UNIVERSITI PUTRA MALAYSIA

GENETIC VARIATION AMONG Stevia rebaudiana Bertoni GENOTYPES UNDER LIGHT TREATMENT ON MORPHO-AGRONOMIC TRAITS AND ISSR MOLECULAR MARKERS

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By

DHULFIQAR AAMER ABDULAMEER

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

To my lovely country Iraq

To my beloved family who always supported and kept praying for me day and night

to be what I am now

And

To my friends who boosted me all these past years

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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February 2018

Chairman : Professor Mohd Rafii Yusop, PhD
Faculty : Agriculture

Stevia rebaudiana Bertoni is a perennial herb with natural sweetening compounds that are 230 times sweeter than sucrose with no calorific value. It belongs to the family *Asteraceae* (Compositae) and is native to Paraguay. It is a good source of carbohydrates, protein, crude fibers and minerals. Stevioside and rebaudioside A are the two major sweetening compounds. Stevia is a short day plant which form flowers when day length is less than 12 hours and it is resulting in low biomass production. In this study, a set of stevia genotypes collected from different sources were evaluated for yield components and flowering characteristics both under short day length (12 hours) and long day length (14 hours) environments. Genetic variation among those genotypes was also investigated using ISSR molecular markers. A total of twenty-six stevia genotypes including two hybrids were collected. The propagation of all collected stevia genotypes was achieved through stem micro-cutting within six months. Eight genotypes namely, CHV1, Morita2, MS012, MS012 (4x), SA18, TPU, and hybrids IIUM-F_1 and UiTM-F_1 which had shown good propagation ability under mist-chamber propagation boxes and were evaluated for the agronomic performance under short day length (12 hours) and long day length (14 hours) environments using a split plot design. In general, all the agronomic characteristics of pooled genotypes were increased significantly under the extended light (14 hours). Stevia plants under sunlight (12 hours) started to flowering after 52 days after transplanted. On the other hand stevia plants under extended light were not flowered up to 134 days at harvesting stage. Combined biomass fresh weight mean of the eight genotypes was increased 728% under extended light compared to genotypes kept under sunlight. The biomass fresh weight mean and plant height of genotype SA18 was the highest in both extended and sunlight conditions (35.82 g and 3.96 g; 92.47 cm and 24.92 cm respectively). The Pearson correlation coefficient showed that biomass fresh weight had strongly positively correlated with all the

characters except number of branches. Genetic variation and cluster analysis based on ISSR markers showed that genotypes CHV1 and H2 were the most closely related genotypes with the highest similarity index (54%). On the other hand, genotypes MS012 and TPU were the distantly related genotypes with the lowest similarity index (24%). In conclusion, stevia plants kept under extended light showed better performance than under sunlight. Three genotypes, SA18, TPU and CHV1 were shown better performance than other genotypes, and are recommended for further utilization in future stevia breeding programs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

VARIASI GENETIK KALANGAN GENOTIP *Stevia rebaudiana* Bertoni DI BAWAH RAWATAN CAHAYA KE ATAS CIRI MORFO-AGRONOMI DAN PENANDA MOLEKUL ISSR

Oleh

DHULFIQAR AAMER ABDULAMEER

Februari 2018

Pengerusi : Profesor Mohd Rafii Yusop, PhD
Fakulti : Pertanian

Stevia rebaudiana Bertoni adalah herba saka yang mengandungi kompaun pemanis semulajadi sebanyak 230 kali lebih manis daripada sukrosa dan ianya tanpa nilai kalori. Ia tergolong di bawah keluarga *Asteraceae* (Compositae) dan yang berasal dari Paraguay. Ia merupakan sumber karbohidrat, protein, fiber mentah dan zat galian. Stevioside dan rebaudioside A adalah dua kompaun pemanis utama spesies ini. Stevia adalah tumbuhan hari-pendek yang mengeluarkan bunga apabila waktu siang kurang dari 12 jam dan seterusnya ianya mengakibatkan pengeluaran biojisim yang rendah. Dalam kajian ini, satu kumpulan genotip stevia yang dikumpulkan dari beberapa sumber yang berbeza telah dinilai dari segi komponen hasil dan ciri pembungaan di bawah persekitaran jangka siang yang pendek (12 jam) dan jangka siang yang panjang (14 jam). Variasi genetik di kalangan genotip juga telah dikaji dengan menggunakan penanda molikul ISSR. Sejumlah dua puluh enam aksesi stevia termasuk dua hibrid telah dikumpulkan. Pembiakan semua aksesi stevia telah dijalankan melalui keratan mikro batang dalam tempoh enam bulan. Lapan genotip iaitu; CHV1, Morita2, MS012, MS012 (4x), SA18, TPU, dan hibrid IIUM-F_1 dan UiTM-F_1), yang menunjukkan prestasi pembiakan yang baik di bawah kotak pembiakan berkabus, dan telah dinilai dari segi prestasi agronomi di bawah jangka siang yang pendek (12 jam) dan jangka siang yang panjang (14 jam) dalam satu eksperimen faktorial. Secara umumnya, kesemua ciri agronomi bagi kombinasi genotip didapati bertambah di bawah pencahayaan yang dilanjutkan. Pokok stevia di bawah rawatan cahaya (12 jam) mulai berbunga 52 hari selepas penanaman. Sebaliknya pokok di bawah pencahayaan lanjutan, pokok stevia tidak berbunga sehingga 134 hari selepas penanaman pada peringkat penuaian. Berat basah biomass keseluruhan bagi lapan genotip tersebut telah meningkat 728% di bawah pencahayaan lanjutan berbanding pokok di bawah cahaya matahari. Purata berat basah biomass dan ketinggian pokok aksesi SA18 didapati adalah tertinggi di bawah

kedua-dua persekitaran, pencahayaan lanjutan dan cahaya matahari (masing-masig l35.82 g dan 3.96 g; 92.47 cm dan 24.92 cm). Pekali korelasi Pearson menunjukkan berat biomass segar adalah berkorelasi tinggi secara positif dengan semua ciri-ciri kecuali dengan bilangan dahan. Analisa variasi genetik dan kluster berdasarkan penanda ISSR menunjukkan genotip CHV1 and H2 mempunyai perkaitan genotip yang paling rapat dengan indeks kesamaan tertinggi (54%). Sebaliknya, genotip MS012 and TPU adalah genotip yang paling berbeza dengan indeks kesamaan paling rendah (24%). Kesimpulannya, pokok di bawah pencahayaan lanjutan menunjukkan prestasi yang lebih baik daripada pencahayaan matahari. Tiga genotip iaitu SA18, TPU dan CHV1 didapati berprestasi lebih baik daripada semua genotip lain, dan dicadangkan untuk digunakan seterusnya di dalam program pembiakbakaan stevia di masa hadapan.

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Dhulfiqar Aamer Abdulameer, 2018

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphisms
bp	Base pair
CV	Coefficient of Variation
DNA	Deoxyribonucleic acid
ISSR	Inter simple sequence repeat
NAA	α- naphthaleneacetic acid
NTSYS-pc	Numerical taxonomy and multivariate analysis system
PCA	Principal component analysis
PCR	Polymerase Chain Reactions
POPGENE	Population genetic analysis
RAPD	Random amplified polymorphic DNA
SSR	Simple sequence repeat
Taq	Thermus aquaticus
TBE	Tris-borate EDTA
UPGMA	Unweighted pair group method based on arithmetic averages

CHAPTER 1

INTRODUCTION

Human interest in natural products is increasing day by day. *Stevia rebaudiana* Bertoni commonly known as candy leaf or sweet leaf (generically as stevia) is an ancient perennial shrub of South America. It belongs to the same family with sunflower (*Asteraceae*) and produces diterpene glycosides that are 300 times lower in calorie sweeteners than saccharose. About 240 stevia species have been identified and characterized, among these species the sweetest essences have successfully been extracted from *Stevia rebaudiana*. Presently, stevia has a great potential as a modern agricultural crop since consumer demand increased for herbal foods, zero-calorie sweetener and appropriate gluco-regulation for consumers afflicted with diabetes and prevention of dental cavities (Thomas, 2010).

The chemical compounds that produce sweetness in stevia are various steviol glycosides while the major components are stevioside and rebaudioside A (Singh and Rao, 2005). Stevioside is the sweet glycoside with the highest concentration found in stevia and contributing about 60-70% of total glycosides (Yadav *et al.*, 2011) but it has licorice aftertaste. However, rebaudioside A contributing about 30-40% of total glycosides in stevia and has no aftertaste (Midmore and Rank, 2002). In addition to sugary compounds, stevia extracts also contain a high level of therapeutic properties that plays an imperative role as antioxidant, antimicrobial, antifungal activities and also serves a good source of fiber and minerals (Abou-Arab *et al.*, 2010).

In Malaysia, stevia was introduced in the 1970s. The initial studies on propagation, cultivation and germplasm evaluation were carried out by Tan *et al.* (2008) under Malaysian Agriculture Research and Development Institute (MARDI). From the study, out of 69 genotypes five genotypes were recommended for local cultivation. However, the major problem for stevia cultivation in Malaysia is photoperiod sensitiveness that affects the early flowering which contributes to low leaf yield and stunted plant growth. Several reports from researchers from Universiti Teknologi MARA (UiTM), Universiti Putra Malaysia (UPM) and International Islamic Universiti Malaysia (IIUM) found that no suitable variety for high biomass production in Malaysia (per comm. Osman 2018). Meanwhile, other countries such as China and Japan, are successful in development of suitable stevia varieties for their local cultivation.

Genetically, *S. rebaudiana* B. is a diploid plant with 22 chromosomes. Several species of stevia have similar characteristics but possess different karyotypes caused by the gain or loss of chromatin due to spontaneous mutation (Frederico and Ruas, 1996). Polyploidy is also recorded in *S. rebaudiana* B. through artificial induction by developing lines containing elevated levels of stevioside (Oliveira *et al.*, 2004). The

stevia has been extensively documented as self-incompatible and pollination assisted by insects (Ramesh *et al.*, 2006). Goettemoeller and Ching (1999) reported that fertile seeds were obtained from self-pollination, however, how much seeds collected was unknown and neither was there any verification of the progeny testing. As a whole, stevia is a highly outcrossing plant that is challenging for breeders to develop inbred lines, whereas, high heterozygosity offers variation to develop hybrid variety.

The agronomical practices for cultivation of stevia needs an optimum temperature of 20-28 °C for good growth, clay sandy soil that rich in organic fertilizers with intensive damping, high illumination and the day length of more than 13 hours per day (Soejarto *et al.,* 1982). The local environment condition of Malaysia can provide the plant with all these conditions except the required day length which leads to early flowering. Because the harvesting of stevia aerial parts need to be carried out before flowering as the level of the sweetness decreases as soon as the plants begin to flower. In addition, the production of stevia varieties with a longer vegetative period in the tropical environment requires a depth study on genetic diversity among the available genotypes.

This study was designed with a set of stevia genotypes collected from different sources evaluated for agronomic traits under short day length (<13 hours) and long day length (>13 hours) conditions. The genetic variation among these genotypes was determined using agronomic characteristics and ISSR molecular markers with the following objectives;

i. To evaluate the agronomic performance of stevia genotypes collected from different sources under short and long day length environmental conditions.
ii. To assess the genetic diversity among stevia genotypes using ISSR molecular markers.
iii. To identify superior genotypes for future stevia breeding programs.

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