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# THE EFFECTS OF PLANTING DENSITY AND FERTILIZER TREATMENT ON THE GROWTH, DEVELOPMENT, YIELD AND SUGAR CONTENT OF *STEVIA REBAUDIANA* BERTONI.) IN NORTHER REGION, VIET NAM

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***Abstract***

*Three factor (N, P, K) and planting density are important major effect to yield and quality of leaf Stevia rebaudiana Bertoni plant after harvest via clustering of High Performance Liquid Chromatography (HPLC). In this reasech, the experiment was carried out to find the fertilizer formula and planting density suitable for growth and development Sweet grass plant in norther Viet Nam. The result indicated that fertilizer combination P2 treatment (300 kg Nitrogen (N) + 100 kg $P_2 0_5$ phosphorus (P2O5) + 240 kg Kalidioxid ($K_2 0$) and D2 planting density obtained the highest glycoside content in Stevia rebaudiana Bertoni. This formular also was recommended for production process of high quality medicinal in the future.*

***Keywords:*** *Stevia rebaudiana Bertoni; Glycoside and RA content; High Performance Liquid Chromatography (HPLC)*

## INTRODUCTION

Sweet grass (Stevia rebaudiana Bertoni) is a genus of about 240 species of herbs and shrubs of the Asteraceae family, also known other name as Sugar Grass, Honey Grass or Sweet Chrysanthemum, is native in the valley between Paraguay and Brazil (Madan S et al., 2010). The botanical characteristics plant and medicinal from left of Stevia rebaudiana Bertoni was described as Fig 1A, B, C.

In recent study indicated that, Stevioside is a major component of the steviol glycoside group - a group of diterpene glycoside derivatives extracted from the Stevia rebaudiana plant. Steviol glycosides have long been used as a source of non-calorie sweeteners, with a very high sweetness (about 200-300 times sweeter than sucrose of sugar cane) (Das, K., R. Dang, and T. N. Shivananda. 2008).

*Figure 1.* Stevia rebaudiana Bertoni plant. **A** Stevia rebaudiana Bertoni plant experiment field. **B** The *Stevia rebaudiana* Bertoni in the fertilizer treatment experiment. **C** The Stevia rebaudiana Bertoni dry leave was used in traditional Vietnam medicine

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According to many documents, stevioside also has antibacterial effects, helping to improve cardiovascular diseases and blood pressure. Stevioside has been used in the food industry in advanced countries such as Japan, China and the United States…(K. Ramesh et al., 2006). In addition, modern medicine show that sweet grass has attracted great attention not only to subjects with little need or forced to limit the amount of carbodrate such as: diabetics, but also can treat obesity and high blood pressure (Crammer B & Ikan R .2003; Das, K., R. Dang, and T. N. Shivananda. 2008).

Stevia rebaudiana species has been cultivated in a number of countries around the world such as China, Japan, Korea, Taiwan, Thailand, Indonesia (Venkata et al., 2011). China has a lead over other countries regarding production capacity and export of stevia, globally (Mordor Intelligence 2017). Stevia rebaudiana has been import to Vietnam since 1988. Currently, according to the national program to develop industrial plant resources (2012), stevia plant is grown in many regions of the country, from Northern provinces such as Ha Giang, Cao Bang, Son La, Phu Tho etc. to southern provinces such as Lam Dong, Dak Lak (Truong Huong Lan et al., 2014) but the ideal climatic of Stevia rebaudiana plant were sub-tropical and tropical with temperature and rainfall suitable.

The basic raw material for the production of stevioside is the leaves of the stevia plant and Fertilizer and cultivar play a major role to yield and quality of glycoside and RA medicinal content, study on *Stevia rebaudiana* plant are very limited and there are still no scientific reports yet in Northwest provinces in Viet Nam. Therefore, how to investigation of N, P, K fertilization are appeared to be quite important for the production of high amount of quality raw material is necessary *in order* to have more research *for completing* the commercial cultivation process in the future for higher yields and quality of leaves and steviol glycosides in the norther Viet Nam conditions. In this research, we study the effect of the fertilizer amount and planting density of *Stevia rebaudiana* plant medicinal Norther Viet Nam province and the formula level of fertilizer had select 300 kg Nitrogen (N) + 100 kg $P_20_5$ phosphorus (P2O5) + 240 kg Kalidioxid (K2O) ~ per hectare ) and planting density D2 (20cm x25 cm) per hectare was suitable for quality of Stevia rebaudiana plants.

## MATERIALS AND METHODS

### *Plant material and Experimental Site*

Field experiments was performed from 2018 to 2019 at Viet Nam national university of agriculture (20°53 N, 105°44E, 20 m above mean sea-level) Ha Noi, Viet Nam. The soil of trial site was selected from 0 to 40 cm soil depth before commencement of the experiment and was analyzed in laboratory of Soils and Fertilizers Research Institute (SFRI), Viet NAM.

The soil character of the experiment sites was analyzed as table 2.in paddy field were taken from the research farm, the pH 6.62

***Table 1*: The soil basic nutrient content in the Stevia rebaudiana experimental filed**

| Depth of Soil (cm) | Oganic matter (mg/100g) | Total N (N%) | Total P - (P2O5%) | Total K- (K2O%) | Available N (mg/100g) | Available P (mg/100g) | Available K (mg/100g) |
|---|---|---|---|---|---|---|---|
| 0-40 | 13,9 | 0,15 | 0,17 | 0,79 | 0,63 | 1,93 | 4,18 |

*Stevia rebaudiana* Bertoni variety Morita 3 (**M3**) was used in this experiment, which was provided by Stevia Ventures Company and evaluated in Japan (Http://Steviaventures.com) regarding productive performance in the Ha Noi climatic conditions of the North region of Viet Nam.

**EXPERIMENTAL DESIGN AND CROP MANAGEMENT**

The experimental design was in randomized blocks to evaluating the effect of seven planting densities with three replications, which was investigated as follows: D1: 20 × 20 cm; 0.04 m2 area per plant (250000 plants·ha-1), D2: 20 × 25 cm; 0.05 m2 area per plant (200000 plants·ha-1), D3:25 × 25 cm; 0.0625 m2 area per plant (160000 plants × ha-1) – control, distance was recommended for *Stevia rebaudiana* Bertoni. M3, D4: 30 × 20 cm; 0.06 m2 area per plant (167000 plants·ha-1), D5: 30 × 25 cm; 0.075 m2 area per plant (133000 plants·ha-1), D6: 35 × 20 cm; 0.07 m2 area per plant (143000 plants·ha-1), D7: 35 × 25 cm; 0.0875m2 area per plant (114000 plants·ha-1). The Irrigation system in this experiments were functioned according to the soil moisture conditions and Drip irrigation consisted of a line of tape with a flow of 2 L h−1.

For fertilizer experiment, the fertilizers method included: (P1) 350 kg N + 200 kg P205 + 150 kg; (P2) 300 kg N + 100 kg P205 + 240 kg K20 K20 (Two combination was given by early research as the best results (Benhmimou A *et al*., 2018; JingtianYang *et al*., 2013); (P3) 225 kg N + 90 kg P205 + 120 kg K20 (The combination was supported after Analyze the nutrient content of stevia plants taked from the soil in Norther region of Viet Nam) ; and (P0): 0 kg N + 0 kg P205 + 0 kg K20 as control respectively for each hectare. Three managements were set for each level and the formula application was adopted follow as: Basal fertilization were applied at the time of planting: 50% N + 50% K20 + 100% P205; Top-dressing fertilizer: phase 1 was applied at 45 days after planting: 25% N + 25% K20; phase 2 (180 DAP): 25% N + 25% K20. A half dose of N and full dose of P and K as per treatment were applied at the time of planting, while remaining half dose of N was applied at 45 days after transplanting.

Area of each plot was set 10 $m^2$ with the size of cultivating places were specified 25cm x 20 cm. The total area of experiment was 120 $m^2$.

In the other hand, first crop was harvested at 95 days after planting (DAP) on August 15, 2018, whereas, the second crop was harvested at 60 days, after fifth cutting and on July 5, 2019. Harvesting times were carried on according to plant phenology, when less than one half of the plants showed flower bud formation, and with no open flower. The sample of plants were cut 5 cm above experimental ground.

**PHYSIOLOGY CALCULATION**

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For physiology experiment, total fresh leaves area and stem length were collected and quantified. After this procedure of leaves were recording the fresh weight of above and below ground parts of plants, the samples were oven dried at 65 ± 2°C until a constant weight and was attained to calculate the percentage of dry matter accumulation.

A total of leaves were weighted from each experimental subplot for the measurement of leaf area (LA) calculated in proportion to the total weight of leaves. Leaf area index (LAI), specific leaf area (SLA), leaf area ratio (LAR) and leaf mass ratio (LMR) were calculated follow as:

LAI = LA / SA (m2/m2)

SLA = LA / LDM (m2 /g)

LAR = LA / TDM, (m2 /g)

LMR = LDM / TDM (g/g)

Explanation:

LA : leaf area; SA: ground area; TDM: total dry mass; LDM: leaf dry mass

**GROWTH AND YIELD ANALYSIS**

Growth indexes included Plant height and stem diameter of stevia plants were recorded at harvest. The plant height was measured with a meter ruler from ground to the base of the fully opened leaf and the stem diameter was measured with slide calipers up to 0.01 mm accuracy. Other hand, Biomass yield (total fresh leaf and stem yield), fresh leaf yield, and dry leaf yield were determined in each plant. We estimated the fresh biomass, fresh and dry leaf yield per plant using one digital scale with precision of 0.01 g. Leaves were dried at 50°C temperature in hot air dryer for 6 hours and stored in clean gunny bags. At this temperature, the quality of dried leaves produced, in terms of colour, sweetness and nutrient content, was better compared with drying at 70°C (Samsudin A& Aziz IA, 2013). Dry leaf had an important role in stevia extract in term of quality (Yadav AK., et al 2011).

**INSTRUMENT AND REAGENT**

For determination of steviol glycosides concentrations experiment, Instrument and Reagent were used include: Waters Acquity UPC2 USA, HPLC, EmPower Color Spectrum Management System, PL203 electronic balance, KQ-5200DE numerical control ultrasonic cleaner, 5µl microliter syringe and 0.45 micro-perforated filter membrane. Acetonitrile and dichloromethane were both chromatographically purified. The steviol glycoside (98%) reference was provided by Chengdu Pufei De Biotech Co., Ltd, batch No 21967-41-9.

**CHROMATOGRAPHIC ANALYSIS CONDITION**

ODS hypersil (Thermo scientific) column ($UPC^2$2-EP 250 mm×4,6 mm, 5 µm), acetonitrile and ultrafilterred water were the mobile phase (70:30 v/v), flow rate was 1.0 ml $min^{-1}$, column temperature was $28^0$C; ELSD parameters: drift tube temperature $70^0$C, spray tube temperature $25^0$C, nitrogen gas flow rate was 1.5mL $min^{-1}$.

**SOLUTION PREPARATION**

Test article solution preparation: 0.5 g powder of *stevia leaf tissue* was crushed and transferred to bolt containing 50 ml of 70 % ethanol and then filtered by Sieve 710 through precise weighing, and added 50 ml mobile phase through precise weighing, soaked the powder of *stevia* for 2 hours and placed it in ultrasonic treatment for 30 minutes and then taken out and cooled and weighed. After all, compensated the weight loss using mobile phase, filtered with 0.45 micro-perforated filter membrane, and finally took the subsequent filtrate as the finish solution.

**Measurement of Steviol Glycosides Content (Glycoside and rebaudioside A)(USP- http://hmc.usp.org)**

The logarithms of peak responses were plotted to compare with the logarithms of steviol glycoside concentrations in mg $mL^{-1}$ from the standard solutions and the regression line was determined using a least-squares analysis; or, a linear regression equation was established using a least-squares analysis according to the logarithms of the peak responses versus logarithms of steviol glycoside concentrations in mg $mL^{-1}$ from the standard solutions.

The concentration, C, in mg $mL^{-1}$, was determined by regression line of the relevant analyze in the sample solution or linear regression equation. The percentages of steviol glycoside in *Stevia rebaudiana* Bertoni were calculated separately follow as:

$$X\ (\%) = \frac{C\ X\ 100\ X\ 10\ X\ 100}{w\ X\ 1000\ X\ (100-A)}\ X\ 100 = \frac{C\ X\ 10000}{w\ X\ (100-A)}$$

**Explanation:**

X= Steviol glycoside content (%)

C= Concentration of the relevant analyte in the sample solution as determined above (mg $mL^{-1}$)

A= volume of the sample solution (oC)

W= weight of *Stevia rebaudiana* Bertoni seed taken to prepare the sample solution (mg)

**STATISTICAL ANALYSIS**

Statistical data analysis using IRRISTAT5.0 and Excel 2007 software, and then strain and variety fertilizing amount were included as experimental factor to calculate the significant difference of steviol glycoside in *Stevia rebaudiana* Bertoni.

**RESULTS AND DISCUSSION**

**Effect of different plant densities and fertilizer methods on Physiology indexes of *Stevia Rebaudiana* Bertoni**

The physiological and growth indexes include height, number of branches, LAI, SLA, LMR and LAR. In this study, the results as shown in Table 2, 3 *Stevia Rebaudiana* Bertoni. plants under different plant densities and fertilizer methods. After treatment, there was no interaction between planting densities and fertilizer treatment. All data are means ± SD calculated from three replicates. Three biological experiments were performed, which produced similar results.

Evaluating the factors isolated, planting density was only significant for LAI ($p>0,005$) with highest content in D2 treatment but did not show significance only for the height, number of branch LMR, SLA and LAR (table 2).

While fertilizer treatment methods were not affected to LMR index but have significant differences in the height and branch of plants, LAI, SLA and LAR, special reached maximum values in the P2 treatment compared to the other fertilizer management (Table 3). Therefore, the result indicated that the fertilizer levels difference and planting density have significant effect on physiological and growth indexes in this experiment which P2 and D2 treatments had the highest value. In order to increase the physiological and growth indexes the formula of fertilization P2 300 kg Nitrogen (N) + 100 kg $P_20_5$ phosphorus (P2O5)+ 240 kg Kalidioxid (K2O)~ per hectare and planting density

D2 ( 20cm x 25 cm) was chosen for experiment.

***Table 2*** **Effect of planting density on physiological and growth indexes**

| Planting densities | Variable | | | | | |
|---|---|---|---|---|---|---|
| | Height (cm) | Number of branches | LAI | SLA | LMR | LAR |
| D1 | 54.79 | 21.15 | 3.61 | 30.11 | 0.62 | 16.54 |
| D2 | 57.12 | 21.77 | 3.98 | 27.75 | 0.66 | 15.66 |
| D3 | 57.82 | 20.96 | 3.64 | 25.77 | 0.55 | 15.23 |
| D4 | 60.67 | 18.77 | 3.66 | 25.71 | 0.56 | 15.05 |
| D5 | 65.15 | 18.28 | 3.29 | 24.11 | 0.51 | 14.65 |
| D6 | 67.51 | 16.55 | 3.45 | 19.67 | 0.51 | 11.18 |
| D7 | 79.27 | 14.66 | 3.02 | 19.61 | 0.5 | 11.01 |
| **LSD (5%)** | **8.56** | **7.23** | **0.3** | **6.56** | **0.08** | **4.54** |

***Table 3*****: Effect of fertilizer treatment on physiological and growth indexes**

| Fertilizer treatment | Variable | | | | | |
|---|---|---|---|---|---|---|
| | Height (cm) | Number of branches | LAI | SLA | LMR | LAR |
| P0 | 44.54 | 14.56 | 0.62 | 14.62 | 0.56 | 9.33 |
| P1 | 61.42 | 16.52 | 1.15 | 18.11 | 0.51 | 13.71 |
| P2 | 61.85 | 19.67 | 1.32 | 20.54 | 0.51 | 11.18 |
| P3 | 60.33 | 19.12 | 1.25 | 22.82 | 0.48 | 14.54 |
| **LSD (5%)** | **2.55** | **6.54** | **0.39** | **10.12** | **0.02** | **5.01** |

**Effect of different plant densities and fertilizer methods on the yield of *Stevia Rebaudiana* Bertoni plants**

In this study, as shown in Figure 2A *Stevia Rebaudiana* Bertoni plants under four fertilization treatment and seven planting density treatment. After treatment, *Stevia Rebaudiana* Bertoni leaves were collected for measurements of yield content. All data are means ± SD calculated from three replicates. Symbols * and ** indicate significant difference between lines at 0.05 and 0.01 levels, respectively. Three biological experiments were performed, which produced similar results.

Figure 2.A result indicated that leaves yield of from D2 to D7 was significant difference and CK (D1) lower than other treatments. However, yield value have no extensive fluctuation between treatment. Simultaneously, based on the economic value and the cost of labor D2 treatment was more suitable than all other treatment.

In this figure 2B demonstrated that the application of fertilizer difference combined could increase yield of Stevia rebaudiana leaves markedly and leaves yield of P2 treatment was the highest. The result proved that decomposed Stevia rebaudiana dregs could provide various kinds of nutrient for the growth, sequentially improved leaf yield of Stevia rebaudiana. Hence, application of P2 with decomposed Stevia rebaudiana dregs was more suitable for Stevia rebaudiana cultivation.

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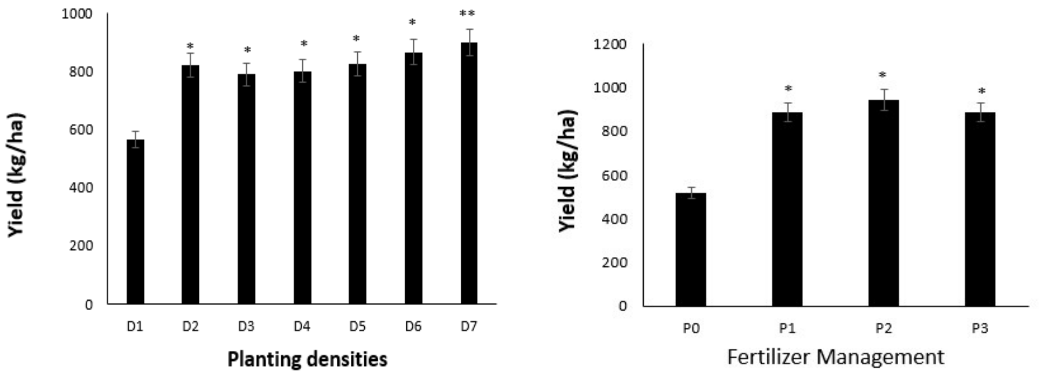
***Figure 2*: A. Effect of planting density on yield of stevia plants.**
**B. Effect of fertilizer treatment on yield of stevia**

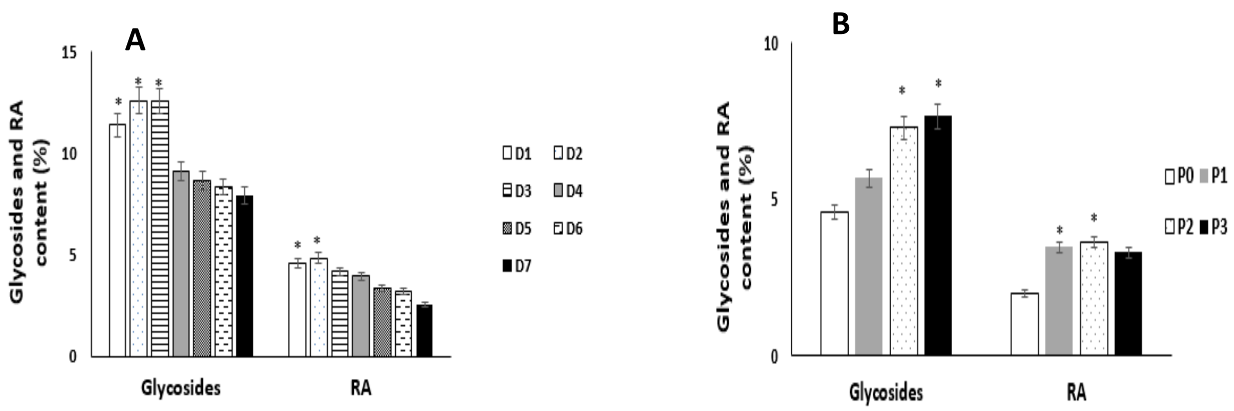



A B

**Effect of different plant densities and fertilizer methods on quality of *Stevia Rebaudiana* Bertoni**Glycosides and RA are two major components in Stevia rebaudiana. There are significant different in glycosides content between different planting density and fertilizer treatment in this study. All data are means ± SD calculated from three replicates. Symbols * indicate significant difference between lines at 0.05 levels, respectively. Three biological experiments were also performed in research.

As show in Fig. 3 A, use of planting density could increase the content of glycosides and RA in Stevia rebaudiana leaves compared with D1 but result not significantly difference and D2 gives the best results in both parameters. Thus, in order to increase the yield of glycosides and RA content of Stevia rebaudiana more than 5% and 3% respectively, the formula suitable was D2 (20cm x25 cm) per hectare was suitable for quality of Stevia rebaudiana plants.

***Figure 3*: A. Effect of planting density on quality of *stevia Rebaudiana* Bertoni.**
**B. Effect of fertilizer treatment on quality of *stevia Rebaudiana***




In contrast, glycosides and RA content (%) in stevia dry leaves was significantly modified by different treatments of NPK combinations (Fig.5) respectively by 7.26 %, 7.63% glycosides content for P2, P3 and 3.61% RA for P2 content higher than P0, means that P2 treatment ( 300 kg Nitrogen (N) + 100 kg $P_20_5$ phosphorus (P2O5)+ 240 kg Kalidioxid (K2O)~ per hectare ) application of decomposed Stevia rebaudiana dregs could enhance the content of glycosides and RA in leaves.

**CONCLUSION AND SUGGESTION**

In recent studies indicated that different treatments of NPK combinations had a significant effect on the growth parameters. Differences fertilizer uptake influent in growth may be because of the higher absorption of water and mineral nutrients due to extensive colonization of roots (Harrier LA & Sawczak J. 2000). Zhao K et al.2008 reported that N, P, K stimulated the leaf production probably due to the increasing production of cytokinin in root tips and involved in *improving* the yield of root crops such as enhancing the yield and the content of herbal plant. According to with the findings of Aladakatti YR., et al 2012, who reported that plant height was significantly higher with nitrogen, phosphorus, and potassium (400, 200, and 200 kg ha-1, respectively) which were on par with 300, 150, and 100 kg/ha respectively. In this study, all treatments increased growth parameters as compared to control experiment. The results show that, the low range of fertilizer level may be decreases the yield of *plant*, however Over fertilizing can even lead to disease resulting in severe crop loss (Li J-H *et al.*, 2009). In this study, maximum plant yield and quality of glycoside and RA medicinal content were attained by 300 kg Nitrogen (N) + 100 kg $P_20_5$ phosphorus (P2O5) + 240 kg Kalidioxid (K2O) per hectare).

In planting density stud recently indicated that both of lower density and higher planting density effect to leaf yield and quality of S. rebaudiana (Kumar et al. 2012, 2014a). Higher yields in high densities are a function of the greater number of plants per area, which compensates the great biomass production per plant (Kumar et al. 2014b). In this study, planting density D2 (20cm x25 cm) per hectare that is suit for growth and development in norther Viet Nam condition.

Optimizing S. rebaudiana on cultivar treatment, it can be targeted for the development to enhance the glicoside content as functional in the traditional medicine. In addition, their ability in the medical treatment need further studies in the future.

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