

EFFECT OF ASCORBIC, HUMIC AND NICOTINIC ACIDS ON CERTAIN BIOCHEMICAL CONSTITUENTS IN FENUGREEK (*Trigonella foenum-graecum* L.)

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ABSTRACT

Two field experiments were conducted to evaluate the effects of ascorbic (AsA), humic (HA) and nicotinic (NA) acid on certain biochemical constituents that influence seed quality of Fenugreek (*Trigonella foenum graecum*, L.). Results indicated that presoaking or presoaking plus spraying in by AsA, HA and NA increased flavonoids, alkaloids, trigonelline, protein, total carbohydrates and oil percentage in seeds. With all tested metabolic inducers (MI), the enhancing effect was more pronounced at its higher adopted level and when it was applied as a combined presoaking plus a foliar spray treatment. Total alkaloid percentage was highest in response to HA at 3000 mg/l whereas trigonelline percentage recorded highest value in response to AsA at 200 mg/l. On the other hand, total phenols percentage was generally decreased with exogenous application of all MI, and the decreased was more evident at the higher level of either AsA, HA or NA. Based on the obtained results, it could be concluded that metabolism of Fenugreek (*Trigonella foenum graecum*, L.) could be modulated toward the accumulation of beneficial components by exogenous application of AsA, HA, and NA.

INTRODUCTION

Fenugreek (*Trigonella foenum graecum*, L.) is known as one of the oldest medicinal plants recognized in recorded history (Acharya *et al.*, 2008). Fenugreek leaves and seeds are consumed in different countries around all the world for different purposes, the most prominent of which are its medicinal uses. Medicinally, it is used as anti-diabetic, lowering blood sugar and cholesterol levels, anticancer, and antimicrobial (Mehrafarin *et al.*, 2011). Fenugreek above-ground organs contain several bioactive Compounds including flavonoids, phenols and alkaloids, the most important of which is trigonelline (Snehloti and Payal, 2012). Trigonelline have several physiological effect the most notably of which is its hypoglycemic effect (Neveen *et al.*, 2007; Abd_Elmawla and Osman, 2011). Secondary metabolites biosynthesis and accumulation could be enhanced through application of plant growth regulators (Danesh-Talab *et al.*, 2014) or certain elicitors (Abd_Elmawla and Osman, 2011). Benzylaminopurine (Ortuno *et al.*, 1998), 2-1-naphthyl acetic acid (Alagukannan and Vijaykumar, 1999), and both GA3 and NAA applied at various concentrations (Danesh-Talab *et al.*, 2014) have been reported to have enhancing effects on certain Fenugreek seeds metabolites. However little is known about the effects of ascorbic and humic acids on the biosynthesis and accumulation of secondary metabolites in Fenugreek. Elicitation and precursor feeding are two common approaches for enhancing secondary metabolites biosynthesis and

accumulation. *Methyl Jasmonate* (MJ) was used as an elicitor for trigonelline induction in Fenugreek 's cell suspension cultures (Abd_Elmawla and Osman, 2011). They reported a 2-fold increase in trigonelline percentage in MJ-elicited cultures. In addition, precursor feeding with Nicotinic acid (NA) was found to enhance trigonelline content in callus cultures of *Moringa Oleifera* (Mathur and Kamal,2012.).On the other hand, the effectiveness of precursor feeding with NA *in vivo* is less investigated and poorly understood. Therefore the present investigation was conducted to assess the effects of *in vivo* application of ascorbic, humic and nicotinic acids on key secondary metabolites as well as major seed constituents of Fenugreek.

MATERIALS AND METHODS

Two field experiments were established during the two growth season 2012 /2013 and 2013 / 2014. in the soaking treatment, seeds ware soaked in solution of AsA and NA either at 100 or 200 mg/L as well as in HA either at 1500 or 3000 mg / L for 5 h. Seeds soaked in distilled water for the same period represent the control treatment. Soaked seeds were inoculated with *Rhizobium sp* before sowing at 15 cm apart in 2x3 m plots. In the combined soaking plus spraying treatment, plants were also received two foliar applications with the same metabolic inducers (MIs) and at the same level used in soaking. The first foliar spray was 30 days after sowing (DAS) whereas the second was 45 DAS. Except for treatment with either MI, all other tillage practices were followed as recommended by The Ministry of Agriculture Egypt. The experiment was laid out in a randomized complete block design. At the end of the growing season, seeds samples were collected to determine certain biochemical constituents. Table (1) shows Physical and chemical analyses of the soil used in the experiment.

Table (1): Physical and chemical analyses of the experimental soils

PH 1:1	EC at 25 °C	Anion and Cation meq/100 g soil									Size distribution %				Texture
		Co ₃ ⁼	So ₄ ⁼	HCO ₃ ⁻	Cl ⁻	Ca ⁺⁺	Mg ⁺⁺	K ⁺	Na ⁺	Sand		Silt	Clay		
										Fine	Coarse				
8.33	1.10	--	0.789	0.28	0.54	0.40	0.65	.007	.40	34.45	1.85	13.6	53	Clay loam	

Determination of total carbohydrate:

A 5 g seed sample was extracted by ethanol 80 % and kept overnight at room temperature. The ethanolic extract was used for the determination of total carbohydrates using anthrone method. The developed green color was measured spectrophotometrically at 630 nm according to Sadasivam and Manickam (1996).

Determination of total alkaloids:

Total alkaloids was determined using the method that described by Aziz et al.(2005) by soxhlet , ten gram of the powdered sample seeds was extracted with 250 mL of ethanol period five hours, extracted of ethanol was evaporated to dryness with a rotary evaporator, under reduced pressure at 40c .dry residue repeat by 150 mL of chloroform and acidify by HCL15% pH3, it let pillow during 30 minutes in the room temperature, the phase acid aqueous were extracted by 150 mL of chloroform, basify by the NaHCO₃ 5% pH 9 and lit it during 15 minutes in the room temperature. The chloroform phase was evaporated to dryness with a rotary evaporator under reduced pressure. The dry residue is the total alkaloid.

Determination of total phenols:

The powdered seeds (2 g) was extracted with methanol, at room temperature overnight. The methanol extracts were combined and concentrated under reduced pressure on a rotary evaporator. Total phenolic percentage of each plants extract was determined with the FolinCiocalteus reagent (FCR) according to Slinkard and Singleton, (1977). Each sample (0.5ml) was mixed with 2.5 ml FCR (diluted 1:10, v/v) followed by 2 ml of Na₂CO₃ (7.5%,v.v) solution. The absorbance was then measured at 765 nm after incubation at 30 C for 90 minute .Results were expressed as Gallic acid equivalent (mg Gallic acid / g dried extract).

Determination of flavonoid:

The total flavonoid percentage of seed extract was determined by a colorimetric method as described in by (Zhissen et al., 1999). Each sample (0.5ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a NaNO₂ solution (15%). After 6 minutes, 0.15 ml of aluminum chloride (AlCl₃) solution (10%) was added and allowed to stand for 6 minute, then 2 ml of NaOH solution (4%) was added to the mixture was thoroughly mixed and allowed to stand for another 15 minutes. Absorbance of the mixture was then determined at 510 nm versus prepared water blank.

Determination of protein:

Percentage of Protein was calculated by multiplying the percentage of total nitrogen by the factor of 6.25 (A.O.A.C.,1980).

$$\% \text{Protein} = \% \text{Nitrogen} \times 6.25$$

It could be mention that chemical determination were made only in the first season.

Determination of Trigonellin:

By Seed percentage of the active ingredient were taken 100 grams of seeds of each experimental unit and underwent grinding and sieving operations since taking 80 grams of seeds crushed in order to remove fat and processed for the purpose of the extraction process for vehicles. After Alkaloids remove fat from seeds according to the method (Wanger *et al.*, 1984) Then it was separated and purified compounds to take 40 grams of residue seeds were extracted defatted Alkaloids and purified according to the method (Tugrul and Ozer, 1985).

Determination of Fixed oil :

At harvesting time anthocyanin percentage was determined in air – dried Fenugreek according to the method described by Du and Francis(1973).The percentage of fixed oil in seeds was determined according to the method mentioned by A.O.A.C (1980).

Statistical analysis:

The obtained data were subject to statistic of variance according to Gomez and Gomez(1984).The treatment means were compared using the least significant difference (LSD).

RESULTS AND DISCUSSION

Effect of AsA:

Ascorbic acid applied at 200 mg /l increased significantly all studied seed biochemical characters whereas decreased total phenols (Fig 4). At 100 mg/l, AsA increased total flavonoids (Figure 3), trigonelline (Fig 1), total carbohydrates and oil percentages (Table 2) whereas did not significantly affect other studied characters. At both AsA levels, its application as a combined presoaking plus foliar spray treatment was superior to its application as a presoaking treatment only. Highest alkaloids and trigonelline percentages were obtained in case of the interaction between the combined application methods with AsA at 200 mg/l.

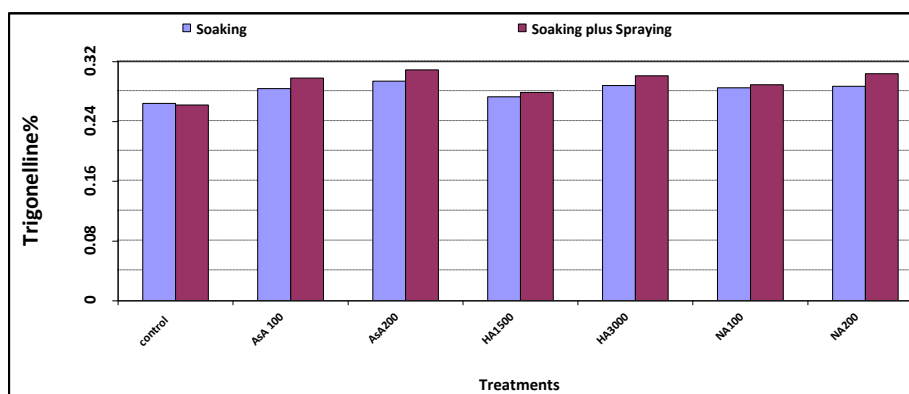


Figure (1) effects of exogenous application with ascorbic (AsA), humic (HA) and nicotinic (NA) acids on Trigonelline percentage in Fenugreek seeds.

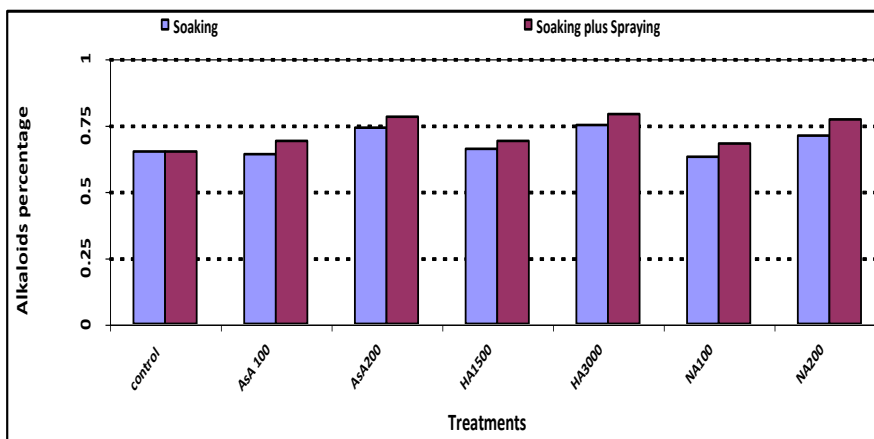


Figure (2) effects of exogenous application with ascorbic (AsA), humic (HA) and nicotinic (NA) acids on alkaloids percentage in Fenugreek seeds.

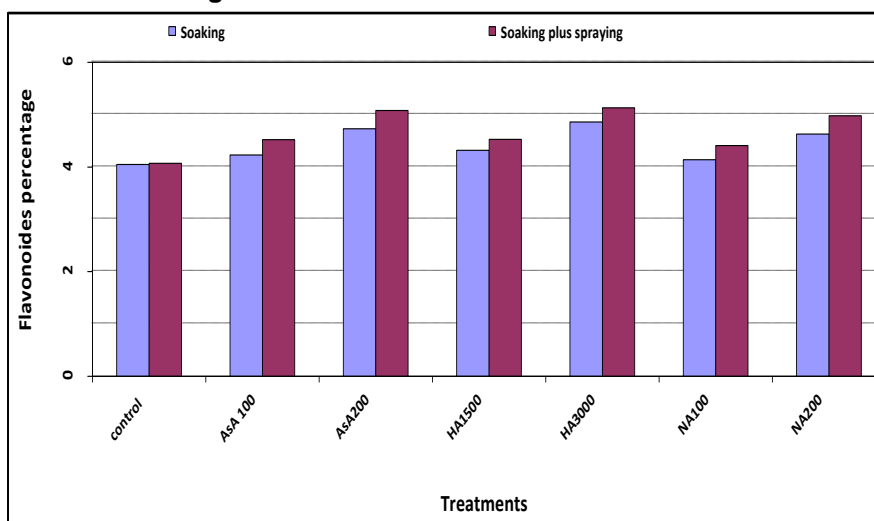


Figure (3) effects of exogenous application with ascorbic (AsA), humic (HA) and nicotinic (NA) acids on flavonoides percentage in Fenugreek seeds.

AsA is involved in a wide array of important functions in plant growth and metabolism. It has a fundamental role as defense antioxidant as well as essential roles in photoprotection, regulation of photosynthesis and growth (El-Lethy *et al.*, 2011). As primary and secondary plant metabolism are closely interconnected, it may be concluded that AsA-induced Flavonoids, alkaloids and trigonelline percentages May be as a consequence to its stimulating effects on plant primary metabolism /metabolites (Table 2). This conclusion is substantiated with the results of previous investigations in which

AsA exogenous application was reported to enhance photosynthetic activity and various plant primary metabolites in *Lycopersicon Esculentum* (El-sayed and Elsayed, 2013). In addition, AsA application was found to increase total phenols and total flavonoids in two different genotype of maize (Salama et al., 2013), as well as various secondary metabolites in *Pelargonium graveolens* (EL-Lethy et al., 2011) and *Jasminum grandiflorum* (Eid et al., 2010) .

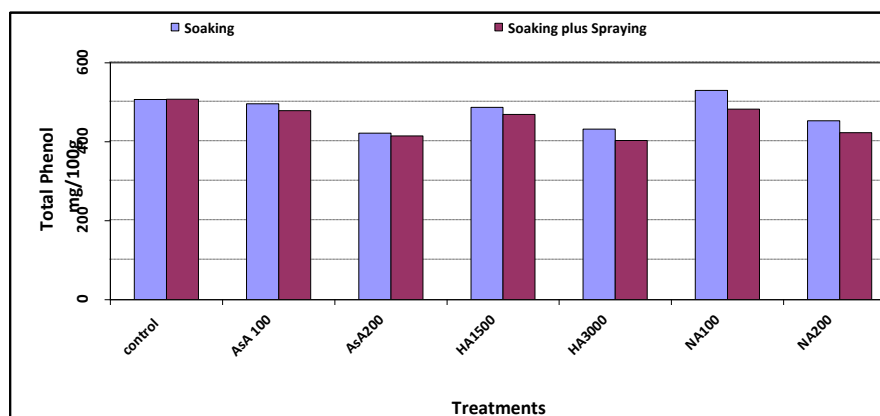


Figure (4) effects of exogenous application with ascorbic (AsA), humic (HA) and nicotinic (NA) acid on Total phenol in Fenugreek seeds.

Effects of HA:

HA applied at 3000 mg/l increased all recorded parameters. On the other hand, total phenols percentage was decreased (figure 4). At its lower adopted level (1500 mg/l), HA increased total flavonoids, trigonelline, total carbohydrates and oil percentage (Table 2). Whereas others parameters were not significantly affected. Higher increment levels were recorded when HA was applied as presoaking in conjunction with a foliar spray treatment. When considering the interaction between HA levels and application methods, the interaction between HA at 3000mg/L and the combined method of application was of superior effect. (Figures 1-4, Table 2)

HA was previously reported to both directly and indirectly affect plant's biochemical processes (Yong et al,2004; Rady,2012). HA was reported to positively influence net photosynthesis along with enhancing chlorophyll percentage and stomatal conductance (Tahira et al,2013). In addition, the activity of two key photosynthetic enzymes, phosphoenolpyruvate carboxylase and ribulose-1,5- bis phosphate carboxylase was enhanced in response to HA treatment (Zhang et al.,2014). Total sugars, total free amino acids and protein concentrations were increased in snap bean treated with HA (Hanafy Ahmed et al., 2010). In their study, total phenols percentage was also increased in response to HA, in contrary to the results of the present investigation. This could be explained by lower HA concentration used in their study, being 2000 mg/l. Inhibition of urease activity by humic acid was reported by Vaughan and Ord, (1991) which may reduce N loss by volatilization, thereby increase N availability to plants which enhance plant

biochemical processes in which N is involved. Trevisan *et al.* (2010) concluded a HA- dependent activation of plant biochemical processes and enzymes synthesis as well as auxin-like activities of HA which resulted in increased root mass, photochemical efficiency and antioxidant levels. In addition, Zhang and Ervin (2004) found that exogenous application of HA increased endogenous cytokinin and auxin levels, which may improve crop productivity .

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Effect of Nicotinic acid (NA):

NA at both levels, increased trigonelline , total carbohydrates and oil percentage .On the other hand, total flavonoids, total alkaloids and protein percentage were increased only when NA was applied at 200 mg/l (Figures 1-4, Table 2). Total phenols percentage was decreased in response to the higher level, whereas it was not affected in response to the lower level NA. Application of NA as a combined presoaking plus spraying treatment was more effective in inducing secondary metabolites accumulation in fenugreek seeds .Nevertheless, more decrease in total phenols percentage was recorded due to the combined treatment (Figure 4). NA and NAD are chemically related (Ali, 2002), so similar effects of both on plant growth and metabolism is envisaged. Both NA and NAD increased ricinine alkaloids and oil percentage in *Ricinus communis* plants grown under salinity stress (Ali,2002). He concluded that adequate supply of NAD through NA

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application is essential for normal plant growth and development. This may explain the positive effects of NA exogenous application on the recorded biochemical constituents. Trigonelline serves as storage form of NA (Blaim and Wanner, 1960; c.f. Mathur and Kamal, 2012). So, trigonelline accumulation due to NA application recorded in the present investigation may be explained on the ground that Nicotinic acid acted as a precursor in the biosynthesis of trigonelline in Fenugreek. This conclusion is supported by the result of previous studies in which trigonelline formation was increased in NA-supplemented tissue cultures of *Trigonella foenum-graecum* (Khanna and Jain, 1972), *Allium cepa* (Khanna et al., 1989) and *Moringa olifera* (Mathur and Kamal, 2012).

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تأثير حمض الاسكوريك والهيوميك والنيكوتينك على محتوى بعض الصفات الكيميائية لنبات الحلبه.

زين العابدين عبد الحميد محمد ، مصطفى فؤاد البنا و أمير صادق عباس محمد شباره
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تم اجراء تجربتين حقليتين بهدف تقييم تأثير المعامله بحمض الاسكوريك والهيوميك و النيكوتينك علي بعض الصفات الكيميائيه التي تؤثر علي جودة بذور الحلبه واوضحت النتائج أن كل من حمض الاسكوريك والهيوميك والنيكوتينك ادى الى زياده نسبه كل من الفلافونيدات و القلويدات والتريجونيللين والبروتين و الكربوهيدرات ومحتوي الزيت في البذور. وقد اشارت النتائج ان اعلى النسب ظهرت عندما تمت المعامله بالنقع اولاً ثم الرش. حيث سجلت اعلى قيمه للقلويدات عند تطبيق الهيوميك بتركيز 3 جم/لتر بينما كان محتوى التريجونيللين اعلى قيمه له عند استعمال الاسكوريك بتركيز 200 ملجم/لتر ومن ناحيه اخري كان هناك نقص ملحوظ في محتوى الفينول عند كل المعاملات . وطبقا للنتائج المتحصل عليها، يمكن الإستنتاج بان التمثيل الغذائي لنبات الحلبه يمكن تحسينه تجاه تراكم بعض المكونات المفيده في البذور وذلك من خلال تطبيق بعض معاملات النقع والرش لكل من حمض الاسكوريك والهيوميك والنيكوتينك.