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# The Effects of Amino Acid and Organic Fertilizers on Some Growth Characteristics and Yield of Dragonhead (Dracocephalum moldavica L.)

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#### ABSTRACT

The present experiment evaluated the influence of L-phenylalanine and different organic fertilizer resources on growth, yield, and essential oil production in Dragonhead. The field experiments were conducted in a factorial based on a Randomized Complete Block Design with three replicates at the University of Maragheh, East Azarbaijan Province, Iran during 2018-2019. The experimental treatments were arranged as the application of fertilizer resources at four levels of the chemical, poultry manure, municipal waste compost, and vermicompost as the first factor and the foliar application of Lphenylalanine at four levels of 0, 50, 100, and 150 mM as the second factor. Results showed that the growth characteristics in Dragonhead plants were significantly improved under foliar application of higher doses of L-phenylalanine (50 and 100 mM), which was revealed by a higher dry matter yield. The highest dry matter yield and essential oil yield were observed for the plants treated with municipal waste compost and vermicompost compared to the plants treated with chemical fertilizer and poultry manure. The application of 100/150 mM L-phenylalanine along with vermicompost in the first year produced the highest dry matter yield (481/531 g.m<sup>-2</sup>). The highest percentage of essential oil was observed under 150 mM of phenylalanine + chemical fertilizer (0.58 %) and 50 mM of L-phenylalanine + vermicompost (0.55%) in the first year. As a result, fertilizing with organic manures and L-phenylalanine can increase the dry matter and essential oil production, and improve the yield of dragonhead.

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# 1. Introduction

Excessively favorable application of chemical fertilizers in agroecosystems has led to environmental problems in recent decades, as a result, the product's quality and quantity have been severely affected and sustainable production has been challenged (Niu et al., 2021). Organic fertilizers are effective in increasing plant growth and productivity by improving soil organic matter, structure, and microbial activities, and hence these fertilizers can be a suitable alternative to chemical fertilizers in agricultural production systems (Verma et al., 2020; Liu et al., 2024). According to several studies, medicinal plants' production quantity and quality have improved under organic nutrition. It has been reported that dry matter and essential oil (EO) production and some compounds of basil essential oil

were improved with the application of organic fertilizers (Singh et al., 2014). According to Bajeli et al. (2016), EO production and menthol contents in Japanese mint (Mentha arvensis L.) were enhanced under livestock and poultry manure application.

Biostimulators as organic substances are used to stimulate plant growth, improve nutrient absorption, and increase plant resistance against environmental stresses (Ghatas et al., 2021). Amino acids, which are known as biostimulators, are nitrogenous compounds that are involved in the structure of proteins, enzymes, and plant growth regulators (Shokunbi et al., 2012). There are different roles for amino acids in plants such improving nutrient availability, stimulating germination, chlorophyll and auxins synthesis, defense mechanisms, cell division, and hormone metabolism

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(Popko *et al.*, 2018; Trovato *et al.*, 2021). Nowadays, the external use of biostimulators based on amino acids has been identified as a new way to supply some nutrients and increase plant performance. Sowmya et al. (2023) found that the use of amino acids in coriander led to increases in chlorophyll, carotenoid, and linalool contents. It is reported that the application of some amino acids had positive impacts on EO in mint (Velička *et al.*, 2022) and basil (Reham *et al.*, 2016).

Dragonhead (Dracocephalum moldavica L.) belongs to the Lamiaceae family and its EO has many uses in the food, pharmaceutical, and health industries (Martinez-Vazquez et al., 2012). This plant has antioxidant, antimicrobial, sedative, antidepressant, antinociceptive, neuroprotective, and cardioprotective properties (Zúñiga et al., 2019; Nie et al., 2021). Active substances (secondary metabolites) of medicinal plants significantly influenced by their characteristics, agronomic management, and climatic conditions where the plants grow (Emami Bistgani et al., 2018; Nasiri, 2021; Tursun, 2022; Nasiri et al., 2024).

Considering the global tendency to produce medicinal plants in a sustainable and low-input production system and the lack of sufficient information regarding the reaction of medicinal plants to other sources of fertilizers and biostimulants, this study aimed to investigate the effect of different sources of organic fertilizers and L-phenylalanine on some growth characteristics, yield, and essential oil production of dragonhead.

#### 2. Materials and methods

## 2.1. Study area and experimental treatments

This experiment was carried out at the Research Farm of the University of Maragheh, Maragheh, East Azerbaijan province, Iran (37°23'N, 46°16'E and 1485 m. a. s. l.) in two crop years of 2018 and 2019. According to the Koppen-Geiger climate classification, Maragheh has moderate climate conditions with a dry summer. Some weather data during the performance of this experiment is presented in Fig. 1. The farm soil had a sandy-clay loam texture, and some of its chemical features (soil depth of 0-30 cm) are shown in Table 1. This research was conducted in a factorial experiment with three replications based on randomized complete blocks design (RCBD). The fertilizers of chemical (CF), poultry manure (PM), municipal waste compost (MWC), and vermicompost (V) were considered as the first factor, and the L-phenylalanine was arranged in four concentrations of 0, 50, 100, and 150 mM as the second factor.

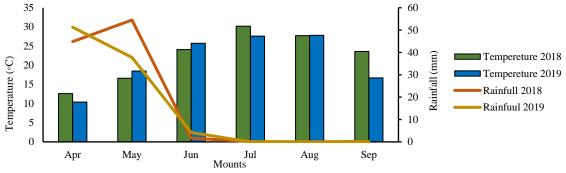


Figure 1. Monthly average temperature and precipitation during the months of the experiment in 2018 and 2019.

Table 1. Texture and some chemical properties of the soil at site of this experiment.

Texture	Total N (%)	Available P (mg kg <sup>-1</sup> )	Exchangeable K <sub>2</sub> O (mg kg <sup>-1</sup> )	EC (dS m <sup>-1</sup> )	pН	OC (%)
sandy- clay loam	0.02	5.65	345		7.75	

# 2.2. Experimental implementation

After initial preparation of the soil, 48 plots (each plot:  $2.5 \times 3 \text{ m}^2$ ) were prepared. Five rows (50 cm apart) were considered in each plot. A distance of 2 m between plots and a distance of 0.5 m between blocks

were considered. According to the results of soil and organic fertilizers analyses (Tables 1 and 2), Urea (equivalent to 200 kg.ha<sup>-1</sup> of pure nitrogen) and Triple Superphosphate (equivalent to 100 kg.ha<sup>-1</sup> of pure phosphors) were used as chemical fertilizers. The consumption amounts of organic fertilizers (PM, MWC, and V) were 8, 10, and 15 t.ha<sup>-1</sup>, respectively. Organic fertilizers were used one month before planting. The phosphorus was applied at the sowing time and nitrogen was applied during seedling emergence, stem elongation, and before flowering.

Table 2. Chemical properties of organic fertilizers used in this experiment.

Ougania fautilizan	N	P	K	Ca	OC	Zn	Mn	Fe	Cu		EC
Organic fertilizer	%				ppm			—рн	(dS m <sup>-1</sup> )		
Vermicompost	1.3	0.89	0.63	2.15	14.6	535	671	1364	97	7.9	6.63
Poultry manure	2.5	1.62	3.2	3.2	27	550	1500	5900	90	7.56	9.78
Municipal waste compost	2.0	1.05	0.9	4.1	21.5	162	335	5800	31	7.2	7.73

N: Nitrogen, P: Phosphorus, K: Potassium, Zn: Zinc, Mn: Magnesium, Fe: Iron, Cu: Copper, OC: Organic Carbone, and EC: Electrical conductivity.

## 2.3. Plant material and evaluation of traits

Seeds of a local dragonhead (*Dracocephalum moldavica* L.) variety, were used for planting. The sowing operation was done on May 14-15 in both years. The distance between the plants was considered 10 cm. After planting, to facilitate seed germination and seedling emergence, sandy soil was manually spread on the surface of the plots. The drip irrigation method was used to irrigate the plots. Light irrigation was performed every other day until the seedlings emerged, and then once a week until harvest. Weeds were manually controlled as needed during the growing period. During the experiment, no pest or disease threatened the plants.

The L-phenylalanine was applied two times (the beginning of the stem elongation period and the beginning of flowering) as a foliar application method on dragonhead plants. Foliar spraying with distilled water was considered as the control. The characteristics of plant height (PH; cm), number of branches (NB), number of inflorescences (NI), number of leaves (NL) (per plant), inflorescence dry weight (IDW; g per plant), dry matter yield (DMY; gm<sup>-2</sup>), EO content (EOC; %), and EO yields (EOY; gm<sup>-2</sup>) were evaluated in this research. At the flowering stage, three middle rows of each plot were considered for sampling and harvesting of plants. A dry, shaded, and ventilated place was considered for drying the collected samples. The dried samples were weighed and their weight was recorded as dry matter yield. Extraction of essential oil was carried out using a Clevenger apparatus and steam distillation method. Fifty grams of dried plant samples were extracted for 2.5 hours in the Clevenger. The percentage of extracted EO (based on weight) was calculated and after multiplying the essential oil percentage and the dry matter yield, the essential oil yield was also obtained.

## 2.4. Statistical data analysis

A statistical analysis system (SAS) software package (version 9. 3) was applied to the analysis of variance

(ANOVA). The least significant difference (LSD) was used to compare the means.

#### 3. Results and discussion

## 3.1. Morphological and growth traits

According to ANOVA (Table 3), the plant height was significantly (p<0.05) affected by fertilization and amino acid. Means comparison (Table 4) showed that the plants treated with vermicompost showed a higher plant height than those treated with PM and CF. On the other hand, the plants were foliar applied with Lphenylalanine at concentrations of 100 and 150 mM. The values were 16% and 19.3% higher than those of the control plants, respectively. The number of branches (NB) was significantly (p<0.05) affected by amino acid and the interaction of year and fertilizer (Table 3). The application of L-phenylalanine (100 and 150 mM) significantly increased the NB by an average of 19.5% compared to the non-application of amino acids. Results also showed that the plants that were treated with vermicompost in the first year had the highest NB (Table 5).

The amino acid, interaction of fertilizer × amino acid, and the interaction of fertilizer × year significantly affected the number of inflorescences. The highest NI were obtained with the use of 50/150 mM of L-phenylalanine + V and with the use of 100 mM of L-phenylalanine + CF (Fig. 2). The lowest NI was obtained with the application of CF without amino acid foliar application. Results (Table 5) also disclosed that the highest NI was gained with V and MWC applications in the first year (2018). The amino acid and the interaction of year and fertilizer had significant effects on the number of leaves (Table 3). The comparison of the means (Table 4) discovered that the application of L-phenylalanine with concentrations of 100/150 mM significantly increased the NL by an average of 19.9% compared with the control. The highest NL (90.7 per plant) was also observed under vermicompost in the first year, with an increase of 22% compared to the other treatments (Table 5).

Table 3. Analysis variance for the traits of dragonhead under fertilizers and amino acid application.

SOV	df	Plant	Number of	Number of	Number of	Inflorescence	Dry matter	Essential	Essential
	aı	height	branches	inflorescences	leaves	dry weight	yield	oil	oil yield
Year	1	23.4ns	2.69 ns	20.7 ns	18.8 ns	0.013	19408.6**	$0.047^{*}$	0.094*
Error a	4	43.7	1.59	3.19	41.9	0.255	879.2	0.0056	0.115
Fertilizer (Fr)	3	51.5*	23.9 <sup>ns</sup>	42.2 <sup>ns</sup>	1274.1 <sup>ns</sup>	6.39 <sup>ns</sup>	27877*	$0.0092^{ns}$	$0.783^{*}$
Amino acid (AA)	3	$378.5^{*}$	$14.8^{*}$	129.4*	1007.9**	3.57*	29628.3*	$0.0707^{ns}$	$2.85^{*}$
$Fr \times AA$	9	43.8ns	$0.505^{\rm ns}$	$10.88^*$	20.99ns	0.11 <sup>ns</sup>	705.5 <sup>ns</sup>	$0.0028^{ns}$	0.041ns
$Year \times Fr$	3	3.169ns	16.4*	53.05**	487.5**	6.54**	2978**	$0.0032^{**}$	$0.077^{**}$
$Year \times AA$	3	38.83ns	1.516 <sup>ns</sup>	9.49 <sup>ns</sup>	27.68 <sup>ns</sup>	0.189ns	3126**	$0.0103^{**}$	$0.117^{**}$
$Year \times Fr \times AA$	9	77.95 <sup>ns</sup>	$0.328^{ns}$	2.631 <sup>ns</sup>	41.65 <sup>ns</sup>	0.151 <sup>ns</sup>	1450**	$0.0023^{**}$	$0.034^{ns}$
Error b	60	61.35	0.73	7.62	44.96	0.54	474.5	0.00045	0.017
CV (%)	•	14.91	9.13	9.01	8.83	12.78	5.35	4.93	7.45

<sup>\*</sup> and \*\* indicate significance at the probability level of 5% (p<0.05) and 1% (p<0.01), respectively, and \*\* indicates non-significance. SOV refers to source of variance.

Table 4. Effect of fertilizers and amino acid application on traits of dragonhead.

Treatments	Plant height (cm)	Number of branches (per plant)	Number of inflorescences (per plant)	Number of leaves (per plant)	Inflorescence dry weight (g)		Essential oil (%)	Essential oil yield (g.m <sup>-1</sup> )
Fertilizer				•				
Chemical fertilizer	51.6±1.9	$10.4\pm0.2$	32.1±0.9	70.1±1.9	$5.3\pm0.21$	$378\pm8.9$	$0.44\pm0.02$	$1.7\pm0.07$
Poultry manure	51.1±1.6	$9.9\pm0.2$	31.4±0.6	$71.0\pm1.8$	$5.4\pm0.13$	$385\pm 9.1$	$0.40\pm0.01$	$1.56\pm0.06$
Municipal waste compost	53.1±1.7	$8.9\pm0.3$	29.6±0.8	77.4±1.8	$6.0\pm0.15$	$419\pm8.2$	$0.43\pm0.02$	$1.77 \pm 0.08$
Vermicompost	54.3±1.5	8.2±0.3	29.4±0.7	86.0±2.1	6.4±0.20	453±9.7	$0.44\pm0.01$	1.99±0.08
LSD (p<0.05)	1.6	3.7	6.69	20.29	2.35	50.13	0.052	0.256
Amino acid concentrations	s (mM)							
0	47.1±1.2	$8.3\pm0.3$	$27.5\pm0.6$	67.5±1.6	$5.3\pm0.15$	$358\pm8.5$	$0.35\pm0.01$	$1.25\pm0.04$
50	52.2±1.6	$9.4\pm0.2$	31.5±0.6	$74.7 \pm 2.4$	$5.6\pm0.20$	$408\pm8.0$	$0.45\pm0.02$	$1.85\pm0.04$
100	54.6±1.7	$9.7\pm0.3$	31.9±0.6	$79.0\pm2.1$	$6.0\pm0.21$	$422\pm9.0$	$0.46\pm0.01$	$1.92\pm0.06$
150	56.8±1.7	$10.1\pm0.3$	32.0±0.8	82.6±1.9	6.7±0.18	439±9.8	$0.47 \pm 0.02$	$2.01\pm0.05$
LSD (p<0.05)	5.725	1.131	2.83	4.833	0.3994	51.37	0.932	0.3141

Each value is mean of twenty-four replicates  $\pm$  standard error.

Table 5. Effect of year and fertilizer sources on some traits of dragonhead.

		Number of	Number of	Number of	Inflorescence
Year	Fertilizer sources	branches	inflorescences	leaves	dry weight
		(per plant)	(per plant)	(per plant)	(g plant <sup>-1</sup> )
	Chemical fertilizer	$7.3\pm0.3$	27.9±1.0	64.6±1.7	$4.64\pm0.1$
2019	Poultry manure	$9.0\pm0.37$	$30.0\pm0.7$	$70.3\pm2.1$	5.33±0.2
2018	Municipal waste compost	$10.5\pm0.27$	$32.6\pm0.6$	$79.9 \pm 2.3$	$6.14\pm0.1$
	Vermicompost	$11.4\pm0.25$	34.0±0.8	90.7±2.4	$7.00\pm0.2$
	Chemical fertilizer	$9.2\pm0.25$	31.0±1.2	75.5±2.6	5.95±0.3
2010	Poultry manure	$8.9\pm0.35$	29.2±0.9	71.1±3.1	$5.49\pm0.2$
2019	Municipal waste compost	$9.4\pm0.45$	$30.2 \pm 1.3$	$74.8 \pm 2.7$	$5.75\pm0.3$
	Vermicompost	$9.5\pm0.34$	30.3±0.8	$80.7 \pm 0.8$	$5.83\pm0.2$
LSD (p<0.05)		0.699	2.254	5.475	0.602

Each value is mean of twelve replicates  $\pm$  standard error.

# 3.2. Inflorescence dry weight and dry matter yield

The results showed that the inflorescence dry weight was significantly affected by the amino acid and the interaction of the year and fertilizer (Table 3). The application of 100/150 mM L-phenylalanine significantly increased the IDW by an average of 14% compared to the control. As seen in Table 4, the highest IDW was obtained with the application of V in the first year, followed by MWC in the first year. According to the ANOVA (Table 3), the tripartite effect of year, fertilizer, and AA on the dry matter yield (DMY) was significant (P<0.01). The highest DMYs (531 and 481

g.m $^{-1}$ ) were obtained with the application of V + 150/100 mM of L-phenylalanine in the first year (Fig. 3). In the second year, the application of all three concentrations of phenylalanine (50, 100, and 150 mM) + V as well as the foliar application of 150 mM phenylalanine + PM showed superiority over the other treatments.

Since organic fertilizers contain macro and micronutrients, their application can increase plant access to nutrients and consequently lead to improvements in plant photosynthetic activity, vegetative growth, and biomass. Organic fertilizers

such as vermicompost are effective in sustaining soil resources and increasing plant growth by improving soil structure and biological activity (Paczka et al., 2021; Voko et al., 2022). It seems that after applying the vermicompost, poultry manure, and municipal waste compost, the plant's ability to absorb nutrients such as zinc, copper, iron, phosphorus, potassium, and nitrogen was increased, and subsequently, greater plant growth and an increase in the number of leaves were observed. (Liu et al., 2021; 2024). On the other hand, it seems that the increase in the number of leaves under organic fertilizer application led to an enhancement in light absorption, an increase in the activity of the photosynthesis process, and an increase in the production of hydrocarbons in the leaves. As a result, plant height, the number of branches, inflorescences, and leaves, the inflorescence dry weight, the dry matter yield, and the essential oil yield were also increased. Other researchers also indicated that the application of vermicompost led to an increase in the plant height, number of branches, and dry matter

yield of hyssop (*Hyssopus officinalis* L.) (Aghaei *et al.*, 2019) and peppermint (Azizi Balabiglou *et al.*, 2022).

Amino acids as growth-promoting substances have different roles in plants. They are involved in stimulating seed germination, increasing plant's access to nutrients, chlorophyll synthesis, plant's defense mechanism against stress, cell division, hormone metabolism, biosynthesis of enzymes, vitamins, terpenoids, and alkaloids, etc. (Popko et al., 2018; Sowmya et al., 2023). They help the development of the plant's root system, increase the absorption of nutrients by the roots, and thus provide suitable conditions for plant growth (Nikiforova et al., 2016). As a result, with greater plant access to nutrients, the activity of photosynthesis is increased, and the growth characteristic and dry weight of the plant will be improved. Consistent with our results, Elshorbagy et al. (2020) have indicated that the growth characteristics in (Lavandula officinalis CHAIX) increased under L-tryptophan and phenylalanine application.

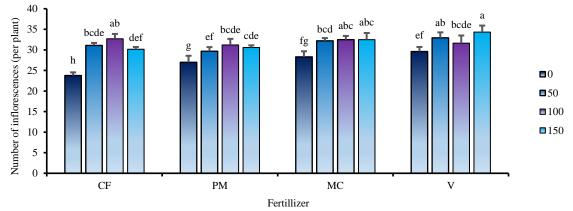


Figure 2. Response of number of flowering branches of dragonhead to fertilizer and amino acid (L-phenylalanine; 0, 50, 100, and 150 mM) application. CF: Chemical fertilizer, PM: Poultry manure, MC: Municipal waste compost, and V: Vermicompost. Different letters on each column indicate a significant difference between treatments according to the LSD test (p<0.05). Figures are expressed as mean  $\pm$  standard error.

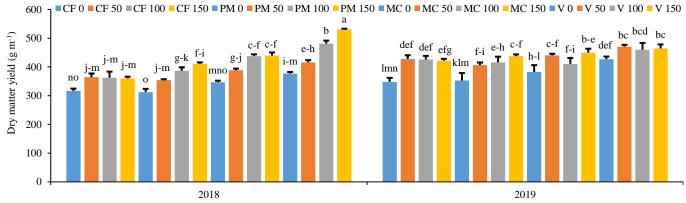


Figure 3. Effect of fertilizer and amino acid (L-phenylalanine; 0, 50, 100, and 150 mM) application on dry matter yield of dragonhead in two years. CF: Chemical fertilizer, PM: Poultry manure, MC: Municipal waste compost, and V: Vermicompost. Different letters on each column indicate a significant difference between treatments according to the LSD test (p<0.05). Figures are expressed as mean  $\pm$  standard error.

#### 3.3. Essential oil content

Dragonhead's EOC was significantly affected by the tripartite effect of year, fertilizer, and amino acid (Table 3). The compression of the means (Fig. 4) indicated that the application of 150 mM of phenylalanine + CF (0.58 %) and 50 mM of L-phenylalanine + MWC (0.55%) in the first year led to

the production of the highest content of essential oil. In the second year, the application of 100 mM of L-phenylalanine + V significantly increased the EOC compared to the other treatments. In both years, the lowest EOC in all fertilizer treatments (CF, PM, MWC, and V) was observed under non-application of the amino acid (water distilled).

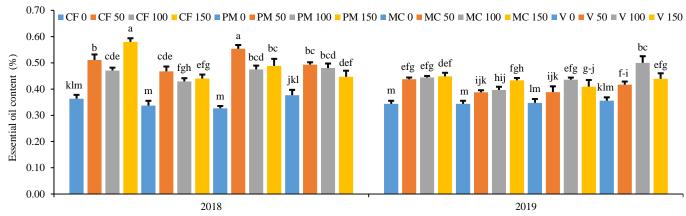


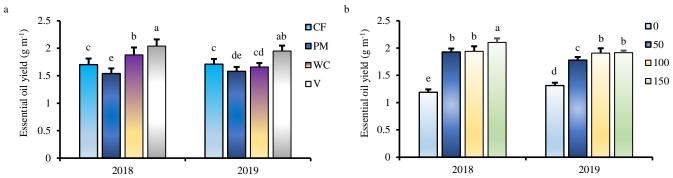
Figure 4. Effect of fertilizer and amino acid (L-phenylalanine; 0, 50, 100, and 150 mM) application on essential oil content of dragonhead in two years. CF: Chemical fertilizer, PM: Poultry manure, MC: Municipal waste compost, and V: Vermicompost. Different letters on each column indicate a significant difference between treatments according to the LSD test (p<0.05). Figures are expressed as mean  $\pm$  standard error.

#### 3.4. Essential oil yield

According to Table 3, the interaction of year and fertilizer and the interaction of year and amino acid on the EOY were significant. As shown in Fig. 5a, in both years, the highest EOY was recorded for V (2.04 and 1.95 gm<sup>-1</sup> for the first and the second years, respectively). The recent treatment increased the EOY by 19.8, 32.4, and 8.5% in the first year (2018) and 14, 23.4, and 17.5% in the second year (2019) compared with the application of CF, PM, and MWC, respectively. Results also showed that the highest EOY (2.1 gm<sup>-1</sup>) was obtained in the first year under 150 mM of phenylalanine (Fig. 5b). Overall, in both years, the application of L- phenylalanine significantly increased EOY compared to control.

Consistent with our results, Dashti et al. (2022) have stated that organic manures (compost and vermicompost) significantly increased essential oil production in salvia (*Salvia leriifolia* Benth). Khalesro et al. (2012) in anise (*Pimpinella anisum* L.) plants have reported similar results. Asadi et al. (2023) also showed that the EO production in peppermint was significantly enhanced by applying chemical fertilizer, vermicompost, and farmyard manure. Since the EOY value is affected by the percentage of EO and DMY,

any increase in the values of these two parameters can cause an increase in EOY. Essential oils are terpenoid compounds, and ATP and NADPH are required to produce the precursors of these compounds. Given that the production of ATP and NADPH requires nitrogen, phosphorus, and other nutrients, the use of fertilizers (chemical and organic) and amino acids can provide additional nitrogen and phosphorus to the plant and help produce greater ATP and NADPH needed for essential oil production (Kapoor et al., 2017; Nasiri, 2021). It is known that amino acids act as precursors of many secondary metabolites and have an essential role in catalyzing the biosynthesis of these compounds (Sowmya et al., 2023; Barros et al., 2016). In this regard, Velička et al. (2022) have reported that the EOC mint was increased under spraying phenylalanine compared to unsprayed plants. Aghaei et al. (2019) in hyssop have also found that the application of amino acids led to an increase in EOC. The results showed a notable difference between the values of some characteristics measured in two years. Overall, the difference in the values of some traits in 2018 and 2019 can be attributed to different humidity and temperature conditions during the growing seasons of the two years (Fig. 1).



## 4. Conclusion

Our results showed that foliar spraying of different concentrations of L-phenylalanine under application of organic fertilizers had valuable effects on growth parameters, dry matter yield, and production of essential oil of dragonhead. Therefore, organic fertilizers such as vermicompost, poultry manure, and municipal waste compost can be used effectively instead of chemical fertilizers in the production of medicinal plants. Overall, the application of Lphenylalanine with a concentration of 50/100 mM in combination with an organic fertilizer such as vermicompost will be a suitable option to increase the quantity and quality of dragonhead production.

#### **Abbreviation**

AA: Amino acid, CF: Chemical fertilizer, PM: Poultry manure, MC: Municipal waste compost, V: Vermicompost, CF: Chemical fertilizer, PH: Plant height, NB: Number of branches, NI: Number of inflorescences, LN: Number of leaves, IDW: Inflorescence dry weight, DMY: Dry matter yield, EOC: Essential oil content, EOY: Essential oil yield.

## **Conflict of interests**

All authors declare no conflict of interest.

## Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

## **Consent for publications**

All authors read and approved the final manuscript for publication.

# Availability of data and material

All the data are embedded in the manuscript.

#### **Authors' contributions**

The first author [L. P.]: Performance of the research, data collection, and writing the initial draft. The second author [Y.N.]: Conceptualization, statistical analysis of the data, writing original draft, reviewing, and editing the manuscript. The third [M. R. M.] and the fifth [F. R.] authors: Advisors.

#### **Informed consent**

The authors declare not to use any patients in this research.

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