoic-derived phenolic acids (C_6-C_1) and cinnamic-derived phenolic acids (C_6-C_3). The two types of phenolic acids are naturally occurring antioxidants that exhibit many functions, including plant growth, development, and defense. Moreover, phenolic acids are considered the main source of building blocks for a wide number of phenolic compounds (Kumar and Goel 2019). There are only a few studies concerning the metabolic profiling of shikimate pathway products in infected hosts and respective parasites. Asan and Ozen (2016) found that the amounts of the two benzoic-derived phenolic acids, *p*-hydroxy benzoic and salicylic acids, decreased due to *Cuscuta babylonica* infestation. In carrot tissues, levels of the individual amino acids in the leaves of Broomrape parasitized plants tended to be similar to those of the healthy leaves, and the free amino acids in parasitic tissues tended to be similar to those of the respective host roots (Nandula et al. 2000). Significant differences in the total phenolic contents and AAAs in *Phelipanche aegyptiaca* collected from different hosts implies the holoparasites can influence the synthesis of these compounds (Kumar et al. 2022). The limitations of these studies were focusing on a single developmental stage, ignoring the role of parasite development, and generally neglecting the differences between parasites and their hosts. Many questions remain unanswered regarding the effect of parasitism on host metabolic profiling and the independence of parasites upon host metabolites during different infestation stages (Clermont et al. 2019). To achieve this, the current study aimed to define the differences in phenolics and AAAs profiling

between infected and non-infected plants of two faba bean (*Vicia faba*) varieties at four stages of *O. crenata* infestation. Moreover, the profiling of these compounds in the parasite tissues was compared across its developmental stages and directly against the respective roots and leaves of the two host varieties.

Materials and methods

Plant materials and samples collection

Seeds of two faba bean (*Vicia faba*) varieties, Nubaria 4 and Nubaria 5 were obtained from The Agricultural Research Centre, Egypt. Parasite seeds were collected from naturally *O. crenata* infested field located at El-Nubaria Experimental Station, National Research Centre of Egypt, in the 2020 season. Plastic pots of 30- cm diameter containing about 5 kg of sandy loam soil (48.4% sand, 41.1% silt, and 10.5% clay; pH 8.0) were used for faba bean cultivation. About 200 mg of *O. crenata* seeds were mixed with the soil of the infested pot, whereas. Ten uniform seeds were sown in each pot, and then the pots were placed in a greenhouse (25 ± 3°C, 12 h photoperiod). Faba bean plants were exposed to the same agricultural practices. Sixteen weeks after planting, healthy (non-infected) and infected faba bean plants with their attached parasites were collected. Infected plants are divided based on the stage of development of the *O. crenata* parasite into four stages of infestation, according to Nativ et al. (2017). The first stage of infestation is host plants attached with parasite tubercles carrying adventitious roots; the second stage is host plants attached with pre-emergent shoots of parasite; the third stage is host plants attached with pre-emergent vegetative shoots of parasite that include very small and condense floral buds; and the fourth stage is host plants attached with post-emergent whole mature flowering parasite. The four developmental stages (S_1 - S_4) of attached parasites were separated manually from host plants. Healthy and infected plants were divided into host roots, host leaves, and attached parasites. The collected samples were dried in an oven at 70°C, ground to a fine powder by mortar and pestle, and kept at -20°C for various analyses.

Analysis of phenolic acid compositions

Phenolic acids were extracted after hydrolysis with sodium hydroxide and determined by High Performance Liquid Chromatography (HPLC), according to McKeehen et al. (1999). Approximately 300 mg of ground sample was added to 15 ml of 4 N NaOH in a 50 ml Pyrex centrifuge tube, purged with nitrogen, shaken for 2 h in the dark with a shaker, and acidified with ice-cold 6 N HCl to reduce pH to 2. The mixture was centrifuged at 3000g, and the supernatant was decanted into a separatory funnel. The supernatant was extracted with ethyl acetate (3 x 50 ml). The ethyl acetate fraction was dried by the addition of anhydrous sodium sulfate, filtered, and concentrated using a rotary evaporator at 40°C to dryness. The residue was re-solubilized in 2.5 ml of methanol HPLC-grade and filtered through a 0.2 µm PTFE filter prior to analysis. Phenolic acids were determined by HPLC, LC-10 AD, Shimadzu, Japan, using a Luna RP-C18 (2) column (250 × 4.6 mm i.d., 5 µm, Phenomenx). HPLC separation of phenolic acids was performed using a mobile phase consisting of a mixture of acetate buffer, acetonitrile (9:1, v/v), and

280 nm for detection. Standard phenolic acids, including protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, caffeic, coumaric, and ferulic, were purchased from Sigma; their retention times were 5.1, 8.0, 9.4, 10.1, 10.7, 19.4, and 24.8 min, respectively.

Analyses of free AAAs

The extraction of the free Phe, Tyr, and Phe from the plant sample was performed according to Matallo et al. (2009). One hundred milligrams of dried powder were placed in a centrifuge tube with 10 mL of acidified water (pH 2.5) and subjected to an ultra-sonication bath with an ultrasonic frequency of 50/60 Hz for 30 min. The extract was subjected to centrifugation at 4000 g for 10 min at 20°C, and then the supernatant was collected and filtered through a 45 µm nylon filter. HPLC analysis was done using the mobile phase of phosphate buffer (pH 6.5) in methanol (90:10, v/v) at a flow rate of 0.9 ml/min, and the detecting wavelength was 220 nm. Standard phenylalanine, tyrosine, and tryptophan were purchased from Sigma-Aldrich (Sweden), and their retention times are 6.08, 9.32, and 11.59 min, respectively.

Analysis of protein amino acids by HPLC Pico-Tag method

Amino acids composition was determined using HPLC-Pico- Tag method according to White et al. (1986). The Pico-Tag method was developed commercially by Waters Associates, as an integrated technique for amino-acid analysis. Phenyl isothiocyanate (PITC, or Edman's reagent) was used for pre-column derivatization. The 6 N acid hydrolysate of the protein sample was diluted with HPLC-grade water and placed into the Pico-Tag amino acid Workstation (Waters, USA) for sample preparation (drying, re-drying, and derivatization) using Waters reagents. The HPLC apparatus was equipped with 600 E Multi-solvent Delivery System and the following gradient of Pico-Tag solvent A and B (Waters Eluent A & B) at 38 ^{0}C, flow rate 1 ml/min. Eluent A comprises 940 ml of 0.14 M sodium acetate, pH 6.40, containing 0.05% triethylamine and 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. Twenty microliter of sample was injected and loaded on Pico-Tag amino acids column (150 x 3.9 mm) stainless steel. The PTC derivatives were detected by at 254 nm.

Determination of total phenolics

The dried sample was extracted with 70% acetone, and the total phenolic content was determined using Folin-Ciocalteu´s reagent (Singleton and Rossi 1965). One milliliter of the extract was mixed with 1.5 ml of deionized water and 0.25 ml of Folin-Ciocalteu´s reagent, mixed vigorously, and allowed to react for 6 minutes. Then 2.5 ml of sodium carbonate 7% was added and allowed to stand for 1 hour, and the blue color was measured at 765 nm using a Shimadzu spectrophotometer. Measurements were calibrated to a standard curve of prepared gallic acid (Sigma), and the total phenolic was expressed as mg gallic acid equivalent g dry sample (mg GAE g^{-1} DW).

Determination of total flavonoids

The total flavonoid content was determined using a colorimetric assay, according to Zhishen et al. (1999). One milliliter of the previous acetone 70% extract was added to 4 ml of distilled water and 0.3 ml

of 5% w/v sodium nitrite. After 5 minutes, 0.6 ml of 10% (w/v) $AlCl_3$ was added, and after 6 minutes, 2 ml of 1M NaOH and 2.1 ml of distilled water were added. Absorbance was measured at 510 nm using a Shimadzu spectrophotometer against deionized water. The quercetin compound Sigma was used as a flavonoid standard, and the total flavonoid content was expressed as mg quercetin equivalents (QE)/g dry sample (mg QE/g DW).

Determination of antioxidant activity

Antioxidant activity was determined in the previous 70% acetone extract according to Brand-Williams et al. (1995) using a 1,1-diphenyl-2-picryl-hydrazil (DPPH) reagent. In brief, 1.5 ml of freshly prepared methanolic DPPH solution (20 µg/ml) was added to 0.75 ml of 70% acetone extract and then stirred. The decolorizing process was recorded after 5 minutes of reaction at a wavelength of 517 nm against blank. The DPPH radical scavenging activity of the extract was measured using the Trolox standard curve. Results were expressed as µmol Trolox g^{-1} dry sample.

Statistical analysis

Analyses were carried out with four replicates. The data were expressed as the means standard error. Comparison of means (LSD, 5% level) was statistically calculated using Stat graphics Plus Version 5.1.

Results

Differences in phenolics and AAAs profiling between infected and non-infected faba bean plants at four stages of Orobanche infestation.

Differences in the phenolic acids and polyphenol profiles

Three benzoic-derived phenolic acids (protocatechuic, *p*-hydroxybenzoic, and vanillic) as well as three cinnamic-derived phenolic acids (caffeic, coumaric, and ferulic) were detected in the roots and leaves of the healthy and infected plants of both faba bean varieties (Table 1). Among the three detected benzoic-derived phenolic acids, *p*-hydroxybenzoic acid was considered the main component in faba bean leaves, while vanillic acid was considered the main component in faba bean roots. The levels of benzoic-derived phenolic acid in the two infected faba bean varieties varied during the different stages of Orobanche infestation (Table 1). The tendency of infected roots of Nubaria 4 plants to accumulate more benzoic-derived phenolic acid than those accumulated by healthy roots was noticed. Roots of this variety produced the maximum increase in protocatechuic (16.6%), *p*-hydroxybenzoic (46.8%), and vanillic (90%) at the first infestation stage. On the contrary, at the fourth stage of infestation, infected roots of Nubaria 4 tended to accumulate less of the three phenolic acids compared with those of healthy roots. Meanwhile, the infected roots of Nubaria 5 showed a significant increase only in the level of vanillic acid at the first three stages of infestation when compared with those of healthy roots. At the fifth stage, infected roots of this variety showed a significant decrease in the levels of protocatechuic and vanillic acids as compared with uninfected roots.

Table 1
Effect of different *O. crenata* infestation stages on benzoic-derived phenolic acids in the roots and leaves of two faba bean varieties. Means followed by the same letter (s) within the same column are insignificantly different at 0.05 level of probability

Orobanche infestation stages	Protocatechuic acid (µg g^{-1} DW)		*P*-Hydroxybenzoic acid (µg g^{-1} DW)		Vanillic acid (µg g^{-1} DW)	
	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5
Roots						
1st Stage	56 ± 2^{a}	54 ± 3^{a}	60 ± 3^{a}	50 ± 3^{a}	312 ± 19^{a}	123 ± 15^{a}
2nd Stage	45 ± 1^{ab}	55.4^{a}	58 ± 3^{a}	45 ± 2^{a}	207 ± 12^{b}	139 ± 9^{a}
3^{ed} Stage	37 ± 2^{b}	55 ± 2^{a}	37 ± 2^{b}	45 ± 3^{a}	149 ± 11^{c}	134 ± 14^{a}
4th Stage	55 ± 3^{a}	45 ± 3^{b}	59 ± 4^{a}	46 ± 2^{a}	191 ± 16^{b}	74 ± 8^{b}
Uninfected	48 ± 2^{ab}	54 ± 2^{a}	41 ± 2^{b}	45 ± 2^{a}	164 ± 15^{c}	81 ± 7^{b}
Leaves						
1st Stage	36 ± 1^{a}	55 ± 4^{a}	97 ± 8^{c}	189 ± 19^{a}	64 ± 7^{b}	73 ± 8^{b}
2nd Stage	38 ± 2^{a}	57 ± 3^{a}	154 ± 12^{ab}	190 ± 13^{a}	84 ± 4^{ab}	120 ± 14^{a}
3^{ed} Stage	37 ± 2^{a}	58 ± 3^{a}	168 ± 18^{a}	190 ± 12^{a}	98 ± 5^{a}	122 ± 9^{a}
4th Stage	36 ± 3^{a}	56 ± 4^{a}	139 ± 15^{b}	191 ± 11^{a}	53 ± 4^{b}	136 ± 16^{a}
Uninfected	36 ± 1^{a}	57 ± 2^{a}	142 ± 26^{b}	204 ± 12^{a}	97 ± 9^{a}	112 ± 5^{ab}

Levels of benzoic-derived phenolic acids in the leaves of two faba bean varieties did not appear to show a clear trend in their effects with different Orobanche infestation stages when compared with those of healthy leaves (Table 1). The protocatechuic acid and *P*-hydroxybenzoic acid in the leaves of both varieties did not show any clear effect with different Orobanche infestation stages. But vanillic acid varied in its response to Orobanche infestation between the two tested varieties. The level of this acid in infected Nubaria 4 leaves tended to exhibit significant decreases, but its level in infected Nubaria 5 leaves tended to increase relative to that of the healthy one at all infestation stages.

Cinnamic-derived phenolic acids in the faba bean roots varied in their levels depending on the faba bean organs, infestation stages, and tested varieties (Table 2). At the first infestation stage, a great increase in these phenolic acids in the roots of both varieties was observed when compared with those of healthy roots. The increase in caffeic, coumaric, and ferulic in this stage constituted 554%, 48%, and 74.5 for Nubaria 4 roots, respectively, compared to healthy roots. Also, at this stage, infected roots of Nubaria 5 increased these phenolic acids by 653%, 23%, and 40%, respectively. Moreover, infected Nubaria 4 roots showed a significant increase in the three cinnamic-derived phenolic acid contents at the second infestation stage. Whereas Nubaria 5 roots showed significant increases in their caffeic and ferulic acid contents at all infestation stages when compared with those of uninfected roots.

Table 2
Effect of different *O. crenata* infestation stages on cinnamic-derived phenolic acids in the roots and leaves of two faba bean varieties (μg g^{-1} DW).

Orobanche infestation stages	Caffeic acid		Coumaric acid		Ferulic acid	
	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5
Roots						
1st Stage	378 ± 22^{a}	213 ± 12^{a}	201 ± 37^{a}	171 ± 32^{a}	69.8 ± 3.4^{a}	53.9 ± 3.6^{a}
2nd Stage	290 ± 18^{a}	207 ± 17^{a}	164 ± 23^{b}	133 ± 25^{b}	57.5 ± 3.1^{a}	42.0 ± 2.1^{ab}
3^{ed} Stage	38 ± 5^{b}	205 ± 13^{a}	152 ± 11^{b}	133 ± 21^{b}	36.8 ± 2.9^{b}	42.0 ± 2.2^{ab}
4th Stage	52 ± 9^{b}	60 ± 8^{b}	156 ± 12^{b}	129 ± 23^{b}	47.1 ± 3.3^{b}	40.2 ± 3.2^{ab}
Uninfected	58 ± 7^{b}	28 ± 6^{c}	135 ± 7^{c}	139 ± 6^{b}	40.0 ± 2.2^{b}	38.4 ± 2.2^{b}
Leaves						
1st Stage	171 ± 11^{a}	90 ± 4^{ab}	215 ± 14^{a}	129 ± 8^{a}	40.9 ± 1.1^{a}	37.5 ± 2.3^{a}
2nd Stage	100 ± 8^{c}	100 ± 7^{a}	188 ± 12^{ab}	140 ± 10^{a}	37.7 ± 2.2^{a}	29.8 ± 1.8^{b}
3^{ed} Stage	114 ± 9^{bc}	102 ± 6^{a}	174 ± 17^{ab}	142 ± 11^{a}	23.8 ± 2.6^{b}	27.8 ± 1.4^{b}
4th Stage	122 ± 10^{b}	103 ± 7^{a}	132 ± 18^{b}	133 ± 9^{a}	31.8 ± 3.2^{ab}	37.8 ± 2.2^{a}
Uninfected	103 ± 7^{c}	74 ± 3^{b}	112 ± 3^{c}	143 ± 11^{a}	25.7 ± 1.2^{b}	37.2 ± 2.0^{a}

Means followed by the same letter (s) within the same column are insignificantly different at 0.05 level of probability.

The levels of cinnamic-derived phenolic acids in the faba bean leaves varied in their levels during different Orobanche infestation stages. The leaves of the parasitized Nubaria 4 plants tended to accumulate more cinnamic-derived phenolic acids than those accumulated by those of un-parasitized plants. Among these phenolic acids, infected Nubaria 4 leaves showed the greatest increase in the coumaric acid content (17.8–192%) at all infestation stages. A maximum increase in caffeic (66%), coumaric (192%), and ferulic acid (59%) contents was found in the infected Nubaria 4 leaves at the first

infestation stage. Whereas, the concentrations of the three cinnamic-derived phenolic acids in Nubaria 5 leaves showed great variations as affected by different infestation stages (Table 2). Infected Nubaria 5 leaves produced significant increases in their caffeic acid contents and an insignificant effect on their coumaric acid contents as compared with those of uninfected leaves. A continuous increase in caffeic concentration was observed at all infestation stages and constituted between 20.6% (at the first stage) and 39% (at the fourth stage). As for ferulic acid in the infected Nubaria 5 leaves, it decreased significantly at the second (19.9%) and third (25.3%) stages of infestation as compared with uninfected plants.

The difference in total phenolics, total flavonoids and antioxidant activity between infected and non-infected plants was observed. With few exceptions, infected roots of two faba bean varieties showed significant increases in total phenolics, total flavonoids, and antioxidant activity compared with those of the healthy roots at all infestation stages (Table 3). The highest increase in these antioxidants tended to be obtained at the first infestation stage. In this stage, increases in phenolics and flavonoids contents and antioxidant activity in infected roots constituted 60.7%, 40%, and 53.5% for Nubaria 4, respectively, compared to 26.6%, 56.3%, and 28.3% for Nubaria 5 roots, respectively, relative to those of uninfected plants.

Table 3

Effect of different *O. crenata* infestation stages on total phenolic, total flavonoids and antioxidant activities in roots and leaves of two faba bean varieties. Means followed by the same letter (s) within the same column are insignificantly different at 0.05 level of probability

Orobanche infestation stages	Total phenolics (mg GAE g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Antioxidant activity (µmol Trolox g^{-1} DW)	
	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5
Roots						
1st Stage	26.2 ± 1.3^{a}	18.1 ± 0.9^{a}	3.15 ± 0.15^{b}	1.0 ± 0.1^{a}	304 ± 14^{a}	345 ± 16^{a}
2nd Stage	20.3 ± 1.1^{b}	16.7 ± 0.9^{a}	4.24 ± 0.21^{a}	0.9 ± 0.3^{a}	231 ± 9^{bc}	291 ± 12^{b}
3^{ed} Stage	15.9 ± 9.0^{c}	16.7 ± 0.8^{a}	2.77 ± 0.11^{b}	0.9 ± 0.2^{a}	200 ± 11^{c}	292 ± 11^{b}
4th Stage	23.8 ± 1.2^{a}	13.0 ± 0.6^{b}	2.95 ± 0.13^{b}	0.9 ± 0.1^{a}	258 ± 12^{b}	232 ± 9^{c}
Uninfected	16.3 ± 0.8^{c}	14.3 ± 0.6^{b}	2.25 ± 0.11^{c}	0.6 ± 0.1^{b}	198 ± 9^{c}	269 ± 9^{bc}
Leaves						
1st Stage	39.2 ± 1.7^{a}	59.8 ± 2.8^{a}	20.1 ± 1.1^{a}	12.9 ± 0.5^{c}	517 ± 28^{a}	725 ± 37^{a}
2nd Stage	33.7 ± 1.6^{b}	53.1 ± 2.3^{b}	18.9 ± 0.9^{ab}	20.2 ± 0.9^{a}	356 ± 14^{c}	743 ± 31^{a}
3^{ed} Stage	33.4 ± 1.4^{b}	53.1 ± 2.5^{b}	20.0 ± 1.2^{a}	20.2 ± 1.0^{a}	352 ± 16^{c}	743 ± 33^{a}
4th Stage	34.4 ± 1.7^{b}	45.1 ± 2.3^{c}	19.0 ± 0.9^{ab}	11.5 ± 0.4^{c}	390 ± 18^{b}	575 ± 24^{b}
Uninfected	35.7 ± 1.7^{b}	53.3 ± 2.9^{b}	17.4 ± 0.8^{b}	15.9 ± 0.7^{b}	390 ± 17^{b}	706 ± 33^{a}

Infected leaves of the two faba bean varieties produced significant increases in total phenolic contents at the first infestation stage when compared with those of healthy leaves (Table 3). However, with one exception, the last three infestation stages did not produce significant effects on the total phenolic content of the leaves of both varieties. Whereas, the total flavonoids in the leaves of two faba bean varieties showed remarkable variations in their responses to different infestation stages. Orobanche infestation increased flavonoid content in the leaves of Nubaria 4 between 8.6% and 15.5% as compared with that of uninfected plants. Meanwhile, the level of flavonoids in the leaves of Nubaria 5 plants

showed variations in their response to different infestation stages. Flavonoid contents in the infected Nubaria 5 leaves showed significant increases at the second and third stages and a significant decrease at the first and fourth stages, as compared with uninfected plants. Antioxidant activity in the leaves of two faba bean varieties varied in their response to different infestation stages as compared with those of uninfected plants. The level of antioxidant activity in infected Nubaria 4 leaves varied in their effects with different infestation stages. It showed a significant increase at the first infestation stage, a significant decrease at the 2nd and 3rd stages, and an insignificant change at the 4th stage. Whereas, different Orobanche infestation stages tended to produce insignificant changes in the levels of antioxidant activity in the leaves of the Nubaria 5 plants as compared with those of healthy leaves.

Differences in free AAA profiles between infected and non-infected plants.

With few exceptions, infected roots of two varieties tended to accumulate more phenylalanine, tyrosine, and tryptophan than those accumulated in uninfected roots (Table 4). However, the levels of these AAAs in the roots of both varieties varied in their degree of response to different infestation stages. The AAA contents in the Nubaria 4 roots tended to respond positively to different infestation stages when compared with uninfected roots. The roots of this variety tended to achieve the maximum increase in phenylalanine (77%), tyrosine (9%), and tryptophan (92%) at the fourth infestation stage. Moreover, the parasitized roots of Nubaria 5 roots produced increases in their phenylalanine, tyrosine, and tryptophan contents ranging between 4% and 10%, 105% and 26% and 125%, respectively, compared with those of healthy ones. The roots of this variety achieved the maximum increase in tyrosine (157%) and tryptophan (124%) at the first infestation stage.

Table 4
Effect of different *O. crenata* infestation stages on aromatic amino acids in roots and leaves of two faba bean varieties (µg g^{-1} DW).

Orobanche infestation stages	Phenylalanine		Tyrosine		Tryptophan	
	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5	Nubaria 4	Nubaria 5
Roots						
1st Stage	216 ± 14^{a}	135 ± 13^{a}	339 ± 17^{ab}	252 ± 12^{a}	72 ± 4^{b}	162 ± 9^{a}
2nd Stage	230 ± 17^{a}	130 ± 11^{a}	388 ± 21^{a}	233 ± 15^{a}	79 ± 5^{b}	114 ± 7^{b}
3^{ed} Stage	116 ± 12^{b}	130 ± 11^{a}	274 ± 12^{b}	233 ± 24^{a}	60 ± 4^{c}	114 ± 6^{b}
4th Stage	208 ± 19^{a}	138 ± 10^{a}	399 ± 18^{a}	201 ± 13^{ab}	141 ± 4^{a}	91 ± 5b^{c}
Uninfected	117 ± 9^{b}	125 ± 9^{a}	366 ± 26^{ab}	98 ± 7^{b}	73 ± 5^{b}	72 ± 4^{c}
Leaves						
1st Stage	1316 ± 77^{c}	1260 ± 55^{a}	364 ± 18^{b}	347 ± 23^{a}	77 ± 4^{b}	110 ± 8^{ab}
2nd Stage	1453 ± 64^{bc}	956 ± 33^{b}	356 ± 23^{b}	239 ± 11^{b}	83 ± 5^{ab}	96 ± 3^{b}
3^{ed} Stage	1865 ± 99^{a}	956 ± 33^{b}	397 ± 22^{a}	239 ± 12^{b}	89 ± 5^{ab}	96 ± 4^{b}
4th Stage	1290 ± 73^{c}	595 ± 42^{c}	404 ± 51^{a}	235 ± 20^{b}	104 ± 7^{a}	120 ± 5^{a}
Uninfected	1665 ± 62^{b}	961 ± 44^{b}	425 ± 24^{a}	331 ± 14^{a}	98 ± 3^{a}	123 ± 5^{a}

Means followed by the same letter (s) within the same column are insignificantly different at 0.05 level of probability

A great variation in the levels of the three AAAs in the leaves of both varieties was observed during the examined infestation stages (Table 4). With few exceptions, infected leaves of two varieties accumulated fewer amounts of phenylalanine, tyrosine, and tryptophan than those accumulated by healthy leaves at different infestation stages. The lowest phenylalanine concentration in the leaves of both varieties was observed at the fourth stage of infestation. Nubaria 4 leaves possessed the lowest values of tyrosine and tryptophan at the first three infestation stages. Meanwhile, leaves of Nubaria 5 produced the lowest tyrosine level at the fourth infestation stage and the lowest tryptophan level at the second and third stages.

Differences in phenolics and AAAs profiling between Orobanche parasites and associated host plants at four infestation stages.

Differences in phenolic acid and polyphenol profiles between the parasite and two host organs

In addition to the three benzoic-derived phenolic acids, protocatechuic, *p*-hydroxybenzoic, and vanillic, that were detected in host plants (Fig. 1), the presence of syringic acid was also identified in parasite during its developmental stages (Table 5). It can be observed that vanillic acid was considered the main benzoic-derived phenolic acid in the parasite and host roots of the two faba bean varieties (Fig. 1). Protocatechuic acid was detected in parasite tissues at all developmental stages in relatively equal amounts ≥ 50 µg g^{-1} DW. Except for the significant increase at the fourth infestation stage, the protocatechuic content of the parasite grown on Nubaria 4did not show any significant increase as compared with its root content. Meanwhile, levels of this phenolic acid in Nubaria 4 leaves tended to decrease significantly when compared with parasite at all infestation stages and with host roots in most stages. On the contrary, leaves of Nubaria 5 plants accumulate more protocatechuic acid than those of parasite and host roots, but variations between them did not reach the level of significance at most infestation stages (Fig. 1). The level of *P*-hydroxybenzoic acid in the Orobanche parasite was between 28.6 and 32.8 µg g^{-1} when attached to Nubaria 4 plants, but its concentration ranged between 29.4 and 52.2 µg g^{-1} when attached to Nubaria 5 plants. Host leaves accumulated more *P*-hydroxybenzoic than those accumulated by either the parasite or the host roots at all infestation stages. Meanwhile, the parasite tissue tended to accumulate a lower concentration of this phenolic acid than that accumulated by the host root of both Nubaria varieties.

Table 5
Levels of syringic acid (benzoic-derived phenolic acid) in plant parts of two faba bean varieties and their attached *O. crenata* parasite at various infestation stages.

Infestation stages	Nubaria 4 variety			Nubaria 5 variety		
	Host root	Host leaf	Parasite	Host root	Host leaf	Parasite
Syringic acid (µg g^{-1} DW)						
1st Stage	ND	ND	47.3 ± 5.4 a	ND	ND	66.5 ± 3.9 a
2nd Stage	ND	ND	45.8 ± 6.3 a	ND	ND	42.1 ± 2.0 b
3^{ed} Stage	ND	ND	43.6 ± 3.9 a	ND	ND	41.4 ± 2.6 b
4th Stage	ND	ND	16.2 ± 2.3 b	ND	ND	11.9 ± 1.0 c

ND = non-detected. Means followed by the same letter (s) within the same column are insignificantly different at 0.05 level of probability

Vanillic acid was considered the major benzoic-derived phenolic acid in Orobanche tissues; its concentration was in the range of 291–360 μg g^{-1} for parasite attached to Nubaria 4 plants and in the range of 169–243 μg g^{-1} for parasite attached to Nubaria 5 plants. Meanwhile, the leaves of host plants tended to accumulate fewer amounts of vanillic as compared with those of parasites and host roots at most Orobanche infestation stages. Except for the insignificant variation at the first stage, the parasite continued to produce a significant increase in vanillic contents as compared with the host roots of the two host varieties at all infestation stages. In comparison with infected Nubaria 4 roots, the parasite exhibited the maximum increase in vanillic content at the third infestation stage (141.6%), but the parasite attached to Nubaria 5 roots showed the maximum increase at the fifth infestation stage (142.5%). Syringic acid was detected only in the *O. crenata* parasite; its concentration in the first three stages of Nubaria 4 infestation was between 43.6% and 47.3% and then dropped to 16.2 μg g^{-1} in the fourth stage (Table 5). Such behavior of syringic acid was observed for the attached parasite with Nubaria 5 plants; its concentration was decreased gradually during the first three stages to be 41.4 μg g^{-1} at the third stage and then dropped to 11.9 μg g^{-1} by the fourth developmental stage.

Among the three detected cinnamic derived phenolic acids, caffeic acid was considered the major phenolic acid in the tissues of the *O. crenata* parasite (Fig. 2). This parasite accumulated more caffeic acid than that accumulated by either the roots or leaves of two faba bean varieties at all infestation stages. Great amounts of caffeic acid were observed in the parasites that grew on Nubaria 4 at the third and fourth stages of infestation, more than 13 times and 104 times that were present in host roots. Moreover, parasites attached to Nubaria 5 plants had more than twice the concentrations of caffeic recorded for host roots. The great accumulation of caffeic in the Orobanche parasite occurred at the third stage of Nubaria 5 infection; its concentration reached more than 400 times that of the attached host roots.

Coumaric acid was detected in parasite tissues in low concentrations as compared with those of the two host parts at all developmental stages (Fig. 2). At all infestation stages, the level of coumaric in the two host parts of both varieties reached more than four times their concentration in the attached parasite. A maximum increase in coumaric acid content was observed for Nubaria 5 roots at the first (16 times) and second infestation stages when compared with those of associated parasites. Ferulic acid in the Orobanche parasite was found in a concentration ≤ 20 μg g^{-1} at all developmental stages, but this compound was present in the host roots and host leaves of both varieties in the range 36.8–69.8 μg g^{-1} and in the range 23.8–40.9 μg g^{-1} at all infestation stages, respectively. The great difference between host roots and attached parasites in their ferulic contents was observed at the first stage of infestation; in this case, host roots of Nubaria 4 and Nubaria 5 accumulated about 7.4 and 3.7 times their concentration in the attached parasite, respectively.

Differences in total phenolics, total flavonoids, and antioxidant activity profiles between the parasite and host organs were observed. The levels of phenolics, flavonoids, and antioxidant activity in the host leaves showed significant increases when compared with those of the parasite and infected roots at all

infestation stages (Fig. 3). Total phenolic content in parasite tissues was changed during the four developmental stages, and the highest level was observed at the first and fifth stages. The parasite accumulated more phenolics than those accumulated in the host roots at all infestation stages. There was an increase in total phenolic contents in parasite tissues as compared with host roots. Total flavonoid content in parasite tissue varied during its developmental stages (Fig. 5). Orobanche parasites tended to accumulate more flavonoids than those accumulated in the associated host roots. As for Nubaria 4 roots, the attached parasite achieved a significant increase in flavonoid content at the fourth infestation stage. Meanwhile, levels of flavonoids in parasites attached to Nubaria 5 reached 4- to 6-fold those of host roots at all infestation stages. The level of antioxidant activity in Orobanche parasites changed during the four developmental stages and showed the highest values at the first and fourth stages (Fig. 5). Antioxidant activity did not change significantly between Orobanche parasites and their Nubaria 4 roots. While in the first and fourth stages of infection, antioxidant activity in the parasite's tissues showed a significant increase in its levels when compared to the infected roots of Nubaria 5.

Differences in free AAA profiles between the parasite and the two host parts

The concentration of free phenylalanine, tyrosine, and tryptophan in the *O. crenata* parasite varied during all developmental stages. (Fig. 4). Phenylalanine in Orobanche tissues still increased significantly when compared with that in the host roots at all stages of infestation. Its concentration in the parasites ranged from 2.2 to 5.5 times that in Nubaria 4-respective roots and 4.3 to 2.7 times that in Nubaria 5-respective roots. Meanwhile, host leaves accumulated higher amounts of free phenylalanine than those accumulated by either the host roots or the attached parasite at all infestation stages. The parasite produced maximum values of both tyrosine and tryptophan at the first stage of development when grown on Nubaria 4 plants and at the fourth stage when parasitizing Nubaria 5 plants. With few exceptions, the parasite accumulates less tyrosine and tryptophan than those accumulated in the respective roots and leaves of two Nubaria varieties at most infestation stages.

Differences in total amino acid profiles between the parasite and the two host parts

At the third infestation stage, the protein in the leaves and roots of the infected two Nubaria varieties and their attached parasites was acid hydrolyzed, and the amino acid composition was determined by HPLC. Among the 17 amino acids detected, the two acidic amino acids aspartate and glutamate were found at the highest concentrations in the roots and leaves of two Nubaria varieties and associated Orobanche parasites (Table 6). Levels of these acidic amino acids varied in their concentrations between the two parts of the host and the attached parasite. The leaves of Nubaria 4 and Nubaria 5 achieved the highest aspartate concentration as compared with those of the host roots and the attached parasite. The roots of Nubaria 4 had the highest glutamate concentration as compared with its leaves and attached parasite. Whereas, the parasite grown on Nubaria 5 plants exhibited the highest glutamate concentration as compared with the roots or leaves of the host plants. Phenylalanine and tyrosine, were detected in the

protein hydrolysate of the parasite, host roots, and host leaves. The parasite developed on Nubaria 4 plants accumulated more Phe than those accumulated by the host roots and host leaves; a contrary result was observed for those of Nubaria 5. The roots of Nubaria 4 exhibited the lowest tyrosine level as compared with those of leaves and attached parasite, but Nubaria 5 associated parasite produced less tyrosine than those of roots and leaves. The parasite that grew on the two varieties accumulated more alanine, valine, methionine, and isoleucine than those accumulated by the roots and leaves of host plants. On the contrary, the parasite accumulated less serine, glycine, and threonine than those of the roots and leaves of both host varieties. The other amino acids showed remarkable variations in their levels between two host parts and attached parasites for both examined faba bean varieties.

Table 6 Profiles of protein amino acids (mg g^{-1} protein) of roots and leaves of two faba bean varieties and their attached *O. crenata* parasite at the third stage of infestation.

Varieties / Amino acids	Nubaria 4			Nubaria 5		
	Roots	Leaves	Parasite	Roots	Leaves	Parasite
Aspartic acid	194.65	232.60	83.80	208.50	285.63	223.25
Glutamic acid	335.92	174.04	280.32	221.45	229.60	313.02
Serine	48.07	44.24	40.40	41.11	47.24	35.84
Glycine	30.08	30.84	26.09	28.82	33.18	15.98
Histidine	30.61	46.18	25.64	40.59	21.65	28.64
Arginine	39.73	50.20	54.88	43.86	38.63	36.47
Threonine	24.77	29.54	23.84	26.96	23.89	19.93
Alanine	40.57	41.51	49.20	32.05	16.79	39.62
Proline	25.64	37.30	36.24	32.07	30.81	26.51
Phenylalanine	52.84	59.80	89.81	58.84	53.04	25.67
Tyrosine	10.85	26.72	26.28	27.49	30.21	19.93
Valine	25.70	46.04	52.04	29.45	32.95	34.80
Methionine	18.93	26.93	38.94	25.77	25.51	34.21
Cysteine	2.52	6.14	8.98	8.46	6.40	6.78
Isoleucine	21.23	28.49	40.09	29.78	26.14	32.98
Leucine	15.85	28.60	35.51	36.27	30.13	35.27
Lysine	30.31	39.97	36.67	56.54	17.00	24.69

Discussion

The shikimate pathway and its products play a role in host-parasite relationships. Phenolic acids and AAAs are the first products of the shikimic acid pathway and are considered the source of building blocks for a very wide number of primary and secondary products in the plant kingdom (Vogt, 2010). In the current study, we defined the profiling changes in phenolic acid and AAAs of the two faba bean varieties and associated *O. crenata* parasites during four stages of infestation. Healthy and infected faba bean plants of the two faba bean varieties were collected 16 weeks after planting. Then, infected plants

were classified into four groups depending on the developmental stages of the attached *O. crenata* parasite.

Differences in phenolics and AAAs profiling between infected and non-infected faba bean plants at four stages of Orobanche infestation.

Phenolic acids are naturally occurring antioxidants that are considered one of the main classes of plant phenolic compounds and serve as a main precursor for the synthesis of polyphenol compounds including flavonoids, tannins, lignin, lignans, coumarins, and other phenolic compounds (Kumar and Goel, 2019). Firstly, we evaluate the changes in phenolic acid compositions, total phenolics, total flavonoids, and their antioxidant activities in the infected and non-infected plants of two faba bean varieties throughout four Orobanche infestation stages. In this study, three benzoic-derived phenolic acids, protocatechuic, *p*-hydroxybenzoic, and vanillic, as well as three cinnamic-derived phenolic acids, caffeic, coumaric, and ferulic acids, were detected in the roots and leaves of the healthy and infected faba bean plants with different concentrations. In line of these results, six phenolic acids, namely, p-hydroxybenzoic acid, vanillic acid, salicylic acid, ferulic acid, benzoic acid, and cinnamic acid, were previously identified in the roots and leaves of faba bean plants (Cen et al., 2023).

The tendency for phenolic acids, total phenolics, total flavonoids, and antioxidant activity to increase in parasitized roots and, to a lesser extent, in parasitized leaves as compared to those of healthy plants was noticed at the most infestation stages. These results are in accordance with those of Zakaria et al. (2015), Briache et al. (2020), and El-Mergawi et al. (2022). In this regard, Karou et al. (2005) reported that the plants responded to pathogen attack by accumulating phenolic phytoalexins, such as hydroxycoumarins and hydroxycinnamates. The enhancement effect of infection on phenolic acids may be connected with their antioxidant defense function against parasites or with their involvement in preventing attached parasite from developing into the host vascular system (Rubiales and Fernández-Aparicio 2012). Our findings showed that the phenolic acids in roots varied in their enhancement effects with parasite infestation. The levels of both vanillic acid and caffeic acid in roots showed a significant increase as affected by Orobanche at the most infestation stages. Variations in the response of phenolic acid in *Carthamus glaucus* plants to the *Cuscuta babylonica* parasite were observed by Asan and Ozen (2016). They found that among the identified phenolic acids, the greatest increase possessed gallic acid (2.4 fold), caffeic acid (1.7 fold), and protocatechuic acid (PCA) (2.1 fold). The results declared that the enhancement effect of Orobanche infestation on the different phenolic constituents varied between the tested varieties. Such differences may be related to the differences between the two varieties in their resistance to the parasite (Zakaria et al., 2015). In this concern, Serghini et al. (2001) found that resistance to the Orobanche parasite is related to increasing the levels of phenolics in host roots, suggesting that the increase of phenolics is a tolerance mechanism to maintain redox homeostasis and improve plant health. Moreover, the identified phenolic constituents in leaves showed variations in their responses to the different stages of parasite infection. The difference between caffeic, ferulic, and coumaric acids that possessed faba bean shoots in their response to Orobanche infestation was previously observed by El-Akkad et al. (2002). Caffeic acid in the infected leaves tended to exhibited a

continuous increase at all infestation stages when compared with the leaves of healthy plants. In accordance with these results, the greatest increase in the levels of caffeic (1.7-fold) was observed when parasitizing *Carthamus glaucus* plants with *Cuscuta babylonica* (Asan and Ozen, 2016). Meanwhile, the maximum increase in the level of all identified phenolics in the roots of both tested varieties due to Orobanche infection was observed at the first infestation stage, and caffeic acid achieved the greatest increase (six fold). The accumulation of phenolic compounds in the infected roots might induce a toxic release near the parasite (Pérez-de-Luque et al., 2009), which may play a defense role against the penetration of the parasite haustorium into the host (Asan and Ozen, 2016).

The results revealed that the levels of free AAAs profiled in the roots and leaves of two faba bean varieties were changed consequently by Orobanche parasitism. Levels of the free phenylalanine, tyrosine, and tryptophan in the roots of the two faba bean varieties showed significant increases as affected by the most stages of Orobanche infestation. In line with these results, a significant increase in the levels of the two amino acids serine and methionine was observed in Broomrape-infected carrot roots when compared with the healthy roots (Nandula et al., 2000). Such enhancement effects of parasite infection on free AAAs in host roots may be due to the higher demand for these amino acids in the infected roots as a result of parasite attachments (Nandula et al. 2000). On the contrary, we observed that the Orobanche parasitism tended to reduce the levels of AAAs in faba bean leaves of both faba bean varieties during different infestation stages. Leaves served as a sink for the metabolites; decreasing levels of free AAAs in infected leaves could reflect the additional susceptible sink that parasite plants represent (Clermont et al. 2019). Meanwhile, we observed differences between the two faba bean varieties in terms of changes in the accumulation of AAAs in roots or leaves upon infestation by *O. crenata*. Variations in the response of *O. crenata* infestation to fatty acid composition between the tested faba bean varieties were reported by Abbes et al. (2020). Such variations in response between the two varieties may be related to the differences between them in their resistance to the parasite (Zakaria et al., 2015).

Differences in metabolic profiling of phenolics and AAAs between host and parasite during four infestation stages.

Orobanche, similar to all other holoparasite plants, are chlorophyll-lacking parasites, unable to undergo photosynthesis, and totally dependent on their hosts for carbohydrates, minerals, and water (Abbes et al., 2020). In the current study, the phenolic components and AAAs metabolic profiling of the parasite was compared across its developmental stages and directly against the respective roots and leaves of the two host varieties. The results of the current study provide many evidences that indicate *O. crenata* is able to self-regulate its phenolic and AAA metabolites during its developmental stages.

The first evidence was concerned with the quantitative differences in the phenolics and AAAs in the parasitic tissues that occurred at four growth stages of the parasite as it developed on two host varieties. We observed that the level of detected phenolic acids, total phenolics, total flavonoids, and antioxidant activities in the parasite's tissues showed a noticeable change during its developmental

stages, and the changes in these compounds did not have the same pattern. We found that the levels of caffeic acid, the major cinnamic-derived phenolic acid, showed to be in the range of 343 μg g^{-1} and 5445 μg g^{-1} during different developmental stages of the parasite. A qualitative change in the phenolic acids, *P*-coumaric, ferulic, and caffeic compositions during various developmental stages of the *O. crenata* parasite was previously observed by El-Akkad et al. (2002). Many investigators showed significant variations in the metabolic composition during different developmental stages of *Phelipanche aegyptiaca* and *Phelipanche ramosa* (Nativ et al., 2017; Clermont et al., 2019). These results indicated that the holoparasite can self–regulate its own metabolites during their developmental stages (Kumar et al., 2022). Another good example of the ability of the *O. crenata* parasite to regulate its own metabolites is that this parasite showed a great variation in its content from phenylalanine (372–663 μg g^{-1}), tyrosine (138–603 μg g^{-1}), and tryptophan (31–170 μg g^{-1}), during the tested developmental stages. In accordance with these findings, a great difference in the levels of AAAs in *Phelipanche aegyptiaca* holoparasite was observed during its developmental stages (Nativ et al., 2017; Kumar et al., 2022). Moreover, a great change in the levels of phenolic compounds and AAAs was observed between the developmental stages of the parasite, which developed on the two different host varieties. For example, caffeic acid in parasite grown on Nubaria 4 showed great variations in their level to be constituted between 343μg g^{-1} at the second stage, and 5445 μg g^{-1} at the fourth stage. Whereas, the level of this compound in parasite grown on Nubaria 5 showed its minimum value at the fourth stage (428 μg g^{-1}) and its maximum value at the third stage (4067 μg g^{-1}). Significant differences were observed in the phenolics identified in *Cuscuta reflexa* that developed on the different hosts, suggesting that the holoparasite can self-regulate its metabolites (Kumar and Amir 2021).

There is much other evidence concerned with the qualitative and quantitative differences in metabolic profiling of phenolics and AAAs between host and parasite during different infestation stages. In addition to the six phenolic compounds detected in host tissues, HPLC analysis showed that syringic acid is considered to be characteristic of the parasite at all developmental stages. Many secondary metabolites were detected only in the parasite tissues; for example, phenylethanoid glycosides are considered to be characteristic of parasites, and they usually do not occur in the host (Scharenberg and Zidorn, 2018). In this regard, Emran et al. (2020) reported that among the detected carotenoids, zeaxanthin only occurred in the holoparasite *Phelipanche aegyptiaca*, which is grown on carrots. Moreover, we observed that the levels of detected phenolic acids in the roots and leaves of the two host varieties did not show permanent relationships with the levels of these compounds that were present in the respective parasite tissue at all infestation stages. The parasite tended to produce a significant increase in vanillic and caffeic acid content as compared with those of the host roots and host leaves of the two host varieties at all infestation stages. The parasite showed a great increase in caffeic acid content at the fourth infestation stage of the Nubaria 4 variety and at the third for the Nubaria 5 variety, making the concentration of this compound in the parasite reach more than 100 times and 20 times its concentration in the respective host roots, respectively. On the contrary, the parasite developed on both host varieties continued to accumulate less coumaric and ferulic acids than those accumulated by the roots and leaves of the respective hosts at all infestation stages. Additionally, the levels of *p*-

hydroxybenzoic acid in parasitic tissues tended to decrease compared with those of host roots at all infestation stages. A difference in the detected phenolic acids between the *O. crenata* parasite and its faba bean host was observed by El-Akkad et al. (2002). The polyphenol profile of *Phelypaea tournefortii* (parasite) was different from that of *Tanacetum polycephalum* (host), indicating the presence of an independent biosynthetic pathway in the holoparasite (Piwowarczyk et al., 2020). Moreover, there was an increase in total phenolic and total flavonoid contents in parasite tissues when compared with those of the respective host roots of the two examined varieties. The parasite developed on Nubaria 5 roots possessed the maximum increase in total flavonoid contents; their levels reached 4- to 6-fold those of host roots at all infestation stages. In line with these results, Hacham et al. (2016) found that total phenols in the Orobanche parasite increased by 3.3-fold compared to the roots of infected tomatoes.

Great variations between the host and parasite in their contents from the free AAAs were observed during different infestation stages. The concentration of free phenylalanine in the parasite ranged between 2.2 and 5.5 times that of the host roots at all stages of infestation. But the parasite tended to accumulate lower amounts of free tyrosine and tryptophan compared to those found in the respective roots and leaves of two host varieties. These findings suggest that the parasite can self-regulate its own AAAs. The difference in AAAs between tomato roots and the *Phelipanche aegyptiaca* parasite attached to them was also observed by Hacham et al. (2016). Who found that the *Phelipanche aegyptiaca* contents from Phe, Trp, and Tyr are higher by 37, 8, and 40-fold, respectively, compared with the host tomato roots. The differences in the results of this study and our findings may be due to the differences between the host and parasite examined in the two studies.

Among the 17 amino acids identified in the two host parts and associated parasite at the third infestation stage, a remarkable variation in their levels was observed. The difference in phenylalanine and tyrosine contents was observed between the parasite and the respective host roots. Also, we found a remarkable variation in the levels of the other amino acids between two host parts and attached parasites. The parasite that grew on the two host varieties accumulated higher amounts of alanine, valine, methionine, and isoleucine, along with lower amounts of serine, glycine, and threonine than those accumulated by the roots and leaves of the two host varieties. In accordance with these results, a significant difference in amino acid contents was shown between the tomato roots and the *Phelipanche aegyptiaca* tubercles attached to them, suggesting that, in addition to amino acids that are transported from the host, the parasite can synthesize its own amino acids (Hacham et al., 2016). The dependence of *P. aegyptiaca* holoparasite completely on its own production of AAAs and the other amino acids was previously implied by Shilo et al. (2016).

Based on these results, we proposed that *O. crenata* has its own metabolic mechanism that enables the parasite to modify synthesize phenolic acids and AAAs according to its own needs, which differ from those of its faba bean host. These findings suggest the presence of an independent biosynthetic pathway for the synthesis of aromatic compounds in *O. crenata* holoparasite (Piwowarczyk et al., 2020). Theoretically, phenolic acids and AAAs are synthesized mainly throughout the shikimate pathway (Kumar and Goel 2019); hence, our findings imply the ability of this parasite to synthesize its own aromatic

compounds through the activity of its shikimate pathway enzymes. Indications of the presence of functional pathways in *O. crenata* were recently noted after studying the effect of glyphosate herbicide targeting the EPSPS enzyme in the shikimate pathway (El-Mergawi and El-Dabaa, 2021). The results of Kumar et al. (2022) indicated that the *Phelipanche aegyptiaca* parasite has its own EPSPS enzyme that is significantly more sensitive to glyphosate herbicide than their host. The presence of activity for shikimate pathway enzymes in the holoparasite tissues can serve as direct evidence that the parasite has its own machinery for AAA and phenolic acid biosynthesis.

Conclusion

In comparison with the healthy faba bean plants, the results indicated that the *O. crenata* parasite enhanced the levels of phenolic acids, polyphenols, and the three AAAs at most infestation stages. The maximum increase in the level of all identified phenolics in the roots of both tested varieties due to Orobanche infection was observed at the first infestation stage. The phenolic components and AAAs metabolic profiling of the parasite was compared across its developmental stages and against the respective roots and leaves of the two host varieties. The results provide much evidence that indicates *O. crenata* is able to self-regulate its phenolic and AAA metabolites during its developmental stages, which differ from those of its host.

Declarations

Author contributions **Ragab El-Mergawi:** Writing – review & editing, Writing – original draft, Investigation, Data curtain, Supervision. **Mahmoud El-Dabaa:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Fathia Elkhawaga:** Data curation, Resources, Methodology, Investigation, Formal analysis Funding acquisition, Conceptualization.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

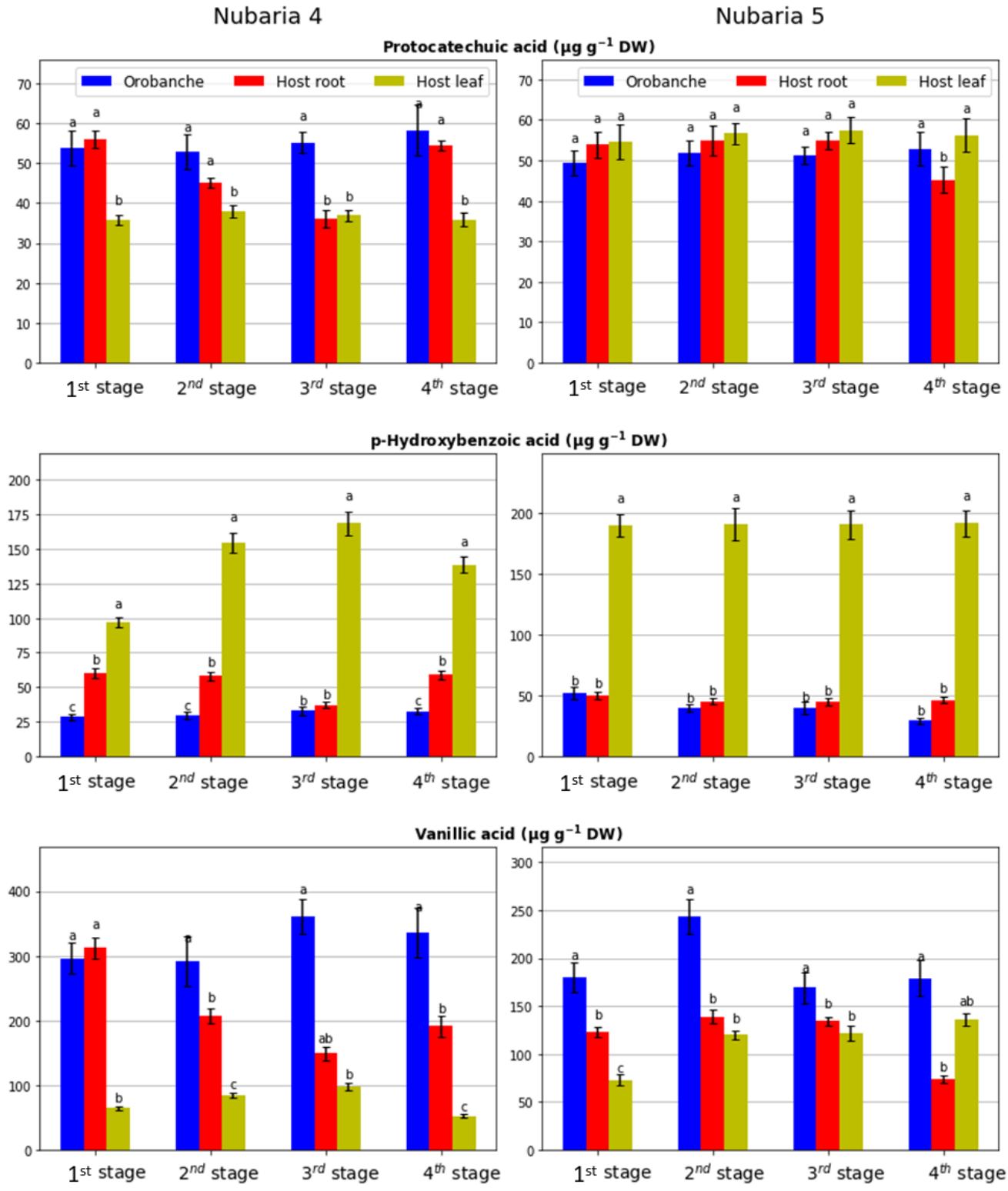

Figure 1

Profiles of the benzoic-derived phenolic acids in plant parts of two faba bean varieties and their attached *O. crenata* parasite at different infestation stages. Means followed by the same letter (s) within the same stage column are insignificantly different at 0.05 level of probability.

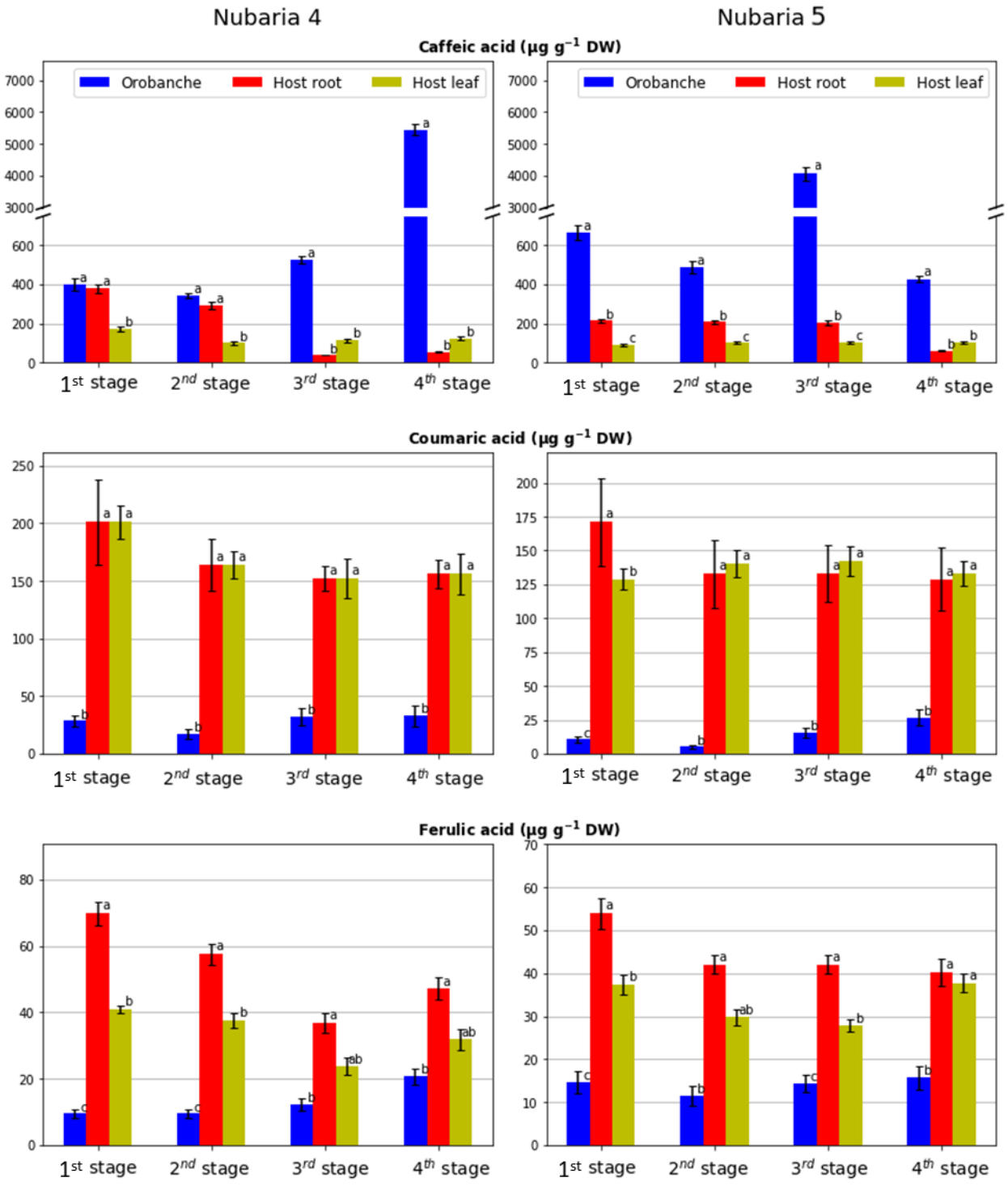

Figure 2

Profiles of the cinnamic-derived phenolic acids in plant parts of two faba bean varieties and their attached *O. crenata* parasite at different infestation stages. Means followed by the same letter (s) within the same stage column are insignificantly different at 0.05 level of probability.

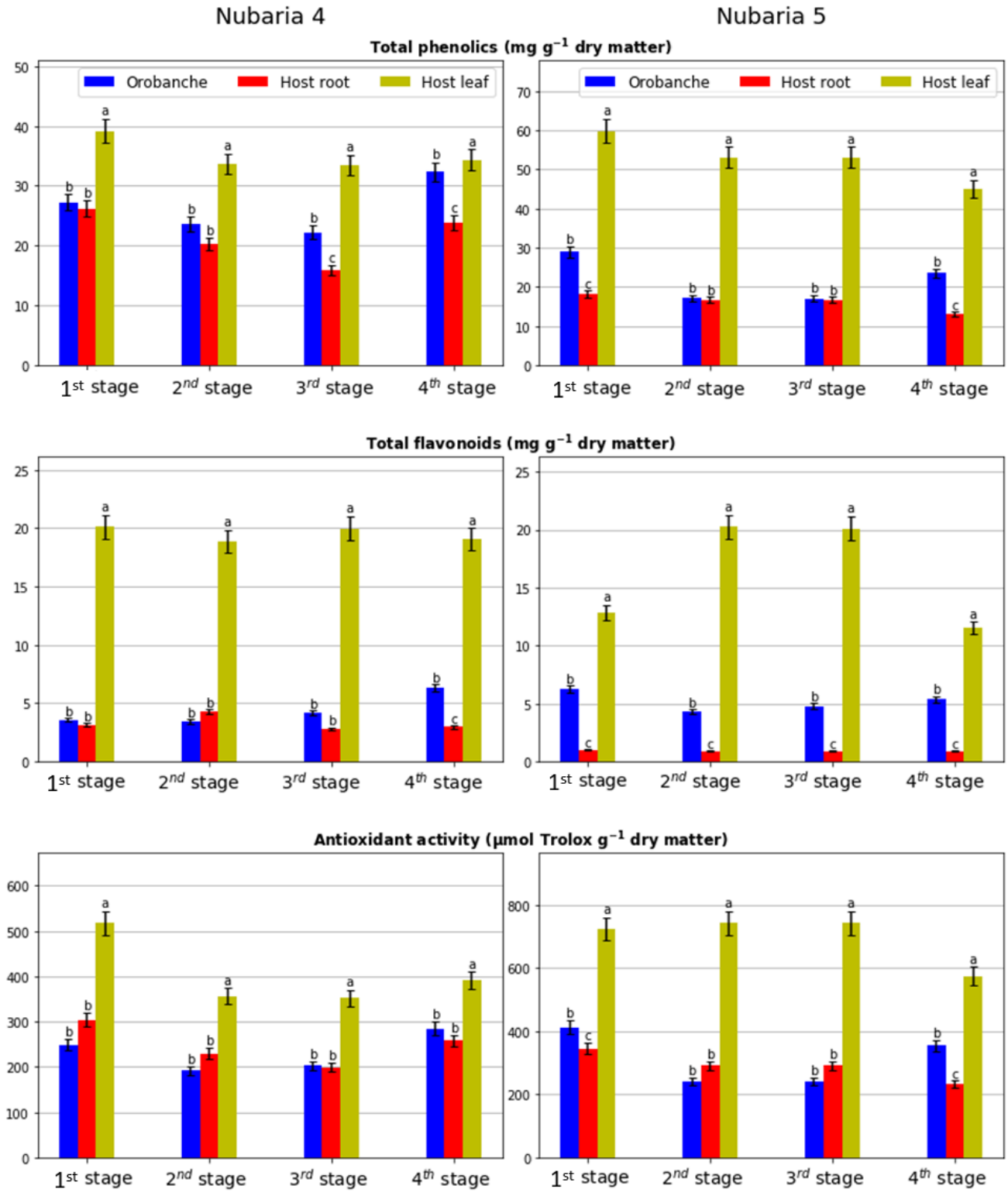

Figure 3

Profiles of total phenolic, total flavonoid, and antioxidant activity in plant parts of two faba bean varieties and their attached *O. crenata* parasites at different infestation stages. Means followed by the same letter (s) within the same stage column are insignificantly different at 0.05 level of probability.

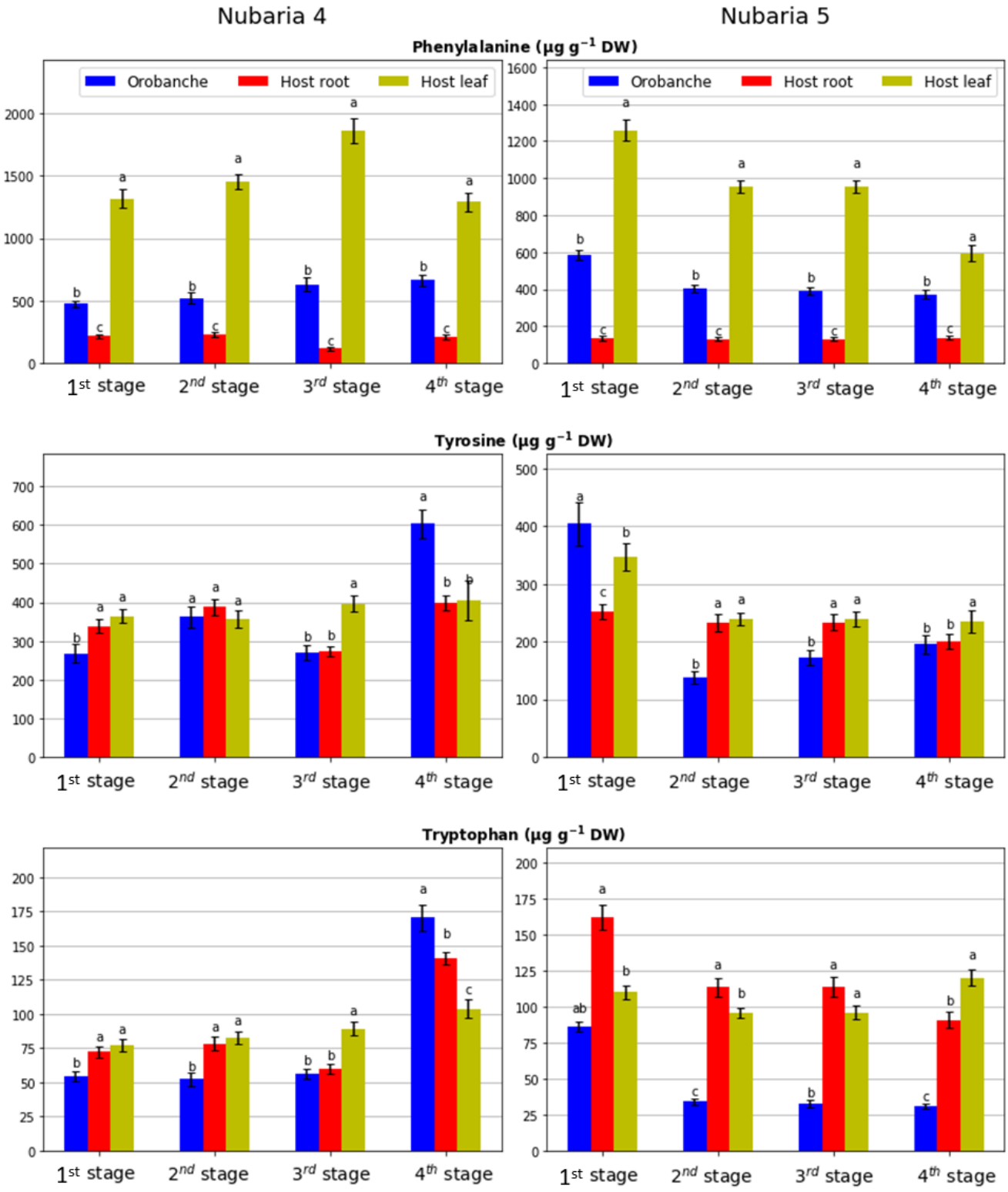

Figure 4

Profiles of the three AAAs in plant parts of two faba bean varieties and their attached *O. crenata* parasite at different infestation stages. Means followed by the same letter (s) within the same stage column are insignificantly different at 0.05 level of probability.