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NITROGEN AND PHOSPHOROUS EFFECTS ON GROWTH OF *Stevia rebaudiana* Bertoni UNDER LIGHT EMITTING DIODE (LED)

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ABSTRACT

Studies on planting of *Stevia rebaudiana*, an important industrial crop that provides non-caloric sweeteners for the beverage sector, is lacking in an indoor controlled environment. In the present study, using a nutrient film technique, nitrogen (N) and phosphorous (P) levels needed for *S. rebaudiana* were determined. The plants were grown under light emitting diodes (LED) at 16h/8h light/darkness photoperiod in an air-conditioned laboratory. The results obtained indicated that in terms of growth performance and leaf production, the half strength Hoagland solution, where N was 100 mg L^{-1} and P was 15 mg L^{-1}, enabled the optimum gain in this crop. The results have implications for economic purposes.

Keywords: controlled environment, Hoagland solution, nutrient film technique

1. Introduction

Stevia rebaudiana (Bertoni), which is commonly known as stevia, candy leaf, sweet leaf or honey leaf, is from the family Asteraceae. It is native to Paraguay in South America but has now been cultivated in many countries for the natural sweeteners in its tissues which are of low calories. The aerial parts of stevia are used as a sugar substitute and the highest abundance of sweet-tasting substances are found particularly in the leaves. These compounds are diterpene glycosides that are 200-300 times sweeter than sugar (Brandle & Telmer, 2007). Stevioside is the major sweetening compound in this plant. Other sweetening compounds in this crop include rebaudiosides and dulcosides (Kaushik et al., 2010; Kurek & Krejpcio, 2019). The extracts yielded from aqueous infusion are utilized in a number of industrial foods including soft drinks, fruit drinks, desserts, confectionery and sauces (Lemus-Mondaca et al., 2012).

The growth and development in *S. rebaudiana* vary by the genetic factors (Yadav et al., 2011), environmental conditions (Tavarini & Angelini, 2013; Khiraoui et al., 2017), agronomic practices (Serfaty et al., 2013; Das & Dang, 2014), processing and storage. Among the agronomic practices, plant nutrition is a crucial determinant for the yield and quality of this crop (Inugraha et al., 2014). Plant growth and assimilate partitioning can be altered with applied nutrients. Promoting the growth of the leaves in terms of increasing the leaf weight ratio is an economic goal of growing this crop. Nitrogen (N) has been playing an important role for this purpose while other macronutrients of phosphorus (P) and potassium (K) required by this species have also been studied (Pal et al., 2013; Inugraha et al., 2014; Behnmimou et al., 2018).

A number of fertilization studies have been conducted on the physiology of *S. rebaudiana* in the field and greenhouses (Kafle et al., 2017). In contrast to the conventional field and greenhouse crop production, there are only some limited recent studies on the growth of *S.*

rebaudiana indoor using light emitting diodes (LED) in the total absence of natural light. LED of certain wavelength was reported to alter its growth form (Yoneda et al., 2017). Nonetheless, we lack information on the manipulation of nutrients for growing this crop indoors. Among the macronutrients, N and P variations were obvious for the growth and yield of *S. rebaudiana* in the field (Mandal et al., 2013; Vafadar et al., 2014; Pal et al., 2015; Rashwan & Ferweez, 2017). This study was, hence, aimed to explore the N and P requirements for raising this crop indoor under commercial Horticulture T8 LED.

2. Materials and Methods

2.1. *Location of study*

The experiments were conducted under a controlled environment in the laboratory of Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA Puncak Alam, Selangor (N 3°11.84', E 101°26.93') in 2018. The laboratory was air-conditioned with average temperature and relative humidity of 25±2°C and 55±5%, respectively.

2.2. *Seed germination*

Stevia rebaudiana (Sahin®, Takii) seeds were purchased from a local nursery. The seeds were germinated on a hydroponic sponge of 2.3x2.3x2.4 cm moistened with tap water in enclosed clear plastic containers. Seed germination started at the end of September 2018. A thin film of tap water was maintained in the germination containers to ensure water supply for the germinating seeds. The containers were placed on 1.5x0.5x2 m wooden racks in the laboratory. Seed germination was carried out under monochromic red LED (660 nm). The hanging LED tubes of 4' in length and 2.5 cm in diameter (18W, 240V) were adjusted to be at about 5 cm above the seed germination containers. Seed germination was carried out at 16h/8h light/darkness. The seeds germinated within one week.

2.3. *Planting procedure*

Two weeks after sowing when the first two leaves fully expanded, the covers of the germination containers were removed and the seedlings were introduced to a thin film of quarter strength Hoagland solution. After two weeks of growth with quarter strength Hoagland solution, the seedlings of approximately 3 cm in height with 4 leaves were transferred to polyvinyl chloride (PVC) troughs of 1.1 m in length and 5 cm in diameter. The PVC troughs were secured on polystyrene holding blocks with hemispheric holes of 5 cm in diameter on the tops. The troughs were placed on the wooden racks. The distance among the seedlings in the PVC troughs was 15 cm.

The seedlings were grown under four units of hanging 2' Horticulture T8 LED tubes (18W, 240V) (MyRay®, B.M. Nagano Industries Sdn. Bhd.). The LED tubes were raised accordingly as the plants grew taller so as to be always at approximately 5 cm above the plants, providing photosynthetic active radiation (PAR) of approximately 100 µmol m^{-2} s^{-1} to the plants. The seedlings were subjected to 16h/8h light/darkness throughout the study.

The seedlings were grown with half strength Hoagland solution in two separate experiments with varying N and P concentrations, respectively, using nutrient film technique in the PVC troughs throughout the study. Each trough was filled with 1.2 L solution, providing a depth of approximately 3 cm in the trough. The nutrient solution was aerated for 15 min hourly using an aquarium pump throughout the experiment.

2.4. *N and P experimental procedure*

In the experiment on N effects on the plant growth and leaf production, the plants were subjected to treatments of 50, 100 (control), 150 and 200 mg L^{-1} N, respectively. Another experiment with P was carried out concurrently. In this experiment, the plants were respectively treated with 15 (control), 30, 45 and 60 mg L^{-1} P. In each experiment, the N or P treatments were combined with other nutrients of the Hoagland solution in half strength throughout the study. Nutrient solutions were at electrical conductivity (EC) in the range of 1,300 to 1,500 µS cm^{-1} and pH of 6 to 7. EC and pH of the solution in troughs were checked twice weekly. The nutrient solution was renewed every two or three weeks when EC and pH were out of range. While EC and pH were found within range, the trough was topped up with similar solution accordingly when the solution level in the trough dropped to below 2 cm.

2.5. *Morphological growth measurement*

Height, number of leaves and flowering of each plant were recorded fortnightly. The height was measured from root collar to the highest point of the plant in its own form and appearance. Leaves with length of ≥ 0.5 cm were counted as the number of leaves.

At 12 weeks after treatment, the plants were harvested by cutting them at their root collars. In addition to the data gathered from height and number of leaves, each plant was also recorded for its stem diameter at root collar, the number of branches and the total leaf area per plant. The stem diameter was measured using a Vernier Caliper while total leaf area per plant was estimated using graph paper.

2.6. *Chlorophyll content determination*

The chlorophyll content of the leaves at harvest was determined according to Lichtenthaler & Wellburn (1983). Mature fresh leaves of each plant were picked randomly and weighed approximately 0.02 g. They were cut into 2 mm strips, and added with 20 mL of 99% methanol in a test tube. A pigment extraction was carried out at room temperature in the dark for 24hrs. The leaf strips turned whitish when the pigments were extracted into the solvent after 24hrs. The chlorophyll concentrations in the test tubes were determined using UV-VIS spectrophotometer (Sastec/ST-UV8000) at 653 and 666 nm. The amount of chlorophyll of the leaves on fresh weight (FW) basis was the total of chlorophyll *a* and chlorophyll *b* as calculated using formulas (1) and (2) below.

$$\text{Chlorophyll } a \text{ (mg g}^{-1}\text{ FW)} = \frac{(15.65\,A666 - 7.34\,A653)\,x\,dilution\,factor\,x\,volume\,of\,product\,(mL)}{1000\,x\,weight\,of\,sample\,(g)} \quad (1)$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1}\text{ FW)} = \frac{(27.05\,A653 - 11.21\,A666)\,x\,dilution\,factor\,x\,volume\,of\,product\,(mL)}{1000\,x\,weight\,of\,sample\,(g)} \quad (2)$$

2.7. *Dry weight determination*

The dry weight (DW) of the plants at harvest was determined after drying at 40°C in a convection oven. The leaves of each plant were separated from the stems. The roots of each plant were removed from the sponge carefully with minimal loss of roots. Then, the leaves,

stems and roots were placed in separate labelled paper bags and dried as mentioned to obtain three consecutive constant DW readings. This was achieved within one week. The leaf weight ratio (LWR) was then calculated using formula (3) below.

$$\text{LWR} = \frac{Leaf\ DW\ (g)}{Total\ plant\ DW\ (g)} \quad (3)$$

2.8. *Statistical analysis*

The experiments were each based on a completely randomized design. Each treatment was replicated thrice with four plants per replicate. In the experiment on N effect, there were only two treatments with living plants at the end of the experiment. The data were, hence, analysed by independent T-test at 5% level of significance. On the other hand, data in the experiment on P effect were subjected to analysis of variance (ANOVA). The least significant differences (LSD) of means at 5% level of significance were calculated. SPSS Version 25 was used for statistical analysis.

3. Results

3.1. *N effect*

In the air-conditioned laboratory, *S. rebaudiana* in nutrient film needed only 100 mg L^{-1} N in combination with half strength of other nutrients in the Hoagland solution. The plants demonstrated significantly better height and number of leaves gain with this N treatment as compared to the lower N treatment at 50 mg L^{-1} (Figure 1). The plants raised with 100 mg L^{-1} N had a height of approximately 50 cm with 130 leaves at the end of the study period of 12 weeks. This crop also had significantly more branching, greater stem diameter, total leaf area and biomass (DW) with 100 mg L^{-1} N, as compared to 50 mg L^{-1} N (Table 1). The mean leaf DW was 1.4884 g/plant with 100 mg L^{-1} N. The LWR, on the other hand, was 0.58, indicating good partitioning of photosynthates to the economic plant part of the leaf with 100 mg L^{-1} N.

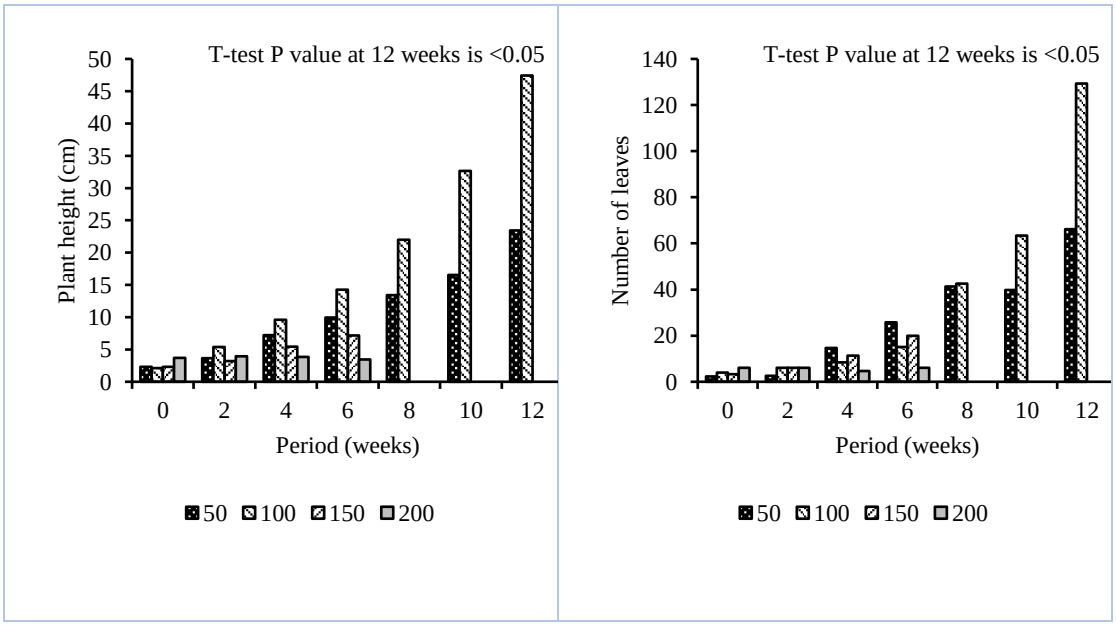

Figure 1. Effects of N (mg L^{-1}) on plant height (left) and number of leaves (right)

The plants supplied with 50 or 100 mg L^{-1} were, however, not significantly different in terms of leaf greenness. The plants treated with lower N at 50 mg L^{-1} showed no chlorosis symptom; they had comparable leaf chlorophyll content as those receiving 100 mg L^{-1} N. Increasing N to 150 mg L^{-1} and 200 mg L^{-1} caused toxicity in *S. rebaudiana*. The plants subjected to these higher N treatments showed stunted growth with height of not more than 10 cm and only approximately 10 small leaves for a period of up to six weeks (Figure 1). They died thereafter.

As for flowering, it is encouraging to note that none of the living plants in this experiment flowered throughout the period of 12 weeks.

3.2. *P effect*

In the experiment on P effect conducted simultaneously, plants grown with P of 15 and 30 mg L^{-1} were not significantly different in height, number of leaves, stem diameter and number of branches (Figures 2 and 3; Table 2). Surprisingly, lower P at 15 mg L^{-1} was better for this crop, enabling higher gain of total leaf area and biomass (DW) in the upper plant parts of leaves and stems (Table 2). There was, however, no significant difference between these two P treatments for the root biomass of the plant.

Table 1. Effects of N on the stem and leaf characteristics and DW at 12 weeks

N (mg L^{-1})	Stem diameter (mm)	Number of branches/plant	Total leaf area (cm^2/plant)	Chlorophyll content (mg/g FW)	DW (g/plant)			LWR
					Leaves	Stems	Roots	
50	1.17±0.10	8.22±0.78	194.33±11.14	2.56±0.27	0.7199±0.0067	0.3584±0.0826	0.0842±0.0171	0.63±0.05
100	2.41±0.39	13.11±1.06	558.72±12.57	2.96±0.28	1.4884±0.1275	0.9014±0.0051	0.1786±0.0270	0.58±0.03
T-test P value	<0.05	<0.05	<0.05	>0.05	<0.05	<0.05	<0.05	>0.05

Mean±SE

Table 2. Effects of P on the stem and leaf characteristics and DW at 12 weeks

P (mg L^{-1})	Stem diameter (mm)	Number of branches/plant	Total leaf area (cm^2/plant)	Chlorophyll content (mg g^{-1} FW)	DW (g/plant)			LWR
					Leaves	Stems	Roots	
15	2.39±0.13	18.17±3.66	670.92±82.94	2.92±0.30	2.2448±0.2922	1.2099±0.1100	0.3630±0.0882	0.59±0.03
30	1.97±0.09	14.22±1.75	489.11±33.30	2.66±0.09	1.5146±0.0856	0.7684±0.0443	0.2712±0.0247	0.59±0.01
45	1.40±0.26	12.33±1.20	274.83±35.13	2.29±0.16	0.8946±0.1318	0.3572±0.0226	0.1046±0.0114	0.66±0.02
60	1.44±0.06	12.00±0.51	279.67±25.35	2.68±0.18	0.7457±0.0384	0.3490±0.0421	0.1046±0.0128	0.62±0.04
LSD	0.51	6.95	162.24	0.64	0.5466	0.2066	0.1578	0.08

Mean±SE

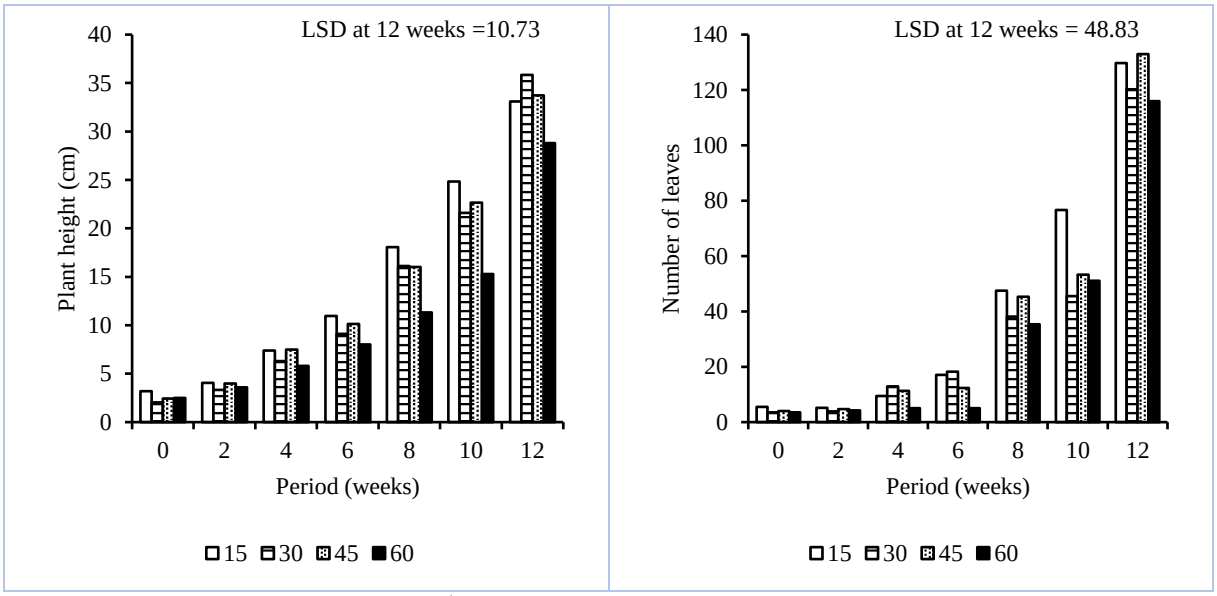

Figure 2. Effects of P (mg L^{-1}) on plant height (left) and number of leaves (right)

This crop generally showed some toxicity symptoms with higher rates of P at 45 and 60 mg L^{-1}. The stem diameter, branching, total leaf area and DW in the plants were reduced at these two higher rates of P (Figure 3; Table 2). Despite differences in the plant growth, all the plants did not differ in leaf chlorophyll content and leaf weight ratio. All the plants also did not flower throughout the study period of 12 weeks with varying P treatments.

Figure 3. Plants following P treatment at 15, 30, 45 and 60 mg L^{-1} (left to right) for 12 weeks

4. Discussion

N is inevitably essential for plant growth. It is the building block for many metabolites in plants making many biochemical reactions possible. N is also a part of chlorophylls that are involved in photosynthesis. Most plants can contain 3-4% N in their aboveground tissues, especially in the green organs of leaves and young stems. Sufficient supply of N to plant is, hence, a significant aspect in obtaining optimum plant growth and yield, in particular for optimum leaf yielding (Rashwan & Ferweez, 2017).

In the current study, *S. rebaudiana* plants also demonstrated the need for appropriate N of 100 mg L^{-1}. It generally had double growth performance and biomass by means of leaf, stem and root DW as compared to those treated at lower N rate of 50 mg L^{-1}, although there was no chlorosis symptom in the plants grown with this lower N level.

Stevia rebaudiana grown indoors with nutrient film technique was, however, very sensitive to higher N levels in our attempts to gain higher leaf production. Increasing N to 150 mg L^{-1} itself was detrimental to this crop. In some field trials, excessive N caused weak stem growth and crop lodging, while increasing plant susceptibility to pests and diseases. Other studies mentioned that over-supply of N fertigation to plants could reduce their quality and antioxidant contents (Tavarini et al., 2015; Elhanafi et al., 2019). Increasing N supply to this sweetener bearing crop must be handled with cautions in efforts to enhance its leaf production.

P, on the other hand, is known for its vital functions in cell division, nucleic acid synthesis, growth of new tissues, energy transmissions and many biochemical regulations in plants (Mamta et al., 2010; Gupta et al., 2011; Behnmimou et al., 2018). Our results indicated that *S. rebaudiana* is contented with low P of 15 mg L^{-1}. Elevated levels of P is unnecessary for this crop for indoor production under LED with nutrient film technique. Although more robust roots were developed on plants treated with higher P of 30 mg L^{-1}, this higher P treatment did not contribute to higher leaf biomass, which is the best plant part for extraction of sweeteners from this crop.

With each plant producing mean leaf DW of 2.2448 g at P of 15 mg L^{-1} as recorded in this study, its estimated yield with a five-layer vertical crop production setup is about 1,000 kg leaf DW Ha^{-1} within 12 weeks, at single layer plant density of 100,000 plants Ha^{-1}, which is possible based on the plant size obtained in the current study. This will fetch equivalent annual field production of 4 tonnes leaf DW Ha^{-1} (Umesha et al., 2011; Kumar et al., 2012; Pal et al., 2015; Rashid et al., 2015). In addition, plants grown indoor under LED are free from pollutants and contaminants that are experienced by the plants in the field (Folta et al., 2005; Massa et al., 2008; Morrow, 2008; Yeh & Chung, 2009; Hogewoning et al., 2012; Gupta & Jatothu, 2013). Flowering of the plants can also be avoided, probably with the 16h light photoperiod as studied, as this crop is an obligate short-day plant (Ceunen & Geuns, 2013). Further study on the sweetener yielded from this crop under LED is needed in promoting the indoor production for this industry crop.

5. Conclusion

Taking into account the results from the two experiments carried out simultaneously, we conclude that N at 100 mg L^{-1} and P at 15 mg L^{-1} in the half strength Hoagland solution were best for *S. rebaudiana* grown indoors with nutrient film technique. With multi-storey crop production scheme on racks, this crop could achieve yield equivalent to the field setup when it is grown under 16h light provided by LED under the controlled indoor environment.

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