Changes of Trigonelline, Nicotinic Acid and Proline Content in Early Growth Stages of *Trigonella foenum-graecum* L. under Saline Condition

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Abstract

Background: Some health-protecting secondary metabolites can be found at a higher concentration in the sprouts compared to other stages of plant growth. In addition, environmental stresses such as salt stress may be resulting in the increase or decrease of specific secondary metabolites in sprouts and plants.

Objective: Evaluation the changes of trigonelline, nicotinic acid, and proline content of fenugreek sprouts under saline and non-saline condition in the different harvesting times.

Methods: This experiment was conducted on the base of factorial experiment in completely randomized design with three replications. The treatments were included three levels of salinity (0, 75 and 150 mM of NaCl), and three harvesting times of sprouted fenugreek (4, 8 and 12 days after beginning the experiment).

Results: In each level of salinity, the highest trigonelline content of sprouts was obtained on the fourth day, and its content was reduced on the eighth and twelfth days. The nicotinic acid amount under moderate (75 mM of NaCl), and severe (150 mM of NaCl) salinity was gradually reduced from the fourth up to twelfth days. Thus, the highest amount of nicotinic acid was observed on the fourth day, and its lowest was obtained on the twelfth day. The proline content of sprouted fenugreek was increased from the fourth up to twelfth day at different levels of salinity, so that the highest proline content of sprouts was observed on the twelfth day especially under 150 mM salinity.

Conclusion: In general, the trigonelline and nicotinic acid content of sprouted fenugreek was reduced from the fourth up to twelfth days, and the highest content of them was observed under 150 mM salinity on the fourth day.

Keywords: ***Trigonella foenum-graecum*, Nicotinic acid, Proline, Sprout, Salinity, Trigonelline**

Introduction

Secondary metabolites are synthesized from primary metabolites (e.g., carbohydrates, lipids and amino acids) in higher plants. They are necessary for protection of plant against herbivores, pathogens and environmental stresses [1]. Plant secondary metabolites are unique sources as food additives, flavors, pharmaceuticals, and industrially important pharmaceuticals [2]. Also, secondary metabolites, are important in plant stress physiology for adaptation [1, 2]. The production of these compounds is often low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant [3]. Accumulation of metabolites often occurs in plants subjected to stresses, including drought, salinity, UV radiation, etc. [4, 5, 6].

Salinity stress involves changes in various physiological and metabolic processes, depending on the severity and duration of the stress [7, 8]. Salinity stress is considered as hyperosmotic stress [9]. Osmotic stress in the initial stage of salinity stress causes various physiological changes, such as cellular dehydration via remove the cytoplasmic water that result is the reduction of the cytosol and vacuole volumes, interruption of membranes, nutrient imbalance, impairs the ability to scavenge reactive oxygen species (ROS), differences in the antioxidant enzymes and decreased photosynthetic activity [9, 10]. Salinity stress is also considered as a hyperionic stress. Entry Na^+ and Cl^- into the cells is caused impair the ion homeostasis and excess uptake might cause significant physiological disorders [7]. The generation of ROS such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radical is elevated in response to salinity stress. ROS formation can lead to oxidative damages in various cellular components such as proteins, lipids, and DNA, interrupting vital cellular functions of plants [11, 12, 13].

Salt stress resulting in the accumulation or decrease of specific secondary metabolites in plants [1, 14]. It was reported that anthocyanin content increased in response to salinity [15]. In contrast, salinity reduced anthocyanin content in the salt-sensitive species [16]. Also, it has been found a significant correlation between salt tolerance and proline accumulation in a number of plants [17, 18]. Total glucosinolate and total phenolic contents of 3, 5, and 7 days old broccoli and radish sprouts treated with low concentrations of NaCl were significantly decreased. However, their content was significantly increased in high concentrations of NaCl. Therefore has been suggested that the nutritional and medicinal features of sprouts could be improved under adequate salt stress [19, 20].

Fenugreek (*Trigonella foenum-graecum* L.), is an annual herbaceous legume crop belonging to the family Fabacecae which is cultivated in parts of Europe, Africa, West and South Asia, North and South America and Australia. The leaves and seeds of fenugreek for medicinal uses (such as anti-diabetic, lowering the sugar and cholesterol level of blood, anticancer, antimicrobial, etc.), food making, and etc. are consumed. Fenugreek seed contains carbohydrates (45 - 60%), proteins (20 - 30%), fixed oils (5 - 10%), pyridine alkaloids, mainly trigonelline (0.2 -

0.38%), saponins (0.6 - 1.7%), glycosides yielding steroidal sapogenins on hydrolysis (diosgenin, yamogenin, tigogenin, neotigogenin), nicotinic acid and, volatile oils (0.015%) [21, 22].

Sprouts are forming from germinated seeds during seedling growth. The sprouts are consumed at the beginning of the growing phase. It has been widely reported that sprouts provide higher nutritive value than raw seeds and their production is simple and inexpensive. Recent research shows that sprouts have important phytochemicals that prevented of disease and the most useful in the respect of the human health. Although a dried seed is characterized by a remarkably low metabolic rate, just sprouting of seed can trigger tremendous and complex changes. These changes consist of three main types that included the breakdown of complex fats into free fatty acids, polysaccharides conversion into simple sugars as oligo and mono-saccharides and breakdown of protein into amino acids and oligopeptides. In addition, vitamins are increased, and essential minerals are supplied in organic form "chelated" for better assimilation and nutrient density is enhanced at the expense of calories. Sprouting also removes some anti-nutrients such as enzyme inhibitors in the seed that make sprout safe for the diet. Therefore, germination can be considered as one kind of predigestion that helps to break down the high-molecular complex materials into their building blocks [23 - 28].

The nutritional value of the sprouts was discovered by the Chinese thousands of years ago. Recently, in the USA, numerous scientific studies suggest that sprouts have an important role in a diet and health. Each sprout may contain as many phytochemicals as an entire plant. Although some health-protecting phytochemicals can be found in the sprout in a higher concentration than in the developed plant [29, 30]. Sprouting in fenugreek is known to improve its essential nutrient contents and reduce the phytic, tannic and trypsin inhibitors [30, 31, 32]. This study was aimed to evaluate the change of trigonelline, nicotinic acid and proline content under saline and non-saline condition in a different harvesting time of fenugreek sprout.

Materials and Methods

This study was conducted in the Eco-physiology laboratory of the Medicinal Plants Institute (MPI), ACECR as a factorial experiment in the completely randomized design (CRD) with 3 replications in 2014. The treatments were included three levels of salinity (0, 75 and 150 mM of NaCl), and three harvesting times of sprouts (4, 8 and 12 days after beginning the experiment by the induction of saline solution).

Fenugreek (*Trigonella foenum-graecum*) seeds with the 985-MPI-SB code were procured from the seed bank of the medicinal plants institute, ACECR. The surface of intact seeds was sterilized with 3% sodium hypochlorite solution for 5 min with ratio 1:5 ($g.ml^{-1}$), and then the seeds thoroughly rinsed with distilled water for 10 min. For seed germination and sprout formation, fifty healthy seeds were put in each sterilized glass petri dish over Whatman filter paper (No. 1) and filter paper was moistened with 15 ml of

distilled water (control) and various concentrations of NaCl. The seeds were incubated under the same conditions in the germination device with a 16 h light and 8 h dark periods and 70 % humidity at 25 °C [33]. The sprouted fenugreek samples were harvested at 4, 8 and 12 days after beginning the experiment. Then each sample was divided into two parts. A part of the sample was frozen in liquid nitrogen and stored in freezer -80 °C till measurement of proline content. Another part of the sample was dried in an oven during 48 h at 70 °C for determination of trigonelline and nicotinic acid content.

Trigonelline and nicotinic acid content

Approximately 1 g powder of dried fenugreek sprouts was weighted, and then with 1 g of magnesium oxide (MgO) and 20 ml distilled water was mixed. The mixture was incubated in a water bath at 100 °C for 20 min. After cooling the mixture was filtered through Whatman paper (No. 4) and its volume was brought to 25 ml with distilled water. Absorbance of the solutions was measured in UV-vis spectrophotometer apparatus at 268 and 264 nm for trigonelline and nicotinic acid, respectively. The trigonelline and nicotinic acid concentration of the sample was determined using a standard curve and was expressed as milligram per gram dry weight [34, 35].

Proline content

Approximately 0.5 g of the frozen fresh plant material was homogenized in 10 ml of 3% aqueous sulfo-salicylic acid by mortar and pestle in ice. The homogenate was centrifuged for 10 min at 15000 rpm and the supernatant was used for proline content measurement. 2 ml of the supernatant was reacted with 2 ml ninhydrin reagent and 2 ml of glacial acetic acid in a test tube for 1 hour in water bath at 100 °C, and the reaction was terminated by placing test tubes in ice bath. In the following 4 ml toluene was added to the reaction mixture and mixed vigorously with a test tube stirrer for 15-20 Sec. Then the reaction mixture was incubated at room temperature for up to two-phase mixtures is formed. The upper phase that consists toluene and proline was separated from the aqueous phase and the absorbance read at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve (0, 4, 8, 12, 16 and 20 $mg.L^{-1}$) and was expressed as micromole proline per gram fresh weight [36].

Statistical analysis

All the data were subjected to variance analysis using SAS software. In cases where interaction effects were significant ($P \leq 0.05$), The SLICE option of the LSMEANS was used to test the simple effects of sprout harvesting time within each level of salinity. However, if slicing of interaction effects were significant ($P \leq 0.05$), the difference between means were compared by Duncan's Multiple Range Test at 5% confidence interval.

Results

Results of variance analysis for the simple and interaction effects of salinity and harvesting time of sprout is shown in Table 1. Different concentrations of NaCl and

harvesting times of sprouts had significant effect ($P \leq 0.01$) on trigonelline, nicotinic acid and proline content. Also, interaction effect of salinity and harvesting time of sprouts was significant ($P \leq 0.01$) on trigonelline, nicotinic acid and proline content (Table 1).

Slice interaction for different harvesting time within each level of salinity was showed that the trigonelline content of sprouts on the fourth, eighth and twelfth days was significantly different ($P \leq 0.05$) under non-saline condition. However, the content of nicotinic acid and proline had no significant difference between various harvesting times of sprouts. The effect of moderate salinity (75 mM of NaCl) on trigonelline and nicotinic acid content in the various harvesting time was significant ($P \leq 0.01$). But, this level of salinity hadn't significant effect on proline content of fenugreek sprouts at the different harvesting time. Also, trigonelline, nicotinic acid and proline content of fenugreek sprouts in the various harvesting time were significantly affected by severe salinity stress (150 mM of NaCl) (Table 1).

The results of means comparison were shown that in each level of salinity include 0, 75 and 150 mM of NaCl, the highest content of trigonelline was obtained in sprouts harvested on the fourth day. In comparison to the fourth day, the trigonelline content of sprouts on the eighth and twelfth days was reduced in each level of salinity. However, the trigonelline content of sprouts on the eighth and twelfth days had not significant difference (Table 2).

Nicotinic acid content of sprouts at the fourth, eighth and twelfth days had not significant difference under non-saline condition. Also, the nicotinic acid content was significantly reduced under moderate salinity (75 mM of NaCl) interval the fourth to twelfth day. Thus, the maximum amount of nicotinic acid was observed in sprouts on the fourth day (0.148 $mg.g^{-1}$), and the lowest of it was obtained in sprouts on the twelfth day (0.099 $mg.g^{-1}$) which had not significant difference with the eighth day (0.113 $mg.g^{-1}$). Under severe salinity stress (150 mM of NaCl), nicotinic acid content of fenugreek sprouts was significantly reduced in the fourth and twelfth days. Therefore, the highest and lowest amount of nicotinic acid was observed in sprouts on the fourth (0.277 $mg.g^{-1}$) and twelfth (0.078 $mg.g^{-1}$) days, respectively (Table 2).

Proline content of fenugreek sprouts in each level of salinity was increased from the fourth day up to twelfth day. Thus, the lowest content of proline under 0, 75 and 150 mM salinity (0.26, 1.45, 3.13 mg.g FW^{-1}, respectively) was obtained in sprouts on the fourth day. Also the highest content of proline under 0, 75 and 150 mM salinity (0.69, 1.97, 5.98 mg.g FW^{-1}, respectively) was observed in sprouts on the twelfth day. Proline concentration in sprouts on the fourth days was 2.36 times less than sprouts on the eighth day under non-saline condition. Under 150 mM salinity, the proline content in sprouts on the fourth days was 63 % less than sprouts on the eighth day (Table 2).

Table 1- Analysis of variance (ANOVA) for simple and interaction effects of salinity and harvesting time on trigonelline, nicotinic acid and proline content of fenugreek sprouts

S.O.V	D.f	Mean squares (MS)		
		Trigonelline	Nicotinic acid	Proline
Salinity (S)	2	0.001 **	24.38 **	4.76 **
Harvesting time (H)	2	0.01 **	61.46 **	0.38 **
Salinity (S) × Harvesting time (H)	4	0.0013 **	9.8 **	0.049 **
Error	18	0.00016	1.76	0.0067
CV %		6.93	14.17	5.89
Interaction slices for different harvesting time within each level of salinity				
0 mM (Control)	2	0.98 *	0.00046 ns	0.15 ns
75 mM	2	1.88 **	0.0019 **	0.202 ns
150 mM	2	41.35 **	0.0327 **	6.39 **

ns, *, ** indicated non-significant and significant at the probability level of the 5 and 1 %

Table 2- Means comparison of different harvesting times within each level of salinity

	Trigonelline (mg.g DW^{-1})			Nicotinic acid (mg.g DW^{-1})			Proline (µM.g FW^{-1})		
Salinity (mM) / Harvesting time (day)	0	75	150	0	75	150	0	75	150
4	5.78^{a}	6.59^{a}	11.27 ^{a}	0.1 ^{a}	0.148 ^{a}	0.277 ^{a}	0.26 ^{b}	1.45 ^{a}	3.13 ^{c}
8	5.11^{ab}	5.46 ^{b}	5.19 ^{b}	0.093 ^{a}	0.113 ^{b}	0.124 ^{b}	0.615 ^{a}	1.79 ^{a}	5.11 ^{b}
12	4.65^{b}	5.06 ^{b}	4.57 ^{b}	0.076 ^{a}	0.099 ^{b}	0.078 ^{c}	0.69 ^{a}	1.97 ^{a}	5.98 ^{a}

Means with the same letters in each column indicate no significant difference between treatments at the 5% level of probability

Discussion

The result showed that the trigonelline content of sprouted fenugreek was reduced from the fourth day up to the twelfth day under non-saline condition. In contrast, proline content of sprouts was increased from the fourth day up to the twelfth day. Moreover, the growth of fenugreek sprouts had no significant effect on nicotinic acid content under non-saline condition. These results correspond with the findings of other authors [6, 19, 20]. Hussain *et al*., (2012) reported the amount of total sugar, reducing sugar and non-reducing sugar in fenugreek sprout were increased up to the fifth day and then were reduced. Also, the amount of iron, magnesium and calcium were increased up to the eighth day after beginning sprout growth [31]. Randhir *et al*. (2004), showed the total phenolic content of fenugreek sprout was increased up in the 3 days after seed germination and then was reduced [23].

Some of the compounds during seed germination and sprout growth to be changed, because it may the complex compounds be decomposed to simpler forms or be converted into other compounds which are essential [37]. However, it has been reported that the trigonelline is stored form for the nicotinic acid. Therefore, the trigonelline content during seed germination decreased via conversion to nicotinic acid and the nicotinic acid is consumed for sprout growth and development

[38]. Simultaneous with water absorption in seeds, the storage protein in cotyledons via proteases activity hydrolyzed to free amino acids for energy production. In the following, free amino acids transferred from cotyledons to the newly emerged shoots and roots for sprout growth [39]. Therefore, the content of free amino acids in the early stages of seed germination and sprout growth increased and then reduced gradually.

In this study, the trigonelline and nicotinic acid content of sprouts grown in 75 mM of NaCl were reduced from the 4 days up to the 12 days. While, the content of proline was not affected significantly by moderate salinity (75 mM of NaCl). Also under severe salinity (150 mM of NaCl), the trigonelline and nicotinic acid content of sprouts were decreased from the 4 days up to the 12 days. But the proline content of fenugreek sprouts between this interval times increased. Amount of free amino acid in many of the plants under saline condition increased. Proline content more than other amino acids accumulated in plants impacted by salt stress. Proline under osmotic stress in plant cells serves as an osmolite and thus plant could be coped with the effects of water shortage. Increasing proline content due to the reduced water potential of the plant can help to maintain the turgor pressure and cell growth. Many studies have been shown that the role of proline, more than linked to maintaining the plant growth is related to its survival. Moreover, the other functions have been proposed for proline include antioxidant for scavenging of free radicals, protection of macromolecule as proteins, reducing the pH inside the cell and carbon and nitrogen storage for over stress period [40, 41].

It had been observed that NaCl exerted a positive influence on the content of alkaloid in vinca (*Catharanthus roseus*), *Ricinus communis* and soybean (*Glycine max*) [42, 43]. In the present study, the content of trigonelline in the fourth day of sprouts growth affected by salinity. While, the trigonelline content in the sprouts eighth and twelfth day had not considerable changes under saline conditions.

Conclusion

Trend changes of trigonelline and nicotinic acid content of the fenugreek sprouts was identical at different harvesting times under non-saline and different levels of salinity condition. Although, the trigonelline and nicotinic acid content of the fenugreek sprouts was significantly decreased from the fourth up to the twelfth day, but in contrast of it, proline content was increased at different salinity levels. However, the highest content of trigonelline and nicotinic was observed under 150 mM salinity on the fourth day.

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