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Effects of rate and application frequency of nitrogen fertiliser on biomass yield and sweetness quality of stevia (*Stevia rebaudiana* Bertoni)

[Kesan kadar dan kekerapan penggunaan baja nitrogen terhadap hasil biomass dan kualiti kemanisan stevia (*Stevia rebaudiana* Bertoni)]

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Abstract
Human life styles have changed so much and awareness on the use of sugar or sweeteners has become an integral part in our daily diet. Sweeteners which are high in calorie like white sugar, brown sugar, corn syrup, maple syrup, molasses and fruit syrups found in natural and processed are hazardous to our health. Stevia is very popular and ideal to substitute sugarcane. A study was conducted to determine optimum nitrogen (N) level and fertiliser application frequency (A) for high biomass yield and sweetness quality (content, yield, ratio) of stevia. A total of seven different levels of N and two application frequencies (after every harvest, A1 and after every two harvest, A2) were tested. Results indicated that biomass yield escalated with increased level of N up to 250 kg N/ha. At this level, total dry matter yield from four times of the harvesting frequency was 6 t/ha. There were interaction between N and A on glycoside content and glycoside yield of stevia. The maximum stevioside (St) content was about 85 mg/g at A2, under control treatment (0 N). Meanwhile, the maximum rebaudioside-A (Reb-A) content was 25 mg/g, better at A1 when plant treated with 40 kg N/ha as compared with A2. The St and Reb-A yield were better with application of 210 kg N/ha and 170 kg N/ha, respectively, at A2. Sweetness index of stevia was calculated by ratio of Reb-A to St because Reb-A possess the sweetest compound in stevia. It was found that application of N fertiliser at 50 kg/ha and at A1, the sweetness of stevia was high with the ratio of Reb-A to the St of 0.4.

Keywords: *Stevia rebaudiana*, nitrogen fertiliser, stevioside, rebaudioside-A, sweetness quality, natural sweetener

Introduction

Stevia (*Stevia rebaudiana* Bertoni) is commonly known as sweet herb, honey leaf or sugar leaf. It is native to Paraguay and has been used as a source of natural sweetener by the Paraguayan for hundreds of years. Stevia is from Asteraceae family and in the similar family with well-known plants such as dandelion, sunflower and chicory. About 240 stevia species are well known, among them *S. rebaudiana,* which been proven to have the sweetest essence (Soejarto et al. 1983). Among the plant parts of stevia, leaf is the economic part, which contains high phytochemical benefits followed by stem and root. The sweet compound and chemical property of stevia is known as diterpene glycosides. There are about 10 sweet

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glycosides in stevia, but only two are of important, stevioside (St) and rebaudioside-A (Reb-A). St is of highest concentration in stevia and has been estimated about to be 250 – 300 times sweeter than sugarcane, with licorice after taste. However, Reb-A, that is another important glycoside present in fewer amounts than St, however assessed as 350 – 450 times sweeter than sugarcane (Crammer and Ikan 1986) with no after taste (Midmore and Rank 2002).

Stevia is an ideal substitute plants for sugar because it has no calorie although it is much sweeter than sugar. The health benefits of stevia make this crop a good potential for commercial production. One of the important factors that influence yield and quality of stevia is agronomic practices including nutrient requirement. Nitrogen (N) is one of the important nutrients needed for vigorous growth and healthy deep green colour leaves (Martin et al. 2006). Increased rates of N fertiliser application enhanced fresh and dry leaves, stem, total soluble carbohydrate (Allam et al. 2001), stem thickness and number of branches (Kawatani et al. 1980). Deficiency of N shortened the vegetative growth in terms of leaf growth and resulted in earlier maturity of the plant (Mangel and Kirkby 2001).

According to several past researches, the fertiliser recommended for growing stevia is 60 kg N, 30 kg P_2O_5 and 45 kg K_2O per ha (Anon 2008). Stevia plant required about 105 kg N, 23 kg P and 180 kg K/ha for moderate biomass yield of 7,500 kg/ha under Canadian condition (Brandle et al.1998). Maheshwar (2005) reported that application of 105 kg N/ha, 30 kg P/ha and 45 kg K/ha into loamy soil yielded higher dry leaf due to optimum number of leaves and branches per plant. Increasing the dosage of N from 200 – 400 kg/ha increases the height of stevia by 14.94% (from 49.6 cm to 57.0 cm), the number of leaf/plant by 12.97% (from 750.4 to 847.7 blade/plant) and leaf area/plant by 15.88% (from 3,628 – 4,204 cm^2) (Aladakati et al. 2012). Research done by Inugraha et al. 2014 found that there were significant interaction between the application of N and K in parameter of growth, number of leaf, leaf area index and chlorophyll level. They also found that significant interaction between the application of N and K occurred on biomass yield and the harvest yield of leaf during the period of harvest I, harvest II, and total. At the period of total harvest, the application of 200 kg N/ha and 225 kg K_2O/ha resulted in fresh leaf and dry leaf yield of about 2,780 kg/ha and 636 kg/ha, respectively. From the literature cited above, it can be concluded that stevia may need about 60 to 400 kg N/ha of fertiliser, depending on the planting region.

Besides the N fertiliser rate, frequency of fertiliser application need to be determined to optimise the use and best timing the fertiliser application. In 2006, Stevian Biotechnology Corporation Sdn. Bhd. collaborated a contract research with MARDI to identify high yielding and good quality stevia accessions for large scale planting. The fertiliser was applied at two-month intervals alternating NPK (15:15:15) and organic fertiliser (Muhamad Ghawas et.al 2009). Earlier study conducted by MARDI on growth and yield performance at different plant spacing showed that the yield of dry leaf at different plant spacing for total seven harvests are between 1.2 to 2.7 t/ha (Rosnani 2008). In this experiment, NPK green fertiliser with ratio 15:15:15 of N: P_2O_5 : K_2O at the rate of 150 kg/ha was applied at three monthly intervals and 1 t/ha of chicken dung were applied after alternate harvests.

Thus, this study was conducted to determine optimum N fertiliser level and fertiliser application frequency for high biomass yield and sweetness quality (content, yield, ratio) of stevia under Malaysian conditions. Impact of N supply to affluence of glycosides, yield and quality of stevia is very crucial to ensure that the plant do not only produce high biomass yield, but also high in sweetness quality.

Materials and methods

The experiment was conducted under controlled environment in the glasshouse at MARDI, Serdang, Selangor. The plants were propagated using stem cuttings. Cuttings with 2 – 3 nodes were taken from mother plants that had been planted in MARDI's field. These cuttings were raised under 25% shade in germination trays (104 plugs) containing mixture of subsoil and Holland peat with a ratio of 3:1. The seedlings were ready to be transplanted after 30 days of propagation. Field soil was used as planting media in this pot experiment in order to obtain results as closed as possible if the plants were planted in the field.

The treatments consisted of seven different rates and two application frequency of N fertiliser. The total N fertiliser rates applied per year were; 0, 50, 100, 150, 200, 250 and 300 kg/ha. The two application frequencies were after every harvest and after every two harvest. Two other fertilisers, phosphorus (P) and Kalium (K) were applied at 50 kg P/ha and 50 kg K/ha in all treatments. Urea (46% N), single super phosphate (20% P) and muriate of potash (60% K) were used as a source of N, P and K fertiliser. The amount of fertiliser used for each fertiliser rate was split according to the number of harvests per year. The total of fertiliser was split for four harvests and harvest interval was between 30 and 35 days after transplanting.

Weeds were controlled manually while pests and diseases were managed with pesticide and fungicide spraying. Pests such as leaf eaters, and aphids were observed and controlled by spraying with chlorphyrifos (Dursban) and diazinon. No critical disease was observed in this experiment. Plants were watered twice daily using drip irrigation system. The sampling plants were harvested 45 days after transplanting when flower buds started to appear in 50% of the plants in a plot and cut at 5 – 10 cm from soil surface using secateurs. Stumps were left to ratoon and were harvested again at harvest interval between 30 and 35 days, when the flower bud started to appear again. The plants were harvested four times during the five-month planting period. The plant growth (plant height, plant canopy, number of branches and number of leaves) was measured every two months, biomass yield and glycosides content were analysed at every harvest. Biomass yield of stevia refers to the total fresh and dry yield of stem, branches and leaves. Fresh samples with moisture content of 80% were dried in convectional oven (Memmert) immediately at 45 ^{o}C until moisture content reduced to 10%. Immediate drying is critical as glycosides content will decline to 33% within three days.

Dried samples were extracted for determination of St and Reb-A content according to method developed by Kolb et al. (2001). Dried plant sample of 0.2 g was weighed and glycosides were extracted using 10 ml of 70% ethanol (ETOH). After the extraction process, the sample was purified before extracting the targeted compound from other compounds (possibly structurally related) or contaminants. The sample was sonicated for 45 min using ultrasonic (1505 Jac Ultrasonic, Jeiotech, Korea) and centrifuged at 5000 rpm for 15 min using centrifuge (5210 R, Eppendorf, Germany). Then, the sample was filtered using a syringe (SS05L, Terumo) and nylon membrane filter with size 17 mm (0.20 µm, Target, USA). After extraction and purification process, the sample was analysed by High Performance Liquid Chromatography (HPLC) to identify the St and Reb-A compounds. These compounds were identified through standard chromatography and finally concentration of St and Reb-A were quantified. The HPLC used NH2 column, a mixture of acetonitrile/water (80:20, v/v) as mobile phase, flow rate at 1 ml/min with injection volume at 200 µl and UV range at 210 nm. When the concentration of these two glycosides in plant samples were determined, sweetness quality was measured by calculated the ratio of Reb-A to the St since Reb-A is much sweeter than St. Presence of Reb-A in higher amount than St make the sweetness of stevia is much higher and quality is better. The glycosides yield also were calculated based on its concentration multiply with biomass yield (dry matter yield).

Statistical analysis
The experiment was designed as a factorial experiment in a randomised complete block design (RCBD) with three replications. Data were analysed using analysis of variance and the significant means were separated by Duncan's multiple range test (DMRT) at $p \leq 0.01$ (SAS 9.1 2002). When first order interaction was significant, regression analysis was conducted to describe the relationship between biomass and glycosides yield at different N fertiliser level and different application frequency. The regression analysis was generated using PROC REG procedures to calculate slopes, intercepts and regression coefficients (R^2).

Results and discussion
Effect on dry yield of plants
Fresh yield was dried to maintain the glycosides content in every plant part especially during storage. In this study, no interaction was observed between N rates and application frequency (A) on dried biomass yield of stevia. However, N did significantly interacted with application frequency on St and Reb-A contents of stevia.

Main effects of nitrogen
Nitrogen (N) rate significantly ($p < 0.01$) influenced dry yield of stevia. Further analysis indicated that the response of dry yields to N rate was quadratically significant. Dry yield (stem and leaf) optimum at the N rate of 250 kg/ha with dried biomass of about 6 t/ha (*Figure 1*). However, research done by Inugraha et al. (2014) in East Java found that at the period of total harvest, the application of 200 kg N/ha and 225 kg K_2O/ha yielded about 0.6 t/ha of dry leaf. The dry biomass yield above was much lower because it recorded only the leaf yield, however, this study included both the stem and leaves. Brandle et al. (1998) found that stevia plant under Canadian condition requires only about 105 kg N, 23 kg P and 180 kg K /ha to yield moderate dry biomass of 7.5 t/ha. Maheshwar (2005) showed that application of 105 kg N/ha, 30 kg P/ha and 45 kg K/ha recorded significantly higher dry leaf yield. The N fertiliser for stevia planted under short-day-length area requires higher input of N fertiliser compared to long-day-length area, which is more than 13 hours.

The biomass yields decreased at N fertiliser beyond 250 kg/ha. This may be due to the effects of excessive N which reduces plant growth and yield. Thus, up to a certain point, uptake of nutrients does not translate into increased biomass yield or plant productivity. Acquaah (2001) described, the use of nutrients at that level was as a luxury consumption which if it is continued might reach a toxic level. In the study, 250 kg/ha of N fertiliser was sufficient for the plant, above this rate effect of N toxicity was observed. Rakesh et al. (2012) reported that higher levels of N in the soil is an essential pre-requisite for photosynthetic ability and contributed to higher dry matter accumulation. The dry matter yield decreased when N fertiliser was increased from 263 kg/ha until 300 kg/ha.

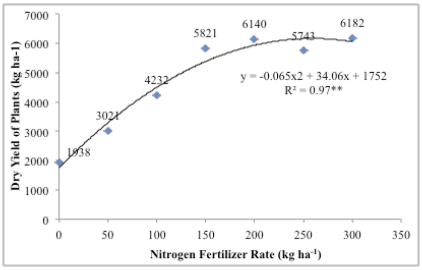

Figure 1. Effect of nitrogen fertilizer rates on dry yield of stevia

Main effects of application frequency

Fertiliser application frequency (A) showed significant effects (p <0.05) on dry yield of stevia. Application of fertiliser at every harvest (A1) yielded higher dried yield of about 5 t/ha compared to after every harvest (A2) of about 4.4 t/ha (*Figure 2*). This probably due to inadequate N fertiliser for unfertilised ratoon crop when fertiliser was applied once in every other harvest.

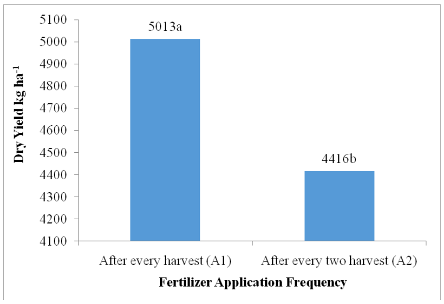

Figure 2. Effect of fertilizer application frequency on dry yield of stevia

Effect on glycoside content

There were significant interaction between N and A on St (*Figure 3*) and Reb-A (*Figure 3*) content of stevia at p <0.01. At the frequency A1, N rate did not show any significant relationship with St content. However, at frequency A2, there was a significant cubic relationship. This cubic relationship on A2 is probably due to the glycosides contents influenced by the fertiliser application at harvest interval. Whereby, when the plant was fertilised with double amount, the glycosides content decreased, and when no fertiliser was added at certain harvest, the content increased. This condition influenced the growth and yield of stevia. As illustrated in *Figure 3*, St content at A2 was higher than A1 when 0 to 30 kg N/ha were applied. At 0N, A2 produce the highest St content (85 mg/g). However, the St content of A1was higher than A2 when N fertiliser was applied between 30 kg/ha and 120 kg/ha. However, application between 120 kg N/ha and 280 kg N/ha, again St content at A2 was much higher than A1. Research done by Inugraha et al. (2014) found that increasing the application of N fertiliser from 100 to 250 kg/ha significantly increased the St level when the plants were fertilised at every harvest. At application of more than 280 kg N/ha, St content at A1 increased and the amount was more than A2. This excessive amount of fertiliser is not good to the plant, soil and underground water. The plants will take fertilisers up to a certain level, beyond this the uptake of nutrients does not translate into increased biomass yield or plant productivity (Acquaah 2001).

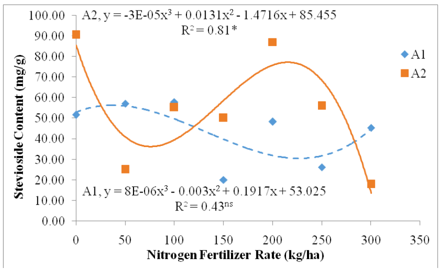

Figure 3. Interaction effect between nitrogen fertiliser rate and fertiliser application frequency on stevioside content of stevia

At the three points of interaction between N and A (30, 120 and 280 kg N/ha) the St. content is 55 mg/g, 45 mg/g and 40 mg/g respectively. This showed that St content is higher at lower N fertiliser rate. This could be probably due to the stress conditions faced by the plants. Usually, stressed plants produce organic compounds as secondary metabolites in abundance. All plants produce and store secondary metabolites, which are not important for primary or energy metabolism of a plant. However, they are important for the ecological fitness and survival of the plant. Apparently, plants evolves with the production and storage of secondary metabolites as a means to defend themselves against herbivores, bacteria, fungi, viruses as well as other competing plants (Michael 2010). Stress condition such as deficiencies of N, P and K stimulate phenolic metabolism in rice, whereby the level of some phenolic acids increase in N-deficient plants (Naoya and Tsuyosi 1997). Levels of p-coumaric acid and ferulic acid in tops also increased noticeably under N deficiency. These findings were in agreement with a study done by Mohd Hafiz et al. (2011) on N fertilisation on Kacip fatimah. They showed that fertilisation with high input N fertiliser could reduce production of total flavonoids and phenolics. When N application was reduced from 270 to 0 kg/ha, these secondary metabolites content increased.

Similar trend as on amount of St content was also showed by Reb-A content as the N rates of A1 and A2 increases (*Figure 4*). The Reb-A content was not significant at A1, but has significant cubic relationship at A2 (*Figure 4*). However, A1 was better in Reb-A content than A2 when applied with N fertiliser between 0 and 160 kg/ha. Reb-A content (25 mg/g) peak at 40 kg N/ha. At A2, Reb-A content increased when N fertiliser was applied more than 160 kg/ha up to 260 kg/ha. At more than 260 kg/ha, the Reb-A content at A2 decreased, and A1 started to increase. From this study, *Figure 3* and *4* show that Reb-A content was lower than St content in stevia. The highest Reb-A and St content at 40 kg N/ha and A1 was 25 mg/g and 55 mg/g respectively. According to Midmore and Rank (2002) the highest concentration of sweet glycosides in stevia is St and the amount is about 60 – 70% of total glycosides. The sweet taste of St has been estimated about 110 – 270 times sweeter than sugar with an after-taste (licorice taste). However, Reb-A is present in lesser amount than St which is about 30 – 40% of total glycosides in stevia, but assessed as 180 – 400 times sweeter than sugar with no after-taste.

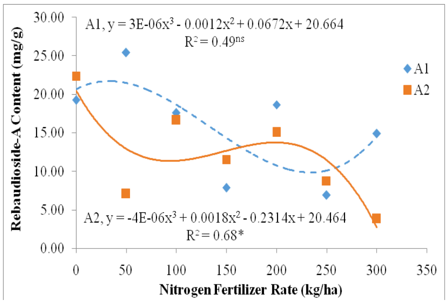

Figure 4. Interaction effect between nitrogen fertiliser rate and fertiliser application frequency on Rebaudioside-A content of stevia

Effect on glycoside yield

St and Reb-A yield were measured and is shown in *Figure 5* and *Figure 6*. In *Figure 5*, the St yield shows significant cubic relationship between N and A at R2 = 0.76 for A2. However, no significant relationship between N and A for A1. Application of N fertiliser between 110 kg/ha and 275 kg/ha gave higher St yield for A2 than A1. The highest yield of 410 kg/ha at 210 kg N/ha.

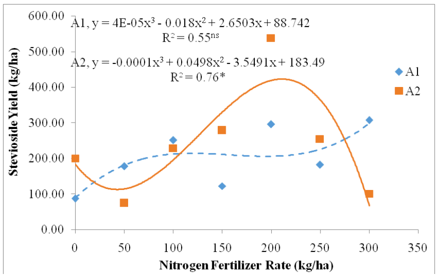

Figure 5. Interaction effect between nitrogen fertiliser rate and fertiliser application frequency on stevioside yield of stevia

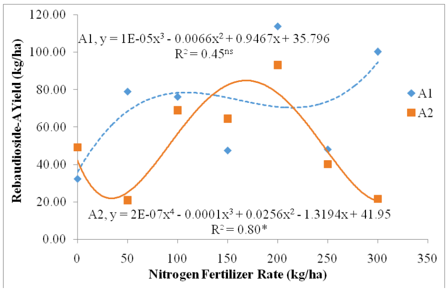

*Figure 6. Interaction effect between nitrogen fertiliser rate and fertiliser application frequency on Rebaudioside-A yield of stevia***.**

In *Figure 6*, Reb-A yield were higher at A1 compared to A2.The relationship between N rate and A was significant at A2, but not at A1. At N rate between 140 and 220 kg/ha, the Reb-A yield at A2 was higher than A1. The highest Reb-A yield was 85 kg/ha at fertiliser rate of 170 kg N/ha. Zahida and Saini (2009) reported that application of fertilizer rate at 40 and 60 kg N/ha is at par and produce significantly higher dry biomass yield. Maximum yield of St and Reb-A is at 60 kg N/ha (applied in urea form). In this study, at high N rate, the St and Reb-A content were low, however the St and Reb-A yield were high due to high biomass yield.

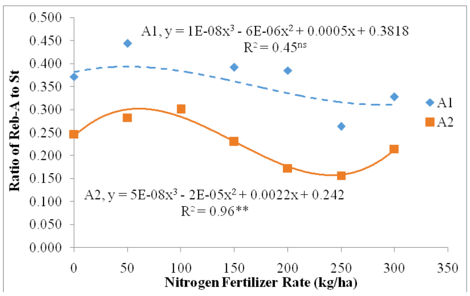

Figure 7. Interaction effect between nitrogen fertilizer rate and fertilizer application frequency on Rebaudioside-A yield of stevia

Effect on sweetness index

The higher presence of Reb-A than other components of glycosides, making it a major component for determining the sweetness of stevia. *Figure 7* shows the ratio of Reb-A to the St at different N rates for each application frequency. Interaction between N and A for the ratio of Reb-A to the St was highly significant. The results showed no significant response at A1, but significant cubic response at A2. However, the ratio was higher in A1 than A2 at all N rates applied. This showed that stevia fertilised at every harvest was sweeter than those fertilised at every other harvest, and the optimum N rate for sweetness index was 50 kg/ha.

Conclusion

The results of this study suggested that the best N fertiliser rate for stevia was 250 kg/ha/year and the best fertiliser application frequency based on biomass yield was after every harvest. However, based on Reb-A and St content, the best N rate and fertiliser application frequency were in the control treatment (0 N) and after every two harvests, respectively. For abundance of Reb-A and St yield, the recommended rate and frequency were 160 – 210 kg N/ha and after every two harvests respectively. Nevertheless, the most important factor in stevia production is its sweetness trait. This study indicated that 50 kg/ha N applied at every harvest was the best practice to get the highest sweetness quality.

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Abstrak
Gaya kehidupan manusia telah banyak berubah di mana pemanis menjadi keutamaan dalam diet seharian kita. Pemanis tinggi kalori yang terdapat dalam gula semula jadi dan gula yang diproses adalah berbahaya kepada kesihatan. Stevia sangat popular dan sesuai bagi menggantikan gula tebu. Satu kajian telah dijalankan bagi mengenal pasti kadar baja nitrogen (N) dan kekerapan pembajaan yang optimum untuk meningkatkan hasil biomas dan kualiti kemanisan stevia. Sebanyak tujuh kadar N berbeza (0, 50, 100, 150, 200, 250 dan 300 kg/ha) dan dua kekerapan pembajaan (selepas setiap kali penuaian, A1 dan selepas setiap dua kali penuaian, A2) telah diuji. Keputusan kajian menunjukkan hasil biomas meningkat dengan peningkatan kadar N sehingga 250 kg N/ha. Pada kadar ini, jumlah berat kering daripada empat kali penuaian ialah 6 t/ha. Terdapat interaksi antara N dan A terhadap kepekatan glikosida dan hasil glikosida stevia. Kepekatan stevioside maksimum adalah sebanyak 85 mg/g pada A2 di bawah rawatan kawalan (0 N). Manakala, kepekatan Reb-A yang maksimum ialah 25 mg/g, lebih baik pada A1 apabila pokok dirawat dengan 40 kg N/ha berbanding dengan A2. Hasil stevioside dan Reb-A lebih baik dengan penggunaan masing-masing sebanyak 210 kg N/ha dan 170 kg N/ha pada A2. Indeks kemanisan stevia telah diukur melalui nisbah Reb-A kepada Stevioside (St) kerana Reb-A adalah kompound paling manis pada stevia. Adalah didapati penggunaan baja N pada 50 kg/ha dan pada A1, kemanisan stevia adalah tinggi dengan nisbah Reb-A kepada St ialah 0.4.