DEVELOPMENT OF NEW STEVIA VARIETY WITH HIGH PARTICULAR TYPES OF STEVIOL GLYCOSIDES FOR GROWING IN MALAYSIA

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By

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DEVELOPMENT OF NEW STEVIA VARIETY WITH HIGH PARTICULAR TYPES OF STEVIOL GLYCOSIDES FOR GROWING IN MALAYSIA

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Chairman : Associate Professor Janna Ong Abdullah, PhD
Faculty : Biotechnology and Biomolecular Sciences

Stevia rebaudiana Bertoni from Asteraceae family is commercially valuable for its steviol glycosides(SGs) contents, which is 300 times sweeter than commercial sugar. However, the bottleneck in Malaysia is the lack of suitable stevia varieties that are able to thrive well under her climatic conditions and still produce high SGs. Mutation induction including gamma irradiation is effective in generating genetic variation and developing new plant varieties with desired traits. Hence, this study was aimed to determine the effects of acute and chronic gamma irradiation on phenotypic changes and enhancement of SGs contents of two selected stevia varieties, AKH L1 and UKMB40408. For acute gamma irradiation, *in vitro* shoot tip explants of stevia AKH L1 variety were subjected to a gamma doses regime of 10Gy to 50Gy, following which phenotypic changes of the irradiated explants and subsequent regenerated plantlets were observed. All irradiated explants exhibited different survival rates, with the lowest at 9.33±8.33% when subjected to 50Gy, while all the control (non-irradiated explants) survived. Acute irradiation at 50Gy was shown to result in poor plants growth and development with necrotic leaf morphology. The LD_{50} (dose resulting in 50% dead) was found to be at 23Gy. Subsequent irradiation of 900 shoot tip explants at 23Gy, and all surviving produced 468 surviving shoot tipswhich were all capable to develop and successfully subculture until the fourth generation, M_4. TheseM_4 *in vitro* mutant plantlets exhibited significant increased in the numbers of leaf (16.07±5.19) and average leaf size (1.12cm±0.26x0.54cm±0.15).High Performance Liquid Chromatography (HPLC) analysis performed in parallel further revealed the mutant plants contained higher concentrations of stevioside (387.04ppm), rebaudioside A (670.18ppm) and rebaudioside D (106.26ppm) compared to the non-irradiated plantlets which exhibited 96.87, 194.42 and 28.25ppm, respectively. Unfortunately, all the mutated *in vitro* stevia variety AKH L1

plantlets exhibited stunted growth after 12 sub-culturing cycles. In another parallel set of experiment with similar irradiation dose performed on 300 plantlets revealed that all samples failed to survive when acclimatized to glasshouse condition. All the roots exhibited poor histomorphological appearances such as loose cortex, loose vascular bundles and broken epidermal cells. Meanwhile, for the chronic irradiation, potted plants of stevia variety AKH L1subjected to a 10Gy to 50Gy doses regime showed that the suitable dose rate (Gy/hour) was 0.17Gy/h with LD_{50} of 35.5Gy. The irradiated potted plants exhibited increased plant height of 75.62±15.06cm and numbers of leaf of 43.5±3.54. Unfortunately, all 54 irradiated potted stevia AKH L1failed to survive one month after the irradiation event. Thus, the chronic experiment was repeated again with another steviavariety UKMB40408, which thrive well in Malaysia but possesses lower SGs contents. This stevia variety, when subjected to 30Gy to 50Gy, showed increased stevioside (149.92ppm, 150.13ppm, and 151.31ppm, respectively) and rebaudioside A (275.60ppm, 271.65ppm, and 272.17ppm, respectively) contents compared to stevioside (112.30ppm) and rebaudioside A (209.73ppm) of the control (non-irradiated). In conclusion, application of gamma irradiation did enhance the plant height, number of leaves, and SGs contents of stevia variety AKH L1, and also the SGs contents of stevia variety UKMB40408. This shows that gamma irradiation proved to be a useful, easy and rapid means of enhancing key traits in different stevia varieties.

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

PEMBANGUNAN STEVIA VARIETI BARU DENGAN STEVIOL GLIKOSIDA TERTENTU YANG TINGGI YANG SESUAI UNTUK DITUMBUH DI MALAYSIA

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Stevia rebaudiana Bertoni dari klasifikasi Famili Asteraceae adalah sangat bernilai kormersial dari segi steviol glikosida yang 300 kali kemanisan daripada sukrosa.Malaysia kekurangan stevia spesies yang dapat berkembang dengan baik di samping menghasilkan steviol glikosida yang tinggi disebabkan oleh keadaan iklim di Malaysia.Induksi mutasi termasuk radiasi gamma adalah berkesan dalam menjana variasi genetik dan menghasilkan tumbuhan varieti dengan sifat yang dikehendaki.Oleh itu, projek ini bertujuan untuk tentukan kesan radiasi gamma atas perubahan fenotip dan peningkatan steviol glikosida pada dua varieti stevia yang telah ditentukan. Untuk iradiasi tunggal, kultur in vitro stevia varieti AKH L1 telah radiasi pada dosis dari 10Gy hingga 50Gy, dan perubahan fenotip atas kultur yang diradiasi and kultur yang tumbuh seterusnya telah diperhatikan. Semua kultur yang telah diradiasi mempamerkan kadar hidup yang berlainan, yang paling rendah pada 9.33±8.33% apabila diradiasi dengan 50Gy manakala paling tinggi pada 100% pada tumbuhan yang tidak diradiasi (0GY). Iradiasi tunggal pada 50Gy membawa pertumbuhan dan perkembagan tumbuhan yang teruk dengan daun yang kebanyakan nekrotik.Berdasarkan hasilan yang didapati, nilai LD_{50} (dosis yang menyebabkan 50% kematian dari populasi yang diradiasi) adalah pada 23Gy. Radiasi seterusnya pada 900 tunas in vitro telah ditentukan pada dosis 23Gy. Tunas yang masih hidup lepas iradiasi telah disubkultur sehingga generasi keempat, M_4. Hasil kajian didapati bahawa kultur in vitro mempamerkan signifikasi peningkatan pada bilangan daun (16.07±5.19) dan purata saiz daun (1.12cm±0.26x0.54cm±0.15). Kromatografi Cecair Prestasi Tinggi (HPLC) juga menunjukkan tumbuhan yang mutasi dicirikan dengan stevioside (387.04ppm), rebaudioside A (670.18ppm) dan rebaudioside D (106.26ppm) yang lebih tinggi berbanding

dengan tumbuhan yang tidak diradiasikan pada 96.87, 194.42 and 28.25ppm. Malangnya, semua mutasi kultur in vitro stevia variety AKH L1 menjadi terbantut selepas disubkulturkan sebanyak 12 kali kitaran. Semua 300 kultur in vitro stevia variety AKH L1 yang telah diradiasi mati apabila penyesuaiikliman pada keadaan rumah kaca. Kesemua perakaran mempunyai histomorphological yang kurang baik seperti korteks yang longgar, bundle vascular yang longgar and sel epidermis yang terkoyak. Untuk iradiasi kronik, stevia variety AKH L1 dalam pepasu telah diradiasi dengan 10Gy hingga 50Gy, dan dosis yang paling sesuai adalah pada 0.17Gy/h dengan nilai LD_{50} pada 35.5Gy. Stevia yang telah diradiasi menunjukkan pertumbuhan ketinggiannya pada 75.62±15.06cm dan bilangan daun pada 43.5±3.54.Kesemua 54 stevia yang telah diradiasi tidak dapat hidup selepas satu bulan.Oleh itu, eksperimen kronik telah diulangi pada varieti stevia variety UKMB40408 yang memang tumbuh di Malaysia tapi dengan steviol glikosida yang lebih rendah. Stevia varietiUKMB40408 menunjukkan peningkatan dalam stevioside (149.92ppm, 150.13ppm, and 151.31ppm) dan rebaudioside A (275.60ppm, 271.65ppm, and 272.17ppm) apabila diradiasi pada 30Gy hingga 50Gy, manakala stevia yang tidak diradiasi mempunyai stevioside pada 112.30ppm dan rebaudioside A pada 209.73ppm. Pada keseluruhan, kajian ini telah menunjukan bahawa aplikasi radiasi gamma dapat meningkatkan ketinggian tumbuhan, bilangan daun dan kandungan steviol glikosida pada varieti stevia variety AKH L1, dan juga kandungan steviol glikosida pada varieti stevia variety UKMB40408.Iradiasi gamma telah terbukti sebagai teknik yang berguna, senang dan cepat dalam meningkatkan ciri-ciri yang dikehendaki dalam varieti stevia yang berlainan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment 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

%	Percentage
˚C	Degree Celcius
µL	Microliter
µm	Micrometer
^{60}CO-γ	Gamma spectrum of cobalt 60
BAP	6-benzylaminopurine
cm	Centimeter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methane sulfonate
g	Gram
g/L	Gram per Litre
Gy	Gray
Gy/h	Gray per hour
H_2O	Chemical formula of water
HCl	Hydrochloric acid
IAEA	International Atomic Energy Agency
IBA	Indole-3-butyric acid
keV	Kiloelectronvolt
L	Litre
LD_{50}	Lethal dose that killed 50% of population
M	Molar
M_4	4th generation
mg/L	Milligram per Litre

ml	Millilitre
mL/min	Millilitre per minute
mm	millimetre
MS	Murashige and Skoog
NaOH	Sodium hydroxide
p.a.	Pascal unit
Reb A	Rebaudioside A
Reb D	Rebaudioside D
RNA	Ribonucleic acid
SD	Standard deviation
SGs	Steviol glycosides
Stev	Stevioside
UV	Ultraviolet
v/v	Volume / volume

CHAPTER 1

INTRODUCTION

1.1 Background of study

Stevia rebaudiana (Bertoni), a perennial sweet herb belongs to the family Asteraceae, is one of the 154 members of the genus *Stevia*. This plant is the most important source of non-caloric natural sweeteners and mainly known as "Sweet Weed', "Sweet Leaf", "Sweet Herbs" and "Honey Leaf". The property of the species that caught attention to the plant is the intense sweet taste of the leaves and aqueous extracts (Uddin *et al.*, 2006). The extract of stevia plant contains mixture of various sweet steviolglycosides (SGs) such as stevioside, rebaudioside A, B, C, D, E and F, dulcoside A, steviolbioside, steviolmonoside, and rubusoside, which accumulate in the leaves and is 300 times sweeter than sugar (Ahmad *et al.*, 2011; Reis *et al.*, 2011; Mathur & Shekhawat, 2012). The compounds that made up the majority proportions of SGs are stevioside and rebaudioside A, and they had been extensively used in the food and beverage industry for decades.

Stevia rebaudiana leaves contain non-cariogenic and non-caloric sweeteners (SGs), which exert beneficial effects on human health (Gardana *et al.*, 2010). According to Summon *et al.* (2008), the powdered form of stevia leaves possessed both hypoglycemic and body weight reducing effect without any adverse effects for diabetic patients. Besides that, stevia can also act as an antimicrobial agent which inhibited the growth of *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis* and *Escherichia coli* (Debnath, 2008). Stevia is also a suitable raw material for the extraction and production of food ingredients. It is a good source of carbohydrates, protein, crude fibre, minerals, as well as dispensable and indispensable amino acids, which are valuable for human nutrition (Madan *et al.,* 2010; Lemus-Mondaca *et al*., 2012).

Jain (2010) proposed that mutation breeding were preferred over traditional breeding methods and genetically modified organisms (GMO) in the past few years. This is because multiple trait mutants can be isolated in mutation induction, but only single trait can be introduced into the crop in transgenic way. Also, mutation induction can help to establish mutant lines range and determine trait specific genes in order to create molecular gene database, for molecular and functional genomics study and improve bioinformatics for future plant varieties development (Hase *et al.,* 2000).Radiation treatment of plants had been reported as one of the most familiar methods for induction of plant mutations where the mutantvarieties were useful in developing new plant

varieties as well as for functional studies of genes (Tanaka *et al.,* 2002; Shikazono *et al.*, 2003).

1.2 Problem statement and justification of study

In Malaysia, stevia is not recognized as a commercial crop due to lack of suitable variety when it was first introduced in the mid-1970s (Armizatul *et al*., 2009). Stevia is a short day plant with a critical day length of at least about 13 hours (Lester, 1999).This was in line with Armizatul *et al*. (2009) report where reduced vegetative growth and lower stevioside content was observed in stevia when day length in Malaysia was less than the critical 13 hours. Stevia plants with greater plant height which brings to more leaves produced are preferred since accumulation of SGs happened in the leaves. Thus, induced mutagenesis especially gamma irradiation has potential to be applied on stevia to developnew mutant varieties with better key traits and suitable for growing under Malaysia's climatic condition.

1.3 Research objectives

There is still limited report regarding the effect of gamma irradiation on the accumulation of SGs contents of stevia in Malaysia. Hence, the main objective of this study was to create new varieties of stevia with high particular types of sweet glycosides *via* gamma irradiation suitable for growing in Malaysia. The specific objectives of the present study were:

1. to determine the effects of gamma irradiation on phenotypic changes of selected stevia varieties,
2. to measure the steviol glycosides(SGs) contents of selected stevia varieties, and
3. to glasshouse acclimatize *in vitro* stevia plantlets.

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