Annals of Agric. Sci., Moshtohor **ISSN 1110-0419**
Vol. 56(2) (2018), 285 – 292 **http://aasj.bu.edu.eg/index.php**

Chemical Evaluation of Some Stevia (*Stevia rebaudiana*, Bertoni) Varieties of under Different Nitrogen Fertilization Levels

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Abstract

Two field experiments were carried out at agricultural research center, Giza, Egypt during 2015/2016 and 2016/2017 seasons to study the effect of nitrogen fertilizer levels (20, 30 and 40 kg N/ fed/cut.) on some stevia varieties (china1, Egy1, spanti and shou2). The highest nitrogen rate of 40 kg. N /fed /cut was significant in all studied yield andChemical characters and gave the highest values of stevioside%. recorded (6.09 and 6.62%), ,rebaudioside A% (7.23 and 8.04%), Stevioside kg /fed (238.9 and 286.1 kg) and rebaudioside A kg /fed (287.2 and 350.5 kg)), respectively as compared with the other two nitrogen treatments in both seasons.

Stevia Varieties were significantly different in all studied yield characters. china 1 Varieties gave the highest values of stevioside% (6.83 and 7.34 %), rebaudioside A% (8.44 and 9.26 %), rebaudioside A kg /fed(300.8 and 363.2 kg), respectively of all studied yield and chemical characters except for dry leaves yield kg/fed where Egy1 varieties gave the highest values in the first and second seasons.

.Concerning the effect of the interaction between nitrogen fertilizer levels and stevia varieties on yield and chemical characters, nitrogen fertilizer level of 40 kg N/ fed/cut applied to china1 varieties gave the highest values in all studied yield and chemical composition except for dry leaves yield (kg/fed) where Egy1 varieties and nitrogen fertilizer level of 40 kg N/ fed/cut gave the highest values in the first and second seasons.

Key words: stevia, nitrogen fertilizer**, varieties**, stevioside, rebaudioside -A **.**

Introduction

Stevia is a new crop in Egypt. It was approved in 1995 for use as a food ingredient by Food Standard for Egypt. This plant is believed to be the most ideal substitute for sugar. Steviol Glycoside (SG) found in the leaves is responsible for the sweetness of stevia. As a new crop to for Egypt, commercial cultivation of stevia is yet to commence.

. Leaves of *Stevia rebaudiana* contains diterpene glycosides which taste sweet but with zero calories and 100–300 times sweeter than sugar (sucrose) **(Megeji *et al* 2005** and **Soejarto *et al* 1983**).

This plant is believed to be the most ideal substitute for sugar and important to assist in medicinal value. Given its many health benefits, recent GRAS acceptance and existing evidence in support of stevia's safety for human consumption.namely stevioside, rebaudioside A, B, C, D and E (**Geuns 2003).** The plant is known to exhibit a wide range of biological activities like hypoglycemic, anti-oxidant, anticancer, antibacterial activities. Stevia contains a high percentage of phenols, flavonoids and antioxidant activity (**Negar *et al* 2012**) . *Stevia rebaudiana*, Bertoni leaves are a natural source of diterpenic glycosides, and various bioactive compounds. characterize antioxidants **(Danijela *et al* 2018).** Stevia now is used as sweeteners to replace sugar in foods, beverages and medicines, stable at wide range of temperature and pH conditions during processing and offer no shelf life limitations,(**Balwinder *et al* 2014).** They are thermostable even at temperatures of up to 200 !C, making them suitable for use in cooked foods.(**Roberto *et al* 2012).**

Materials and methods

The present work was designed to study the respons of (yield and chemical properties of some stevia (*Stevia rebaudiana*, Bertani) varieties under different nitrogen fertilization levels. Two experiments were carried out at Agricultural Research Center , Giza, Egypt during 2015/2016 and 2016/2017 seasons.

Treatments: **Two factors were studied as follow:**

Factor A: nitrogen fertilizer levels (N) : N1= 20 kg N fed/cut, N2:=30 kg N fed/cut and N3= 40 kg N fed/cut.

Factor B: Varieties (v): V1: china 1, V2 : Egy 1, V3 : spanti, V4 :shou 2.

Nitrogen fertilizer was applied in the form of urea (46.5% N) in two equal rates, the first rates was applied after 15 days of planting (or after cutting in the following cuts) and second rates was applied after 15 days from the first rates.

Field Experimental Design:

The experimental design used was a split plot design with three replicates, Fertilizer levels were arranged in the main plots and cultivars in the sub plots. The sub plot area was 10.8 m2 (6 ridges) 3.0 meters in the length and 60 cm in width

The treatments (12) were the combinations of three nitrogen levels and four varieties

Table (A) Physical and Chemical analysis of experimental soils in 2015/2016and 2016/2017 seasons.

Analysis	Seasons	
	2015/2016	2016/2017
Mechanical analysis		
Coasre sand %	0.20	0.50
Fine sand %	12.16	14.2
Silt %	48.85	45.7
Clay %	38.99	36.5
Textual class	Cilt clay loam	Cilt clay loam
Chemical analysis		
Organic matter %	1.08	1.32
CaCo3 %	1.10	2.0
PH(1:2.5)**	8.10	8.0
Soluble Ca++ (meq /100 g soil)	11.2	16.2
Soluble Mg++ (meq /100 g soil)	10.7	14.7
Soluble Na + (meq /100 g soil)	8.3	11.7
Soluble K + (meq /100 g soil)	4.1	2.6
Soluble Co3 --(meq /100 g soil)	--	--
Soluble Hco 3--(meq /100 g soil)	3.5	5.6
Soluble Cl--(meq /100 g soil)	13.3	17.9
Soluble So4--(meq /100 g soil)	17.3	31.7
Available N(ppm)	11.00%	13.9%
Available P(ppm)	9.12%	11%
Available K(ppm)	35.86%	34.1%

Studied characters

-Dry leaves yield kg/fed/ year
- Stevioside%-
- Rebaudioside A% -
- Stevioside yield (kg / fed / year) -
- Rebaudioside Ayield (kg / fed / year)

Statistical analysis:

All collected data were statistically analyzed according to technique of analysis of variance for split- plot design by " GENSTAT Version 12th 2009 " computer software package (VSN International, Hemel Hempstead, UK) to determine The differences among treatment means were compared by L.S.D. test at $P \leq 0.05$ **Payne, *et al* (2009).** Combined analysis for the two seasons of experimentation was done according to the homogeneity of experimental error variance (**Bartlett, 1937).**

Extraction and estimation of Stevia sweeteners in relation to total

Sweeteners

. Preparation of leaves for extraction

Stevia leaves were dried in an electric oven (E. Schulz & Co. Inh. Franz. Skorezewsh KG) at 50°C.

2. Stevia sweeteners

Stevia sweeteners were obtained from Stevia International Company for Agra industrial Projects (SKAP)

Stevioside standard preparation

Stevioside standard preparation was carried out according to **Nishiyama *et al*. (1992)** as follows: Dried Leaves (10 g leaves of *Stevia rebaudiana*, Bertoni obtained from Sigma) were extracted by soaking leaves in 1.0 liter of water nearly boiling (85°C) for 30 minutes. The resulting liquid fraction was separated by Buchner filtration and the residue was washed with an additional volume of hot water (50 ml). The aqueous solution was concentrated by lyophyilization (Edwards Model EF03, England) to be 50 ml and defatted by ethyl acetate, then extraction with isobutyl alcohol (150 ml) was carried out.The aqueous phase was discarded and the organic solution was evaporated by rotary evaporator (Type 349, James Jobling and Co. Ltd., England) at 70°C until drying was obtained. The dried extract was dissolved in hot methanol (100 ml) and kept over night to crystallize. The crystals were separated by filtration and re-dissolved again in boiling methanol (50 ml).This solution was clarified with active charcoal (B.D.H. Laboratory Chemicals Division. Poole, England) and left to recrystallize. The procedure was repeated three times until the formation of colorless crystals was observed. The pure solution of the stevioside prepared (pH of 9) was subjected to HPLC.At the same time an authentic pure stevioside was obtained from Nu Naturals Inc. U.S.A.

. Extraction of Stevia sweeteners from Stevia leaves with

methanol (MeoH)

Extraction of Stevia sweeteners from leaves were carried out by 0.5gm of dry stevia leaves was ground and dissolved in 0.5 ml methanol and put in shaking and heating for 30 minutes at 70°C then kept in room temperature for cooling then abukhner funnel was used for filtiration using Afilter paper one time after

that we used Activated charcoal for filtration another time finally we kept the filtrate frozen until analysis

Identification and quantitative analysis of Stevia sweeteners by HPLC in Stevia leaves extract

Stevia (*Stevia rebaudiana*Bertoni) leaves extract was separated and identified on HPLC as follows: Stevioside and other sweet components standard as prepared above were filtered through a millipore membrane (13 mm. diameter, 0.5 µm pore size) were subjected for determination by chromatography with stevioside standard as internal standard. Different extracts of Stevia leaves were injected for chromatography Acetonitrile with HPLC grade (Fisons Co. England) was used in this study as mobile phase.HPLC separation was carried out on 210nm (Agilent 1200PDA detector); Eclipse plus C18 column (3.5µm 4.6x250 mm); linear gradient over 20 min (84:55% CH3CN in H2O/ 0.1% TFA); flow rate 2.0 mL/min. Injection volume: 70 µl at ambient temperature (25°C). All the conditions used were according to **Makapugay *et al.*, (1984).** For each sample identification, quantification and the retention time were as described by **Makapugay *et al.* (1984).** Area under each peak was used to calculate the percent of each compound**.**

Results and Discussion

Effect of nitrogen fertilizer levels

Results in Table (1) indicate that dry leaves yield (kg/fed),.Stevioside yield (kg/ fed / year) and rebaudioside A yield (kg / fed / year) were significantly affected by increasing nitrogen fertilizer levels in the first and second seasons. The highest nitrogen rate of 40 kg N /fed/ cut gave the highest values as compared with the other nitrogen treatments of all studied characters in both seasons. The highest values of dry leaves yield were (3640 and 4025kg), respectively, in 2015/2016 and 2016/2017seasons. These results are in agreement with **Attia (2005)**, **Lee *et al* . (1980), Chalapahti *et al.* (1999)** and **Ângelo *et al* (2017).** Plants fertilized with 40 N kg / fed / cut had the highest values of stevioside%. recorded (6.09and 6.62%) respectively, in 2015/2016 and2016/2017seasons. Similar results were obtained by **Attia (2005)**. Adding 40 kg. N/fed/cut increased the values of rebaudioside A% (7.23 and 8.04%) in 2015/2016 and 2016/2017season,respectively. Similar results were obtained by **Geeta and Midmore (2017).** Nitrogen fertilizer levels of 40 kg .N/fed/ cut gave the highest values of Stevioside yield (kg /fed/ year) (238.9 and 286.1 kg) respectively, in the first and second seasons. Plants fertilized with 40 N kg / fed / cut had the highest values of rebaudioside A (kg /fed/year) (287.2 and 350.5 kg)) respectively, in the first and second seasons. On the other hand, the lowest nitrogen rate of 20 kg N/fed cut produced the lowest values of all studied characters in both seasons.

Discussion Nitrogen levels from 20to 30 and 40 kg .N/fed/ cut these increases may by attributed to the increase in content percentage Steviol Glycoside (SG), rebaudioside- A, in the leaves .These results are in agreement with those obtained by **Rodrigues *et al* (2017) , Attia (2005)**. **Geeta and Midmore (2017).**

Effect of cultivars

Data presented in Table (2) indicate clearly that stevia varieties were significantly different in dry leaves yield (kg/fed), Stevioside yield (kg / fed / year) and rebaudioside A yield (kg / fed / year) in the first and second seasons. China1 varieties gave the highest values of all studied characters except dry leaves yield kg/fed in the first and second seasons.

Egy1 Varieties gave the highest values dry leaves yield in the first and second seasons respectively. China1 varieties produced the highest values ofstevioside%, respectively in 2015/2016 and 2016/2017seasons. Similar results were obtained by **Asmaa Haraz (2016).** The highest values of rebaudioside A% were produced from china1 varieties in the first season .The current work is in agreement with **Asmaa Haraz (2016) and Anami *et al*., (2016).** China 1 varieties produced the highest values of stevioside kg /fed yield/ year, in 2015/2016 and 2016/2017season. Similar results were obtained by **Thiyagarajan and Venkatachalam (2015) and Anami *et al*., (2010)**. The highest values of rebaudioside A (kg /fed/ year) was produced from china1 varieties respectively in the first seasons. The current work is in agreement with **Asmaa Haraz (2016) .** Mean while, spanti varieties gave the lowest values of all studied characters in both seasons.

Discussion

Such variation might reflect the efficiency of plant building metabolites or might be ascribed to genetical differences. These results are in agreement with those found by Asmaa **Haraz(2016) and Thiyagarajan and Venkatachalam (2015)**

E**ffect of interaction between nitrogen fertilizer levels and stevia cultivars**

Data in Table (3) show that the interaction effect between nitrogen fertilizer levels and stevia varieties were significant for dry leaves yield kg/fed, Stevioside yield (kg / fed / year) and rebaudioside A yield (kg / fed / year) . Nitrogen fertilizer levels of 40 kg N/ fed/cut and China1 varieties gave the highest values of all studied characters except dry leaves yield in the first and second seasons. Nitrogen fertilizer level of 40 kg N/ fed/cut and egy1 varieties gave highest values of dry leaves yield kg/fed in 2015/2016 and 2016/2017season. Adding 40kg. N /fed / cut and China1 varieties recorded the highest values of stevioside% in 2015/2016 and 2016/2017seasons.The highest values of rebaudioside A% were obtained by treating China1 varieties with nitrogen rates of 40 kg N /fed / cut ,respectively in the first and second seasons.

Table 1. Effect of nitrogen fertilizer levels on yield and chemical composition of stevia in 2015/2016 and 2016/2017 seasons.

characters	**Dry leaves yield kg /fed/ year**		**Stevioside%**		**Rebaudioside A%**		**Stevioside kg /fed/ year**		**Rebaudioside A kg /fed/ year**	
	2015/2016	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**
N1	**2088**	**2651.**	**2.98**	**3.28**	**3.69**	**3.93**	**69.6**	**99.4**	**87.6**	**119.3**
N2	**2743**	**3088.**	**4.59**	**4.91**	**5.44**	**6.01**	**135.3**	**162.2**	**159.7**	**199.3**
N3	**3640**	**4025**	**6.09**	**6.62**	**7.23**	**8.04**	**238.9**	**286.1**	**287.2**	**350.5**
Mean	**2824**	**3255**	**4.55**	**4.93**	**5.45**	**5.99**	**148.0**	**182.6**	**178.2**	**223.0**
L s d 0.05	**262.2**	**253.8**	**___**	**___**	**___**	**___**	**22.03**	**9.57**	**28.15**	**11.44**

N1: 20 kg N fed/cut. , **N2:** 30 kg N fed/cut , **N3**: 40 kg N fed/cut.

Table 2. Effect of cultivars on yield and chemical composition of stevia in 2015/2016 and 2016/2017 seasons.

characters	**Dry leaves yield**		**Stevioside%**		**Rebaudioside A%**		**Stevioside kg /fed yield/ year**		**Rebaudioside A kg /fed yield/ year**	
	2015/2016	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**
Treatment										
V1	**3355**	**3717**	**6.83**	**7.34**	**8.44**	**9.26**	**242.7**	**286.1**	**300.8**	**363.2**
V2	**3894**	**4442**	**5.00**	**5.52**	**6.04**	**6.63**	**204.4**	**256.3**	**245.3**	**307.5**
V3	**1729**	**1889**	**2.07**	**2.32**	**2.22**	**2.61**	**39.7**	**50.2**	**41.7**	**56.0**
V4	**2316**	**2970.**	**4.31**	**4.56**	**5.12**	**5.47**	**105.0**	**137.8**	**124.9**	**165.5**
Mean	**2824.**	**3255.**	**4.55**	**4.93**	**5.45**	**5.99**	**148.0**	**182.6**	**178.2**	**223.0**
L s d 0.05	**340.8**	**294.1**	**___**	**___**	**___**	**___**	**20.25**	**13.74**	**24.85**	**16.59**

V1: China 1 **V2**: Egy 1 **V3**: **S**panti , **V4** : **S**hou 2

Table 3. The interaction effect of between nitrogen fertilizer levels and stevia cultivars on chemical composition of stevia in 2015/2016 and 2016/2017 seasons

Characters	**Dry leaves yield**		**Stevioside%**		**Rebaudioside A%**		**Stevioside kg /fed yield/ year**		**Rebaudioside A kg /fed yield/ year**	
Treatment	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**	**2015/2016**	**2016/2017**
N1V1	2222	2701	4.89	5.22	5.87	6.26	108.7	141.0	130.3	169.2
N1V2	3009	3693	3.79	4.32	5.15	5.18	114.0	159.6	154.9	191.5
N1V3	1346	1351	0.34	0.33	0.33	0.41	4.6	4.5	4.4	5.4
N1V4	1773.	2860	2.89	3.23	3.42	3.88	51.2	92.5	60.6	111.0
N2V1	3380	3735	7.06	7.61	8.93	9.63	238.7	284.1	301.8	359.6
N2V2	3848.	4155	4.28	4.66	4.52	5.59	164.8	193.7	174.0	232.4
N2V3	1443	1764.	2.76	2.95	3.26	3.54	98.0	52.0	47.1	62.4
N2V4	2302	2698	4.26	4.42	5.04	5.3	380.6	119.1	116.1	143.0
N3V1	4463	4716	8.53	9.18	10.54	11.89	380.6	433.1	470.2	560.8
N3V2	4824	5477	6.93	7.59	8.44	9.10	334.5	415.5	407.0	498.6
N3V3	2399	2552.	3.11	3.69	3.07	3.92	74.7	94.2	73.7	100.1
N3V4	2875.	3353.	5.77	6.02	6.88	7.23	165.8	201.8	197.9	242.5
mean	2824.	3255.	4.55	4.93	5.45	5.99	148.0	182.6	178.2	223.0
L s d 0.05	541.8	475.1	___	___	___	___	34.24	21.60	42.45	26.07

N1: 20 kg N fed/cut., **N2**: 30 kg N fed/cut , **N3**: 40 kg N fed/cut.
V1: China 1 **V2**: Egy 1 **V3**: **S**panti , **V4** :**S**hou 2

Nitrogen fertilizer level of 40 kg N/ fed/cut and china 1 varieties gave highest values of stevioside (kg /fed / year) in 2015/2016 and 2016/2017seasons, respectively. Adding 40kg. N /fed / cut to China1 varieties recorded the highest values of rebaudioside A (kg /fed/year) respectively in the first and second seasons.

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التقيم الكميايئ لبعض أصناف الاستيفيا تحت مستويات مختلفة من التسميد الازوتي

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اجريت الدراسة في محطة بحوث الجيزة مركز البحوث الزراعية موسمي 2015- 2016 و2016-2017 لدراسة تاثير التسميد النتروجينى (20 ، 30و40 كجم نتروجين للحشة)وبعض الاصناف
(egy 1، china 1، spantil و shou 2) وتفاعلهما علي الصفات تحت الدراسه في الموسمين.
* اعطت معدلات التسميد الازوتي اختلافات عاليه المعنويه لجميع الصفات تحت الدراسة عند مستوي معنويه 5 % و اعطي معدل تسميد40 كجم نتروجين/ فدان / حشة اعلي القيم لجميع الصفات محتوى الاوراق من المحليات مثل(الStevioside وrebaudioside-A) وكذالك محصول الفدان من المحليات مثل(ال Stevioside وrebaudioside-A) في السنه مقارنة بالمعاملات الاخري في الموسمين وكذلك المحصول الجاف الاوراق .
*أوضحت النتائج وجود فرق معنوى كبيرة بين جميع الاصناف عند مستوي معنويه 5 % وسجل china 1 أعلى تركيزمن الrebaudioside-A) و أعلى تركيزمن ال (Stevioside) وكذالك محصول الفدان من المحليات مثل(الStevioside وrebaudioside-A) في السنه في الموسمين مقارنه بالاصناف الاخري
* أوضحت النتائج أيضا ان التفاعل بين اضافة معدل40 كجم نتروجين/ فدان / حشة والصنف china 1 اعطي اعلي القيم لجميع الصفات ماعد كميه محصول الفدان من الاوراق الجافه حيث سجل التفاعل بين اضافة معدل 40 كجم نتروجين للحشة والصنف egy1 اعلي كمية محصول الفدان من الاوراق الجافه في الموسمين .