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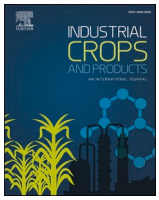

Potential of ultrasound and nicotinic acid to improve physiological responses and trigonelline biosynthesis in fenugreek (*Trigonella foenum-graecum* L.)

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) is an important source of trigonelline, a valuable anti-diabetic metabolite. The main objective of this study was to investigate the possibility of ultrasonication and nicotinic acid feeding in stimulating trigonelline biosynthesis and antioxidant system. For this purpose, the seeds were ultrasonicated in three groups for 0, 5, and 10 min at 40 kHz at 25 °C. Seedlings were treated after the emergence of the first true leaves (10 days) with Hoagland's solution complemented with 0, 2, 4 mg L^{-1} nicotinic acid. The results showed that the 5 min ultrasound priming of fenugreek seeds improved germination-related traits compared with the control culture. However, the 10 min ultrasound priming negatively affected and reduced the germination traits. The highest values of growth indices of seedlings were recorded under effect of 5 min ultrasound and 4 mg L^{-1} nicotinic acid. Ultrasound and nicotinic acid individually and as combined decreased photosynthetic pigments (chlorophylls and carotenoids). The interaction between the two factors increased the antioxidant compounds and biochemical levels of seedlings compared with the control treatment. The highest increase in trigonelline content (170.9 mg/100 g FW) was observed under 5 min ultrasound and 2 mg L^{-1} nicotinic acid feeding. Generally, it appears that ultrasound potentiated the effect of nicotinic acid feeding on defense responses and the production of secondary metabolites, including trigonelline biosynthesis. Overall, it can be concluded that 5 min ultrasound seed priming and 2 mg L^{-1} nicotinic acid can improve growth indices, and therefore to achieve optimal biomass yield and trigonelline production is recommended in fenugreek cultivation.

1. Introduction

Fenugreek (*Trigonella foenum-graecum* L.) medicinal plant is mainly cultivated in India, Pakistan, Egypt, China and the Middle East countries, including Iran (Varghese et al., 2019). In addition to treating some cardiovascular diseases and free radical scavenging activity, this plant has also been shown to have several medicinal properties, including anti-diabetic, anti-cancer and antimicrobial (Varghese et al., 2019; Baba et al., 2018). The high dietary fiber content of the plant makes it a good candidate for reducing the risk of cancerous diseases (Srinivasan, 2019). In addition, fenugreek also contains the trigonelline metabolite and it has been revealed its role in nerve fiber elongation, which is thought to have anti-Alzheimer's properties, (Tohda et al., 1999).

The first known source of trigonelline is fenugreek seeds (Jahns, 1885). Trigonelline has attracted the attention of biologists for proving its role in inducing G2 arrest at the root and shoots of a wide range of plants (Evans and Tramontano, 1981). In addition to the nitrogen storage form for the plant, trigonelline has also been suggested as a signal for a symbiotic relationship with leguminous bacteria (Boivin et al., 1990), nyctinasty (Ueda et al., 1995) and response to various stresses (salinity, ultraviolet, and oxidative stress) (Tramontano and Jouve, 1997). Extensive therapeutic effects for trigonelline have been reported in the human body, causing this metabolite to receive more attention (Ashihara et al., 2015; Zhou et al., 2012). In many plants, especially the

Abbreviations: US, Ultrasound; NA, Nicotinic acid; CVG, Coefficient of the velocity of germination; FGP, Final germination percentage; GI, Germination rate index; FW, Fresh weight; LA, Leaf area; MDA, Malondialdehyde; TBA, thiobarbituric acid; DPPH, 2,2–diphenyl-1–picrylhydrazyl; BSA, Bovine serum albumin; PAL, Phenylalanine ammonia-lyase.

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legume family, nicotinic acid is converted to trigonlin (N-methyl-nicotinic acid) by the enzyme trigonline synthase (EC 2.1.1.7) (Matsui et al., 2007). However, in seeds, it is possible to convert trigonillin to nicotinic acid by the trigonelline demethylase (Zheng et al., 2005).

NA is derived from pyridine nucleotides and is used as a precursor for secondary pyridine metabolites (Ashihara et al., 2015). The strategy of precursor or intermediate compounds feeding has undergone extensive studies to increase the production of plant metabolites (Namdeo et al., 2007). Mathur and Kamal (2012) showed that supplementing the culture medium with 500 mg L-1 NA leading to a 1.10-fold enhancment in trigonelline production in cell culture of Moringa oleifera. Feeding precursors studies have been performed mainly in vitro conditions. In this study, we added NA to the nutrient solution to provide it to the plant roots.

Low-energy ultrasound, in addition to inducing the defense system, also increases the permeability of cell membranes and the transport of molecules through them (Rezaei et al., 2011). Accelerating molecules, increasing the transport of substances and reagents to active sites (Sinisteram, 1992) and enzymatic activity are other mechanisms of ultrasound in a biological systems. Various studies have shown that low-energy ultrasound waves significantly increased the production of valuable secondary metabolites including ginsenosides (Lin et al., 2001), shikonin (Lin and Wu, 2002), taxol (Rezaei et al., 2011) and resveratrol (Yu et al., 2016a) in plant cells, tissues and organs.

The main purpose of this study was to investigate the possibility of ultrasonication and NA feeding in stimulating trigonelline biosynthesis and antioxidant system in hydroponically growing fenugreek (as a fast-growing source of trigonelline). To this end, in addition to measuring different physiological and biochemical indices, the yield of hydroponically-grown fenugreek biomass was also evaluated in terms of trigonelline biosynthesis.

2. Material and methods

2.1. Plant material and culture conditions

Fenugreek seeds (Pakan Seed Company, Isfahan, Iran) were surface-sterilized with 2% sodium hypochlorite solution for 10 min and then rinsed four times with sterilized distilled water. After that, the seeds were exposed to ultrasound treatment in three groups for 0, 5 and 10 min at 40 kHz at 25 °C. Then, 20 seeds for each treatment were planted in a greenhouse with sunlight PAR, at 27 ± 0.5 °C, with a humidity of 28 ± 3% in the plastic pots (17 ×19 cm) containing pumice. After establishment and growth of seedlings following irrigation with Hoagland's solution, the 10 most uniform seedlings that had more uniformity were selected and the rest were thinned. Seedlings were treated after the emergence of the first true leaves (10 days after planting) with Hoagland's solution supplemented with 0, 2, 4 mg L^{-1} NA (pH = 6.5 and EC = 1.2 dS/m) which was applied daily. After 30 days of feeding with a solution containing NA, three plants from each treatment were kept at − 80 °C for further analysis. Growth indices were also measured immediately.

2.2. Ultrasonication

The procedure proposed by Rezaei et al. (2011) was used to sonicate the seeds. An open ultrasonic bath (Ultrasonic bath FALC Instruments, Italy) with two constant frequencies (40 and 59 kHz) and variable levels of power (peak power: 300 W) with a useful volume of 10 L (internal dimensions: 300×240×150 mm) was used to treat the seeds. The seeds were moved to 250 mL Erlenmeyer flasks including 100 mL distilled water. To apply the ultrasonication, the flasks of containing seeds were immersed in the ultrasound bath. The exposure times were 0, 5 and 10 min at peak power level (300 W). The temperature was maintained at 25 ± 0.5 °C during the seed ultrasonication.

2.3. Measurement of germination parameters

In this study, three different parameters related to germination were investigated, which were calculated as follows: Coefficient of the velocity of germination $(CVG) = N1 + N2 + .+Ni/100\ x\ N1T1 + .+NiTi$; where N is the number of germinated seeds per day, and T is the number of days from seed sowing corresponding to N; Final germination percentage (FGP) = the total seeds germinated at end of trial/number of initial seeds used 100 times. Germination rate index $(GI) = G1/1 + G2/2 + .+Gi/i$; where G1 is the germination percentage on day 1, G2 is the germination percentage at day 2; and so on (Al-Mudaris, 1998).

2.4. Growth analysis

Thirty days after seedling emergence, root and shoot length, and fresh weight of roots and shoots were measured. The number of leaves was also counted. Leaf area (LA) was determined with a leaf area meter.

2.5. Measurement of photosynthetic pigments

The method proposed by Baskar et al. (2015) was used to measure the content of photosynthetic pigments. In order to determine chlorophyll content, 100 mg of frozen leaf tissue from each treatment was grounded and extracted in 95% (v/v) ethanol for 72 h at 4 °C in the darkness. After centrifugation at 10,000 rpm, the obtained supernatant was analyzed spectrophotometrically at 664 and 648 nm. Chlorophyll a and b concentrations as well as total carotenoid, were measured based on the subsequent calculations:

Chla = Chlorophyll a (μg mL^{-1}) = 12·21 (A663) - 2·81 (A646) × V/1000 W

Chlb = Chlorophyll b $(\mu g\ mL^{-1}) = 20{\cdot}13\ (A646) - 5{\cdot}03\ (A663) \times V/1000\ W$

Cartenoids = $(1000 \times A470 - 1{\cdot}82 \times Chl\ a - 85{\cdot}02 \times Chl\ b)/198$

The final results were expressed as mg g^{-1} FW.

2.6. Biochemical analysis

Total anthocyanin was measured by means of the technique designated by Jeong et al. (2010). After grounding 100 mg of the sample tissue in liquid nitrogen, it was isolated with acidic methanol (99:1 v/v) followed by darkness incubation of 24 h at 4 °C. The resulting extract was mixed with chloroform and spun at 13,000 rpm for 2 min. The supernatant was spectrophotometrically analyzed at 530 and 657 nm and the results were calculated using the following equation: A530–1/4 A657. The data were stated as mg g^{-1} FW.

The total phenolics content was evaluated based on Folin–Ciocalteu reagent (Shen et al., 2009). Grounded plant samples were homogenized with reagent and then with sodium carbonate (15%). Absorbance rate was read spectrophotometrically at 755 nm. Gallic acid was used as the standard calculation ($r^2 = 0.975$) of total phenolics and the data were expressed as the equivalent of gallic acid (GAE) per gram of sample.

The method proposed by Djeridane et al. (2006) was used to measure total flavonoids. For this, 200 mg of plant samples were homogenized with 3 mL of acidic ethanol. After extraction, the supernatant was placed in a hot bath at 80 °C for 10 min. After sample cooling, the supernatant absorbance was measured at 450 nm. A quenching coefficient of 33,000 cm mol^{-1} was used to calculate the concentration of flavonoids.

The DPPH (2,2 – diphenyl-1–picrylhydrazyl) test was used to assess the antioxidant capacity of the leave tissue samples (Brand-Williams et al., 1995). For this purpose, 0.2 g of the sample leaves were homogenized with 3 mL of acidic ethanol. It was then centrifuged at 8000 rpm for 12 min. Then, the supernatant was completed with DPPH radical methanolic solutions (10^{-4} M). The sample absorbance was spectrophotometrically quantified at 517 nm after 30 min. Finally, the radical scavenging activity (%) was estimated according to the next equation:

Table 1
Analysis of variance of the effect of ultrasound and Nicotinic acid on the studied traits in fenugreek.

S.O.V	df	Mean square									
		HR	HS	NB	LB	WFR	WFS	Chl.a	Chl.b	Carotenoid	Phenolics
US	2	21.37**	21.70**	204.59**	63.44**	372,160.70**	120,152.70**	0.12**	0.48**	215,324.90**	4564.75**
NA	2	1.07 ns	0.92 ns	43.82 ns	3.11 ns	19,373.15**	11,738.93**	0.22**	0.34**	216,600.19**	365.60**
US×NA	4	2.61**	1.09 ns	343.48* *	7.22 ns	11,158.87**	19,886.20**	0.04**	0.20**	74,113.46**	1759.29**
Error	18	0.42	0.58	27.93	4.04	6.41	209.33	0.00	0.00	9030.86	5.56
S.O.V	df	Mean square									
		Flavonoid	Anthocyanin	DPPH	Vi	MDA	H_2O_2	Protein	PAL	Trigonelline	
US	2	0.82**	31,377,325.94**	1236.81**	901.66**	1232.59 ns	5.27**	36.41**	1.75**	3789.76**	
NA	2	0.74**	43,621,647.79**	553.77**	824.03**	238,629.88**	27.52**	406.49**	0.18**	3815.15**	
US×NA	4	1.61**	28,254,407.90**	876.70**	446.67**	113,833.52 **	6.29**	169.03**	0.27**	13,426.98**	
Error	18	0.01	25,518.19	0.02	0.56	696.49	0.00	0.85	0.00	1.72	

**, * and ns denote significant differences at 0.01, 0.05 levels, and not significant, respectively.

Radical scavenging activity (%) = [(Ao - As)/Ao] × 100, where, Ao is the absorbance of control blank, and As is the absorbance of the sample.

Ascorbic acid was measured based on color reduction of compound 2,6-dichlorophenolindophenol by ascorbic acid (Desai and Desai, 2019). In this assay, 5 g of plant sample was homogenized with 15 mL of 3% metaphosphoric acid, and the resulting homogenate was then centrifuged at 10,000 rpm at 4 °C for 12 min. Supernatant solution (0.5 mL) was added to 0.5 mL of the dichlorophenolindophenol solution (100 mg L^{-1}) and the absorbance rate of the resulting solution was spectrophotometrically determined at 520 nm. The ascorbic acid concentration was intended based on a standard curve prepared from different concentrations of ascorbic acid.

The amount of damage to membrane lipids was revealed by measuring the content of the ultimate product of lipid peroxidation, malondialdehyde (MDA). In this method, previously described by Health and Packer (1968), 200 mg of the plant sample was homogenized with 3 mL of trichloroacetic acid (TCA). Next, centrifugation homogenate was performed at 8000 rpm for 10 min. Then 750 µl of solution and 750 µl of thiobarbituric acid (TBA) were mixed and kept at 100 °C for 30 min. After cooling the samples, their absorbance was spectrophotometrically quantified at 532 and 600 nm.

The content of plant tissue H_2O_2 was conducted using titanium tetrachloride assay as previously designed by Brennan and Frenkel (1977). Leaf samples were incubated with cold acetone followed by mixing with

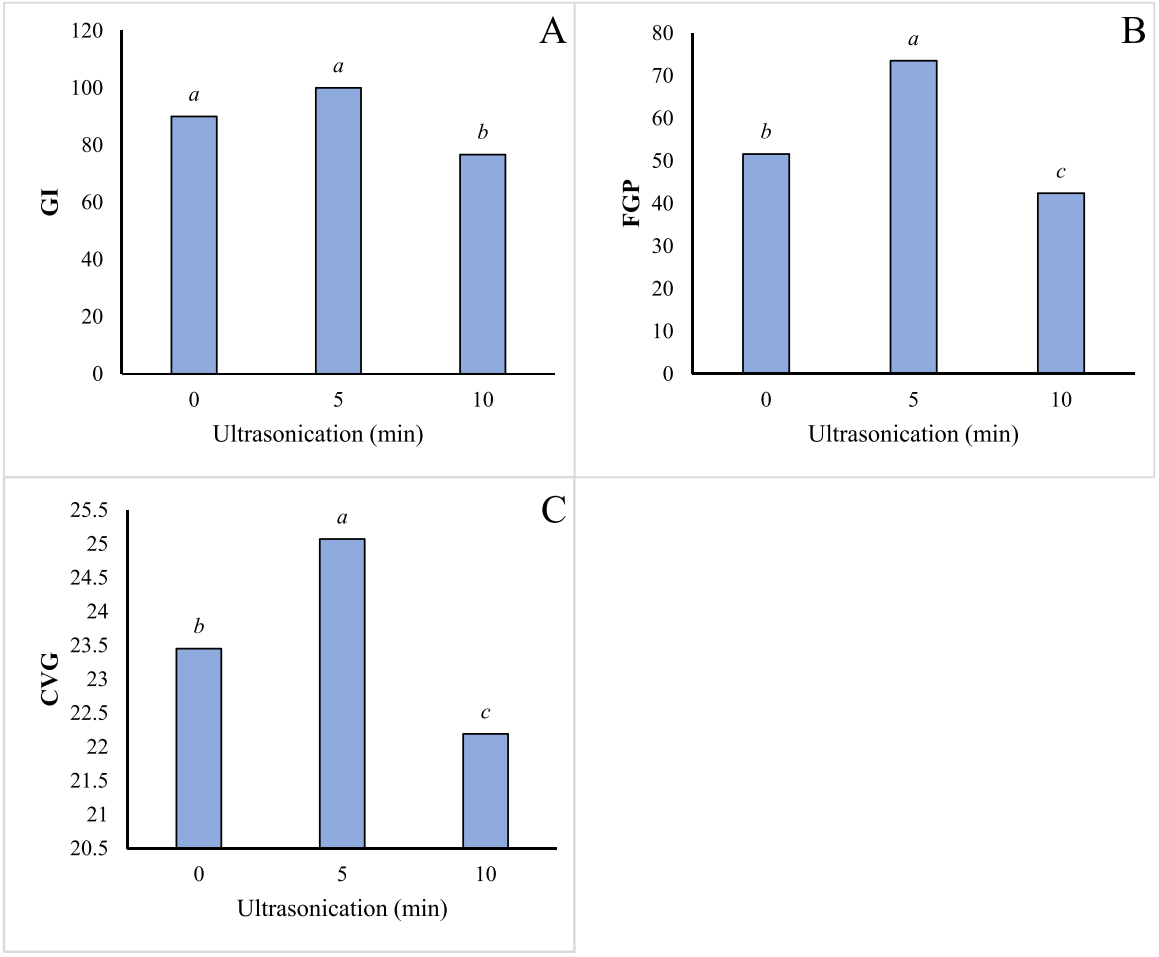

Fig. 1. Effect of ultrasound exposure time on the fenugreek seed germination. A) Germination rate index, B) Final germination percentage and C) Coefficient of the velocity of germination. Values are means of four replicates. Based on Duncan's multiple range test (MRT), the means with the same letter(s) are not significantly different ($P \leq 0.05$).

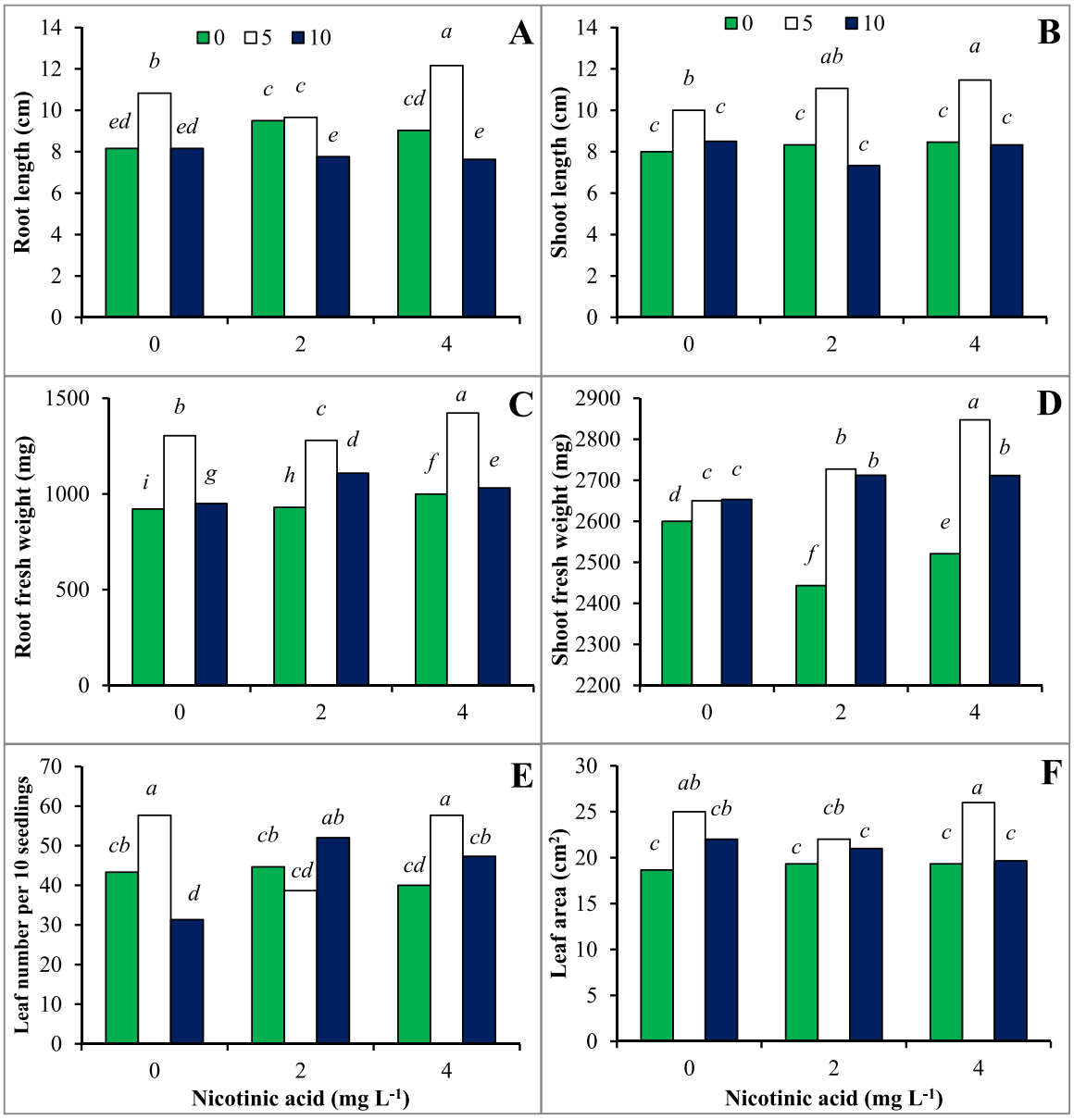

Fig. 2. Effect of ultrasound exposure time and NA on vegetative growth parameters of fenugreek seedlings at 30 days after seed germination. A) Root length, B) Shoot length, C) Root fresh weight, D) Shoot fresh weight, E) Leaf number, and F) Leaf area. Values are means of four replicates. Based on Duncan's multiple range test (MRT), the means with the same letter(s) are not significantly different ($P \leq 0.05$).

titanium chloride (20%) in concentrated HCl. Ammonia was added to the solution and centrifuged at 5000 rpm for 30 min, and the ultimate precipitate was dissolved in 2 N sulfuric acid. Supernatant adsorption at 415 nm was quantified with a spectrophotometer·H_2O_2 generation was estimated based on a standard curve drawn from different levels of H_2O_2.

Total protein content was estimated by Bradford's (1976) assay through bovine serum albumin (BSA) as standard. For this purpose, leaf samples were incubated in 80% acetone and the pellets were dissolved in 1 N NaOH for 24 h. Next, the supernatant was boiled at 90 °C for 30 min and then centrifuged. The supernatant absorbance was spectrophotometrically measured at 595 nm. Then, using the standard curve of BSA, the protein concentration of the samples was quantified.

Phenylalanine ammonia-lyase (PAL) activity was spectrophotometrically revealed by producing trans-cinnamic acid from L-phenylalanine at a wavelength of 270 nm by Jung and Choi (2020) method. Incubated samples were mixed with 6 mL extraction buffer [50 mM Tris Hydrochloride (Tris HCl) buffer, beta-mercaptoethanol, 10 μM leupeptin, 5.0 mM EDTA, 1.0 mM PMSF, 5.0 mM ascorbic acid, 0.15% (w/v) PVP, and pH 8.8]. Centrifugation was performed at 12000 rpm for 25 min at 4 °C. The volume of the reaction mixture was [3.6 mM Sodium Chloride, 16 mL L-phenylalanine, 50 mM Tris-HCL buffer (pH 8.8), and 0.5 mL of enzyme solution], which was reached to 3 mL with distilled water, was incubated for 2 h at 37 °C. It was then mixed with 500 μl of 6.0 M HCl. Finally, PAL activity was spectrophotometrically measured.

2.7. Trigonelline determination

The amount of trigonelline alkaloid was evaluated by UV-spectrophotometry. For this purpose, 1 gr of plant sample was combined with 1 gr of MgO (magnesium oxide) and 20 mL of deionized water and placed in a bain-marie at 100 ± 2 °C for 18 min. After cooling the sample at room temperature, it was filtered through Whatman paper and its volume reached 25 mL with deionized water. Then it was centrifuged at 1200 rpm. The supernatant absorbance was read at 268 nm and the trigonelline content of the samples was quantified using a

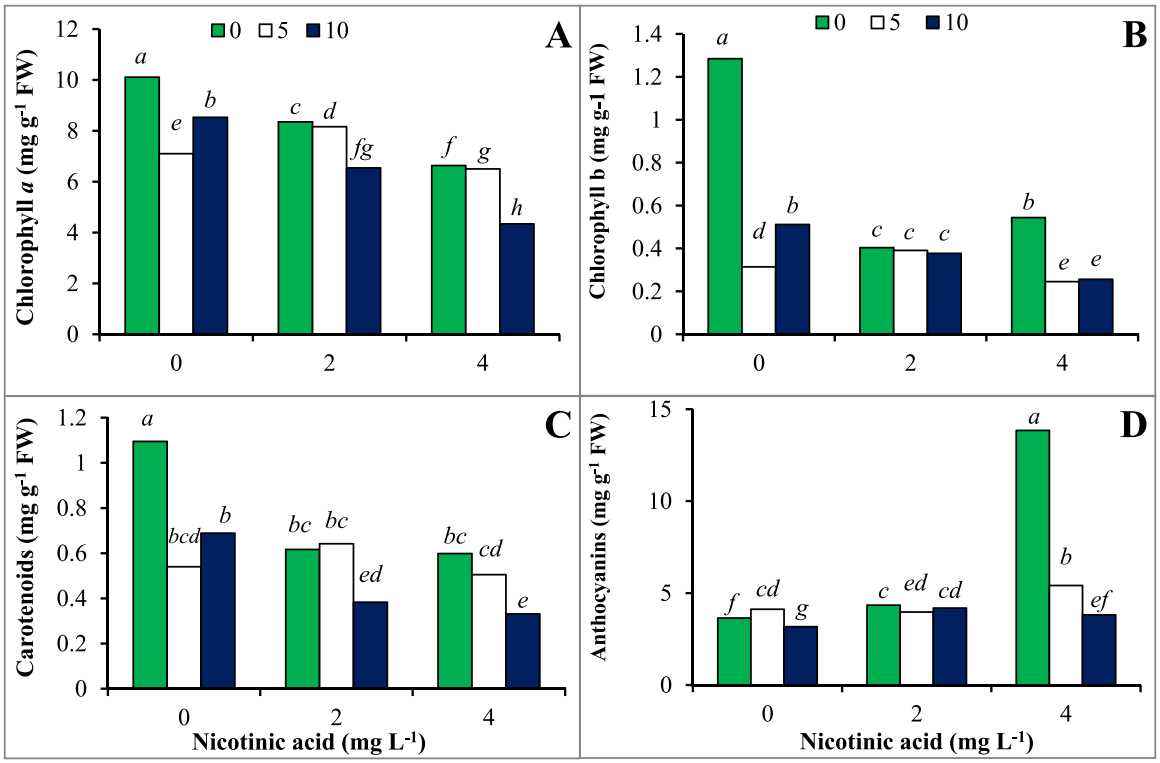

Fig. 3. Effect of ultrasound exposure time and NA on pigments content of fenugreek seedlings at 30 days after seed germination. A) Chlorophyll *a*, B) Chlorophyll *b*, C) Carotenoids and D) Anthocyanins content. Values are means of four replicates. Based on Duncan's multiple range test (MRT), the means with the same letter(s) are not significantly different ($P \leq 0.05$).

standard curve of trigonelline and stated in mg 100 g^{-1} FW (Badi, 2018).

2.8. Statistical analysis

The experimental design was factorial based on completely randomized design (CRD) with factors; ultrasonication and nicotinic acid fertigation and four replications in two years. Two way ANOVA and Duncan's multiple range tests were performed using SAS statistical software version 9.3. The Excel (ver. 2013) was also used to draw the graphs.

3. Results

3.1. Germination Parameters

The variance analysis of the effect of ultrasound and Nicotinic acid levels revealed high significant differences for most of the studied traits (Table 1). The influence of ultrasound on seed germination-related indices is shown in Fig. 1. Germination index (GI) was higher under 5 min ultrasound of fenugreek seeds, although there was no statistically difference with control treatment (the non-primed ultrasound and NA samples). The 10 min ultrasound priming negatively affected and reduced the germination index (Fig. 1 A). ultrasound also affected FGP. The highest FGP was observed under 5 min ultrasonicated seeds. Increasing the priming time to 10 min reduced the FGP (Fig. 1 B). The germination rate coefficient was higher than the control under 5 min sonication, and 10 min ultrasound priming reduced this coefficient to less than the control levels (Fig. 1 C).

3.2. Plant growth measurements

Growth analyses were performed 30 days after seed germination. As shown in Fig. 2, the root length was longer under 5 min sonication and untreated NA samples. The maximum root length was found under 5 min sonication and 4 mg L^{-1} NA feeding. The lowest root length was noted at all NA levels under 10 min ultrasound priming. Shoot length was also affected by the interaction of both factors. The maximum shoot length was recorded under 5 min ultrasound priming and 4 mg L^{-1} NA feeding. As shown in Fig. 2B, the minimum root length was observed under 10 min ultrasound priming and no priming, respectively, in combination with different levels of NA feeding. The highest fresh weight of roots and shoots was obtained under 5 min seed sonication and 4 mg L^{-1} NA feeding. The lowest root length was observed under the control plants. On the other hand, the minimum root length was found under 2 mg L^{-1} NA feeding and unprimed samples. The number of leaves was also evaluated in this study. The highest leaf number was found under 5 min ultrasonication and 0 mg L^{-1} NA, as well as 5 min ultrasonication and 4 mg L^{-1} NA. Under 10 min ultrasound and 0 mg L-1 NA, the number of leaves was less compared with other treatments. Leaf area (LA) was higher under 5 min the ultrasound and 4 mg L-1 NA followed by 5 min ultrasound and 0 mg L^{-1} NA, compared with the other treatments.

3.3. Photosynthetic pigments content

Analysis of different pigments content was performed under ultrasonication and NA feeding. As Fig. 3 shows, the content of chlorophyll *a* in the control treatment was higher compared with the other treatments. Also, the content of chlorophyll *b* and carotenoids under control treatment was higher than other treatments. The lowest content of chlorophyll *a* and carotenoids was recorded under 10 min sonication and 4 mg L^{-1} NA. Also, the lowest chlorophyll *b* content was observed under 5 and 10 min ultrasound and the addition of 4 mg L^{-1} NA to the nutrient solution. As presented in Fig. 3, the interaction of both factors significantly decreased the content of chlorophyll *a*, *b* and carotenoids compared with the control treatment. Besides, total anthocyanin was higher under 4 mg L^{-1} NA and non- ultrasound primed samples, followed by 4 mg L^{-1} NA and 5 min ultrasound priming than other

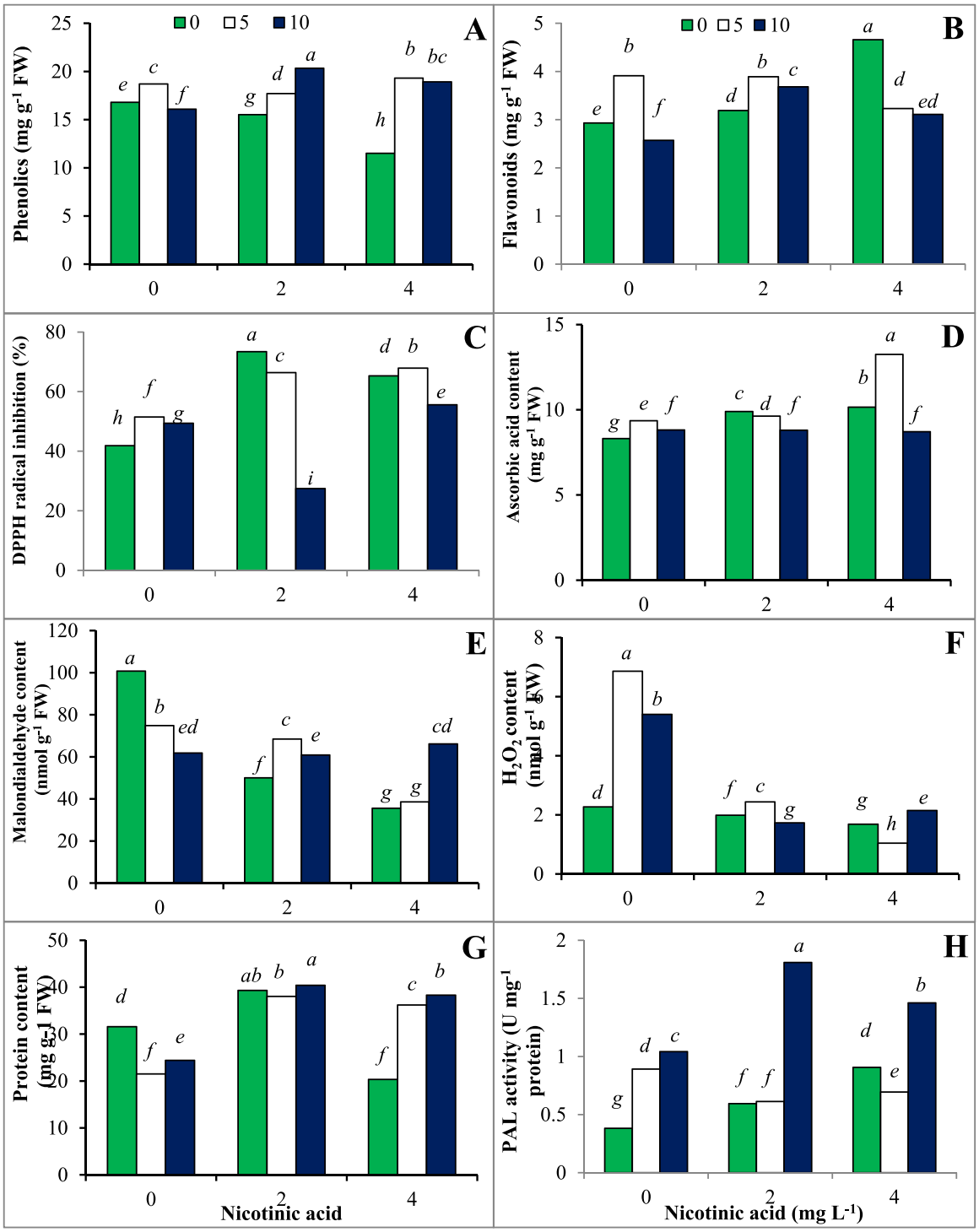

Fig. 4. Effect of ultrasound exposure time and NA on biochemical parameters of fenugreek seedlings at 30 days after seed germination. A) phenolics content, B) flavonoids content, C) DPPH radical inhibition, D) Ascorbic acid content, E) Malondialdehyde content, F) H_2O_2 content, G) Protein content, and H) PAL activity. Values are means of four replicates. Based on Duncan's multiple range test (MRT), the means with the same letter(s) are not significantly different ($P \leq 0.05$).

treatments. The lowest anthocyanin content was observed under control treatment.

3.4. Biochemicals content, antioxidant potential and PAL enzyme activity

The concentration of phenolic compounds in fenugreek leaves showed significant changes in response to the levels of both factors. It was found the treatment of 10 min ultrasound priming and 2 mg L^{-1} NA followed by 5 min ultrasound priming and 4 mg L^{-1} NA lead to the maximum amount of phenolics. The lowest content of phenolic compounds was recorded in non- ultrasound primed seedlings with 4 mg L^{-1} NA (Fig. 4 A). Maximum of flavonoid compounds were noted in non-ultrasound primed seedlings and 4 mg L^{-1} NA. The lowest content of flavonoid compounds was obtained under the interaction of 10 min ultrasound and 0 mg L^{-1} NA (Fig. 4 B). Completion of Hoagland's solution with NA showed an increase in antioxidant capacity (percentage of radical inhibition of DPPH) compared with the untreated solution so that treatment of 2 mg L^{-1} NA and non- ultrasound primed samples

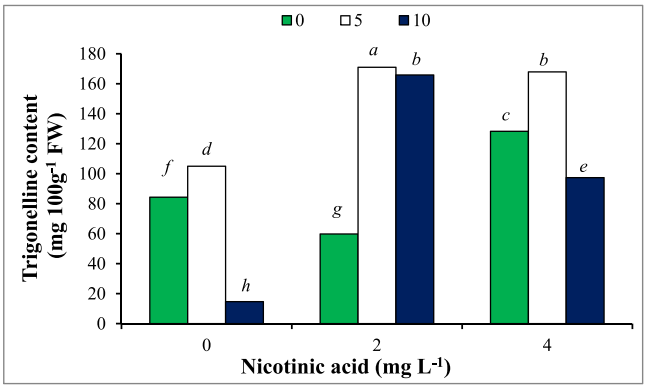

Fig. 5. Effect of ultrasound exposure time and NA on trigonelline content of fenugreek at 30 days after seed germination. Values are means of four replicates. Based on Duncan's multiple range test (MRT), the means with the same letter(s) are not significantly different ($P \leq 0.05$).

resulted in the highest antioxidant capacity. The lowest percentage of DPPH radical inhibition was recorded under treatment of 2 mg L^{-1} NA and 10 min ultrasonication followed by control treatment (Fig. 4 C). Ascorbic acid content was also affected by the interaction of both factors. AA levels increased under treatment of 4 mg L^{-1} NA and 5-min ultrasound priming. The lowest AA content occurred under 10 min ultrasound regardless of NA application levels (Fig. 4 D). MDA content was highest under control treatment. Increasing levels of NA nutrition decreased MDA content (Fig. 4 E). Besides, H_2O_2 accumulation was highest in treated samples with 5 and 10 min ultrasound and non-application of NA, respectively. In general, H_2O_2 accumulation was significantly lower with increasing NA levels (Fig. 4 F). In this study, the total protein was also investigated. The total protein was higher under 2 mg L^{-1} NA, regardless of the time of sonication than other treatment levels. In general, the lowest value of protein was obtained with non-NA treatments (Fig. 4 G). PAL enzyme activity was also evaluated as a vital enzyme in the biosynthesis pathway of antioxidant defense compounds. The activity of this enzyme was higher under 10 min ultrasound priming than other sonication times. The maximum activity of this enzyme was recorded under 10 min sonication and 2 mg L^{-1} NA. The lowest PAL activity was observed under the control treatment (Fig. 4 H).

3.5. Trigonelline accumulation

The amount of trigonelline in fenugreek tissue, which is very important in the human diet due to its health properties, was also analyzed. As Fig. 5 shows, increased trigonelline content was observed under 5 min ultrasound priming and supplementing Hoagland's nutrient solution with 2 mg L^{-1} NA. The lowest trigonelline yield was found under 10 min sonication and non-NA application. In general, supplementing Hoagland's solution with 2 mg L^{-1} NA and 5 min ultrasound priming is suggested to increase trigonelline yield for fenugreek cultivation under hydroponic conditions.

3.6. The correlation between treatments and traits

The correlation between ultrasound exposure time and Chl.a, Chl.b, carotenoide, antocyanin, and antioxidant potential were significant and negative, while the correlation between ultrasound exposure time and phenol was significant and positive at $p < 0.01$. The correlation between NA and Chl.a, Chl.b, carotenoids, MDA and H_2O_2 contents were significant and negative, while the correlation between NA and antocyanin and vitamin C were significant and positive at $p < 0.01$ (Table 2).

4. Discussion

Ultrasound with physical, chemical and thermal effects on living things can be a simple, fast and low cost method for plant seeds priming. The ultrasound effect in stimulating the germination of a number of legume family crops including mung bean (*Vigna radiata*) (Kumara and Gautama, 2019), pea (*Pisum sativum*) (Chiu and Sung, 2014), soybean (*Glycine max*) (Yang et al., 2013), adzuki bean (*Vigna angularis*) (Chiu, 2021) has been revealed. An increase in the germination of fenugreek seeds was also shown in this study under the effect of ultrasound priming. One of the reasons for the improvement of germination due to ultrasound priming is the microstructural changes in the seed testa and subsequent increase in water absorption and enhances α-amylase activity (Chiu, 2021).

Root and shoot length, fresh weight of roots and shoots, number of leaves, and leaf area in total under 5 min ultrasound -priming of seeds and 4 mg L^{-1} NA were higher than other treatments. Improving germination-related indices can be the reason for improving plant growth due to the application of 5 min ultrasound priming. In the study of Ma et al. (2018), the best level of gibberellic acid (GA_3) in terms of improved germination resulted in improved subsequent growth and development (fresh and dry weight, and plant length) in the false wheatgrass (*Leymus chinensis*) plant. In a forgoing study, Farooq et al. (2010) obtained the maximum roots length, roots number, and fresh and dry mass in rice seedlings derived from primed seeds. It seems to be a correlation between improved germination and subsequent growth and development. Our finding is in line with previous studies.

It was found improvement of the fresh and dry weight of onion foliage due to foliar application of NA under salinity stress (Hussein et al., 2014). NA is involved in many physiological pathways in all plant species and is a component of several enzymes involved in main biochemical pathways such as protein biosynthesis and/or IAA oxidase (Noctor et al., 2016), amylase, and proteinase (Hussein et al., 2014). In the present study, most of the growth traits including root and shoot length and fresh and dry weight treated with 4 mg L^{-1} were higher compared with the control treatment. However, there are results that show the inhibitory effect of NA on growth and division of cells which is inconsistent with our results.

The content of chlorophyll *a*, *b* and carotenoids considerably decreased and anthocyanin increased under the influence of the interaction of both factors. However, the decreasing effect of both factors on the content of chlorophyll *b* and carotenoids was greater than chlorophyll *a*. The effect of ultrasound on plant pigments has been less evaluated. Sonication with the sound wave is a form of non-living stress for plants (Wang et al., 2006; Da Silva and Dobránszki, 2014). Decreased contents of chlorophyll *a*, *b*, and carotenoids have been described in the

Table 2
Correlation coefficient (r) among measured traits in fenugreek.

Treatment	Chl.a	Chl.b	Carotenoid	Phenolics	Flavonoid	Anthocyanin	Antioxidant potential
US	-.514**	-.493**	-.558**	.625**	-0.322	-.464*	-.489**
NA	-.714**	-.483*	-.547**	-0.101	0.360	.529**	.387*
Treatment	Vi	MDA	H_2O_2	Protein	PAL	Trigonelline	
US	-0.198	0.018	0.248	0.213	.773**	-0.107	
NA	.549**	-.705**	-.717**	0.309	0.237	0.333	

** and * denote significant differences at 0.01, 0.05 levels, respectively.

legume family under salinity stress (Taïbi et al., 2016). Low-frequency ultrasound has thermal and chemical effects on living organisms (Da Silva and Dobránszki, 2014), therefore, it seems that part of the stress caused by ultrasound is due to the effects of heat shock. Hassanein et al. (2012) showed that heat shock reduces the chlorophyll *a* and *b* content in fenugreek. Vitamins have been shown to regulate plants metabolism and induce their activities (Bronzetti et al., 2001).

Decreased carotenoid content and improved chlorophyll content have been observed due to foliar application of nicotinamide under drought stress (Abdelhamid et al., 2013). It was revealed that nicotinamide improved the plant pigment concentration in faba bean (*Vicia faba* L.), which is inconsistent with our results (Dawood et al., 2019). Despite the role of NA (vitamin B_3) in various physiological processes, few studies have examined its effect.

According to forgoing studies, the effect of ultrasound on the stimulation of the antioxidant system is characterized by the increased amount of MDA (Safari et al., 2013), PAL activity (Wang et al., 2006), increased phenolic compounds (Yu et al., 2016b), higher generation of H_2O_2 and increased secondary metabolism (Rezaei et al., 2011). In this experiment, phenolic compounds, ascorbic acid, and PAL enzyme activity levels were increased after the priming of seeds by ultrasound. These results are supported by previous results. It seems that the seeds priming with ultrasound lead to establishing a form of stress memory that improved performance of the antioxidant system after ultrasound stress condition. Lukić et al. (2020) stated that the induction of the antioxidant mechanisms caused by drought stress may continue for several weeks.

The results showed that PAL enzyme activity, the main enzyme for adjusting the entrance of phenylalanine to the production of phenolics, and a branch point enzyme between the primary and the secondary metabolism (Dixon and Paiva, 1995), stimulated by ultrasound and NA feeding in seedlings. In this study, increasing the stimulation of secondary metabolites such as phenolics and decreasing primary metabolites such as photosynthetic pigments are also consistent with this finding. This may show that eliciting seeds by ultrasound downregulated primary metabolism in favor of secondary metabolism.

It was shown that foliar spray of lemongrass with nicotinamide considerably increases the protein content (Tarraf et al., 1999). Improving the amount of protein was thought to be due to the increase in organic nitrogen storage. Such a result was also obtained in this study. In addition, NA affected the antioxidant status of cell cultures of garden pea (*Pisum sativum*), carrot (*Daucus carota*), European aspen (*Populus tremula* L.), hybrid aspen (*Populus tremuloides*), and Madagascar periwinkle (*Catharanthus roseus*) (Berglund et al., 2017). Their result showed that NA defend plant cells from oxidative stress. The interaction of ultrasound and NA caused a substantial increase in trigonelline content in fenugreek seedling tissue. Badi et al. (2018) reported increased levels of trigonelline and NA under the arginine amino acid treatment. The feeding experiment showed the trigonelline biosynthesis from some of its precursors such as quinolinic acid, nicotinamide and NA in cotyledons and embryonic axes of mungbean seedlings (Zheng et al., 2005). Although NA is a precursor to the valuable metabolite trigonelline, few studies *in vitro* and *ex vitro* have evaluated its role in trigonelline biosynthesis. However, various stresses such as drought have been shown to increase trigonelline content (Irankhah et al., 2020). It seems that the increase in stress level induced by various factors such as ultrasound can enhance trigonelline content.

5. Conclusion

The present study showed that 5 min exposure to mild ultrasound can improve the germination of fenugreek seeds and the interaction between 4 mg L^{-1} NA and 5 min ultrasound increased subsequent growth of seedlings. The use of NA and ultrasound reduced the photosynthetic content of pigments and promoted anthocyanin and antioxidant responses. An increase in antioxidants was associated with a decrease in lipid peroxidation and potentially damaging H_2O_2 radicals. Considering the trigonelline content, the best treatment was 5 min ultrasound and supplementation of Hoagland's solution with 2 mg L^{-1} NA. The effect of seed ultrasound -priming on biochemical traits and trigonelline content one month after sonication may be stress memory-related, although more studies are needed at the molecular level. In general, it can be concluded that 5 min ultrasound priming of fenugreek seeds improved germination-related traits compared with the control treatment. Then, 5 min ultrasound priming of fenugreek seeds is recommended to induce germination. Overall, it can be concluded that 5 min ultrasound seed priming and supplementing Hoagland's nutrient solution with 2 mg L^{-1} NA can improve growth indices, and therefore to achieve optimal biomass yield is recommended in fenugreek cultivation.

Credit authorship contribution statement

Raheleh Najafi: Methodology, Investigation, Writing, Formal analysis. **Ayatollah Rezaei:** Conceptualization, Supervision, Methodology, Writing. **Daryush Talei:** Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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