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A Review on Induced Mutagenesis of *Stevia rebaudiana* Bertoni

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Abstract – *Stevia rebaudiana* Bertoni in the Asteraceae family is commercially valuable and cultivated throughout the world due to the great demand for its steviol glycosides (SGs) contents particularly rebaudioside A. Previous studies confirmed that maximal content of SGs in stevia was achieved at or just before flowering, and delayed flowering with long days provide longer duration for steviol glycosides accumulation. However, there is no suitable stevia variety to be cultivated in Malaysia due to her short day length. Mutation induction, including gamma irradiation, had been shown to be useful for generating genetic variations as well as developing new plant varieties from which desired mutants were successfully selected. The use of mutagens, both physical and chemical, has helped in creating mutants that expressed the selected desirable traits. This paper presents some selected essential data available in extant scientific studies on stevia with the focus on application of gamma irradiation on stevia. Both established achievements and recent publications of gamma radiation on stevia were reviewed. Emphasis is on the exceptional potential of stevia through induced mutation approach especially by using gamma rays.

Keywords: Gamma irradiation, induced mutagenesis, *Stevia rebaudiana*, steviol glycosides

Introduction

Stevia rebaudiana Bertoni (hereinafter designated as "stevia"), the indigenous plant of Paraguay, is a perennial herb of the Asteraceae family. *Stevia rebaudiana* stands out among 230 species in the *Stevia* genus because it gives the sweetest taste, about 300 times sweeter than sugar (Soejarto, Compadre, Medon, Kamath, & Kinghorn, 1983; Debnath, 2008). Early studies revealed that stevia was known to the Spanish in the 16th century. However, it remained insignificant until it was 'rediscovered' by Dr. M. S. Bertoni in 1888 in Paraguay. Then in 1905, the plant was scientifically described and named in honour of a Paraguayan chemist, Dr. Rebaudi (Ramesh, Singh, & Megeji, 2006). Due to its natural extreme sweetness, it was given several names, among them honey leaf, sweet leaf of Paraguay, sweet leaf, sweet herb, candy leaf, and honey yerba (Carakostas, Curry, Boileau, Brusick, 2008).

The compounds that provide the sweetness of stevia are steviol glycosides (SGs, i.e. sweet glycosides). To date, the sweetening compounds that had been discovered in the leaves of stevia are stevioside, steviolbioside, rebaudiosides (A, B, C, D, E, F, M and N), rubusoside and dulcoside A with their structures described by Brower III et al. (2014). Out of various SGs reported, stevioside and rebaudioside A are the main metabolites as they render sweetness to stevia (Yadav et al., 2011). Stevioside comprises 60-70% of the total glycosides content and has been evaluated as being 110-270 times sweeter than sugar (DuBois, 2000). It is believed to give a somewhat bitter aftertaste, or reported as a "licorice" taste or lingering effect (Jakinovich, Moon, Choi, & Kinghorn, 1990). Meanwhile, rebaudioside A was present in 30-40% of the total sweet content and assessed to be 180-

400 times sweeter than sugar with more pleasant sweet taste and no bitter aftertaste (DuBois, 2000). Therefore, a higher ratio of rebaudioside A to stevioside indicates a better sweetness quality, and is hence preferred as reported by Yadav, Singh, Dhyani, & Ahuja, (2011). The stevia leaves contained the highest SGs content, followed by flowers, stems, and seeds, whereas the root was the only organ that did not contain SGs. Also, the maximal content of SGs was observed during formation of the flower buds and it then gradually decreased (Bondarev, Sukhanova, Reshetnyak, & Nosov, 2003; Yadav et al., 2011).

As a result of its sweetness and advantageous effects on human health, stevia has attracted economic and scientific interests these days. Japan was the first Asian country to market stevioside as a sweetener in the food and drug industries (Chatsudthipong and Muanprasat, 2009). Since then, stevia has been adopted by other Asian countries as a natural sweetener and commercialised in China, South Korea, Thailand, Singapore and Malaysia (Chatsudthipong and Muanprasat, 2009; Lemus-Mondaca, 2012). In addition to being a non-caloric sweetener, stevia possesses several therapeutic benefits such as anti-microbial (Debnath, 2008), anti-fungal (Silva, Oliveira, Prado, Carvalho, & Carvalho, 2008), anti-virus (Kedik et al., 2009), anti-hypertensive (Hsieh et al., 2003), anti-hyperglycemic (Benford et al., 2006), anti-inflammatory (Takahashi et al., 2001; Gupta, Purwar, Sundaram, & Rai, 2013), anti-tumour (Jayaraman, Manoharan, & Illanchezian, 2008), anti-diarrhoeal (Takahashi et al., 2001; Sattigeri, 2012), diuretic (Takahashi et al., 2001), and immunomodulatory effects (Chatsudthipong and Muanprasat, 2009). Stevia is postulated to have great potential to become a major source of natural sweetener for the growing natural food market in future as the leaves of stevia contain functional and sensory properties more superior than those of several other high-potency sweeteners available (Goyal and Goyal, 2010).

Need for Induced Mutagenesis of *Stevia rebaudiana*
Sucrose is the most widely used sweetener worldwide, yet a high daily intake of sucrose will lead to several metabolic disorders and ailments, significantly obesity, diabetes and dental caries (Philippe, De Mey, Anderson, & Ajikumar, 2014). Malaysians have been ranked as the most obese of all South East Asian populations and the sixth in Asia-Pacific (Patrick, 2014). Also, there were 3.3 million cases of diabetes in Malaysia in 2015 (International Diabetes Federation, 2015). Presently, consumption of alternative sugar such as low calorie sweetener is on the rise due to increasing awareness about the effects of sugar intake. Low-caloric sweeteners are believed to help in improving population heath by inducing weight loss and weight management (Wiebe et al., 2011).

Artificial sweeteners are mostly developed from synthetic compounds in the laboratory and most of them have safety issues and are harmful to human as previously reported (Kim, Seo, & Cho, 2011; ŠicŽlabur et al., 2013).Aspartame is an example of a widely used artificial sweetener in the food and beverage industries (American Cancer Society, 2014). However, it is not heat stable and thus unsuitable for baking or cooking (DuBois and Prakash, 2012). It has been reported that aspartame ingestion has serious negative side-effects associated with carcinogenicity and brain disorders as observed in aspartame-fed zebrafish with acute swimming defects (Kim et al., 2011). Thus, natural sweeteners are preferred. Stevia is the only natural sweeteners in the market and can be used as a complementary to sugar. Stevia possesses high commercial potential as it has excellent taste profile, synergistic with sugar and other high intensity sweeteners, non-calorie, suitable for consumption by diabetic patients, and no major safety concerns (ŠicŽlabur et al., 2013). Summon, Mostofa, Jahan, Kayesh, & Haque, (2008) revealed that the powdered form of stevia leaves had both hypoglycemic and body weight reducing effects without negative effects for diabetic patients.

Unfortunately, stevia failed as a commercial crop when it was first introduced into Malaysia in the mid-1970s due to the lack of a suitable variety (Armizatul, Azhar, & Nazrul, 2009). Stevia is a short day plant with a critical day length of at least about 13 hours (Lester, 1999). Short day length in Malaysia (less than 13 hours) leads to early flowering in stevia, with lower percentage of sweeteners content. Previous studies demonstrated that the vegetative growth and stevioside content of stevia were decreased when day length was less than the critical 13 hours (Armizatul et al., 2009). Through induced mutation, improvement of crops quality and bioactive metabolites production were developed in rice with low amylase and protein contents for special dietary needs (Amono, 2006), in rice with high-resistant starch preferred by diabetes patients (Chen, Liu, Wu & Shu, 2006), in maize with protein improvement (Tomlekova, 2010), and in cassava with high amylase content (Ceballos et al., 2008). All these studies showed that induced mutagenesis has potential to be applied on stevia to create mutants that are suitable for cultivation under Malaysia's climatic condition.

Mutation Breeding

Mutation refers to the process in which heritable change in the genes of an individual that occurs under environmental conditions. It has been the principal factor in evolution as the changes are transferred between generations, to give rise to the development of a new individual, species and genera (Mba, Afza, Bado, & Jain, 2010). It can take place in two ways: as changes in gene bases and/or alterations in the amount or structure of the chromosomes. Besides alterations in nature, induced mutagenesis has been widespread in developing science areas for the creation of variants within crop varieties by induced mutagenesis with physical and chemical mutagens (Jain, 2010).

Mutants are defined as living creatures with permanent hereditary changes that may be displayed using molecular means or distinguished by phenotypic tools (Forster, Shu, & Nakagawa, 2012). Mutation can be generated in plants by exposure to mutagens, including physical, chemical and biological means with mutagenic properties (Janick, 2015). In general, mutagen treatment damages the nuclear DNA and new mutations are induced randomly and heritable during the process of DNA repair mechanism (Mba, 2013). These changes can also happen in the cytoplasmic organelles, which result in chromosomal or genomic mutations that allow plant breeders to pick out favourable mutants (Jain, 2010). However, mutation breeding is typically focused on the use of physically and chemically induced mutagenesis, while other types of experimental mutagenesis are usually found in functional genomics studies (Novak and Brunner, 1992; Ulukapi and Nasircilar, 2015).

Mutation breeding practices are favoured over traditional breeding methods and genetically modified organisms (GMO) practices due to several beneficial properties as reported by Jain (2010). One of the dominant advantages in induced mutations is that multiple traits mutants can be isolated unlike the transgenic way whereby only a solitary trait can be introduced into the crop. In addition, mutation induction can help to establish mutant lines range and determine trait specific genes for the creation of a molecular gene database to support molecular functional genomics study and improve bioinformatics for future plant varieties development. Freisleben and Lein (1914) coined the term "mutation breeding" ('Mutationszüchtung') to describe the deliberate induction for plants product development and the establishment of mutant lines for crop development. As a result of low mutation frequency rate of spontaneous mutations, plant breeders seek new breeding technologies. Induced mutation is aimed at increasing the mutation frequency rate for the sake of selecting suitable variants for plant breeding (Jain, 2010). Thus, mutation breeding provides an alternative way in getting desired traits or features that either do not exist naturally or have been missing during the evolutionary process (Novak and Brunner, 1992).

According to the Joint Division of the Food and Agriculture Organization and International Atomic Energy Agency (Joint FAO/IAEA), at least 3233 mutant varieties had been reported in the Mutant Varieties Database (MVD). Interestingly, about 77% of these mutant crop varieties were seeds propagated which reflected the ease of using seeds instead of other vegetative propagules as starting materials for mutations induction (Mba, 2013). More than half (60%) of the mutant crop varieties were produced in Asia, while China alone contributed in excess of 25% of all mutant varieties officially reported globally (Mutant Varieties Database of Joint FAO/IAEA). Cereals made up almost half (48%) of all mutant crop varieties reported in the MVD, followed by flowers (20%), legumes and pulses (14%) and vegetables, forge, edible oil plants and trees, and others (3%) (Mutant Varieties Database of Joint FAO/IAEA).

Induced Mutation Technology

Agents that artificially induced mutations can be categorised into physical and chemical mutagens (Mba, 2013). In order to widen the genetic diversity, mutations had been induced to enhance productivity of plants in both seed- and vegetatively-propagated crops by exposing botanical seeds and vegetative parts including stem cuttings, twigs buds, and tubers to mutagenic agents (Jain, 2010; Ulukapi and Nasircilar, 2015). Mutations induction in vegetatively propagated crops was reported by Mba (2013) to be more effective as researchers imposed upon the nature of totipotency in plants.

Physical mutagens are the most frequent mutation induction technique used to develop direct mutant varieties. The types of radiation used to induce mutations are divided into ultraviolet radiation and ionising radiation (Mba, 2013). Ultraviolet radiation (UV) with wavelengths of 250-290nm is limited to treatment of cells free from extraneous tissue (i.e. pollen grains and generative cells in developing pollen tubes) as they have moderate penetration capacity and cause negligible physiological damage. On the other hand, ionising radiations (X-rays, gamma rays, fast and thermal neutrons, alpha and beta particles) have better capacity to penetrate plant tissue and can induce more chemical changes (Jain, 2010). Ionising radiations have their own unique properties and vary in their ionisation capacities as reported (Forster et al., 2012; Mba, 2013). The two key factors that influence the efficiency of physical mutagen in plant mutagenesis are energy and ability to penetrate the tissues. However, other parameters, for instance, the source availability and accessibility, the suitability of the mutagens used, the safety of both treatment and post-treatment management, and the cost of treatment can impact the performance of a particular mutagen (Forster et al., 2012). Yang et al. (2013) commented that ^{60}CO-γ and ion beam injections had mutagenic impacts on S. *rebaudiana* hybrid progenies whereby they exhibited lower height, consistent leaf shape, fewer branches, shorter internode length, lodging and were cold-resistant. Khalil, Zamir & Ahmad, (2014) observed higher biosynthesis of stevioside content from *in vitro* stevia shoots than other *in vitro* and *in vivo* grown tissues after gamma irradiation. Awad (2009) also reported gamma radiation dose induced an increased in total carbohydrate with doses of 20 Gray and 30 Gray (Gy), increment in total protein content with 10 Gy and 20 Gy, and increment in nucleic acid RNA but reduction in total DNA in stevia.

Chemical mutagenic agents are effective as they resulted in high rates of mutation especially point mutation (Jain, 2005). Base analogues, alkylating agents, nitrous acid, intercalating agents and chemicals that alter the structure of DNA are considered as chemical mutagens. They vary in their mode of action towards DNA as explained by Toker, Yadav, & Solanki, (2007) and Forster et al. (2012), such as deamination, transitions and insertions induction, the stoppage of transcription and replication or strand break. More than 80% of the mutant varieties recorded in the IAEA mutant varieties database were the result of alkylating agents (Mutant Varieties Database of Joint FAO/IAEA). Among these compounds, the most popular and widely used chemical mutagen for mutation induction is ethyl methane sulfonate (EMS; $CH_3SO_2OC_2H_5$) (Forster et al., 2012). EMS is classified as a mono-functional alkylating agent, and it leads to high gene mutation frequency and low chromosome aberration frequency (Kodym and Afza, 2003; Lai, Huang, Wang, Li, & Wu, 2004). The selection of an efficient dose of the chemical mutagens to induce mutation of the starting material depends upon the intensity and period of treatment, the solvent employed or the pH of the solution (Jain, 2010). Khan et al. (2016) successfully developed stevia mutants with higher stevioside and Reb-A content through EMS and gamma ray. Higher expressions of several important enzymes from UDP Glucosyltransferase family involved in the steviol glycoside biosynthesis pathway were observed in the mutant strains of stevia (Khan, Rahman, Verma, & Shanker, 2016). Colchicines was also found to be effective as a polyploidizing agent in developing new stevia variants with two-fold increase in steviol glycosides contents including steviosides and Reb-A (Rameshsing, Hegde, & Vasundhara, 2015). Ke-qin et al. (2012) proved that 9 mmol L^{-1} NaN_3 mutation treatment was able to cause stress effect in *S. rebaudiana* in a short period of time.

Overall, physical mutagen offers less risk compared to chemical mutagens as it is non-toxic and no detoxification or cleaning of mutagens from the materials is required after the practice (Khan et al., 2000; Mba, 2013). Similarly, Ali, Aboshosha, Kassem, EL-Dabaawy, & EL-Banna,. (2015) concurred that chemical mutagenic agents are not usually successful and not a choice to consider for inducing mutation in vegetatively propagated plants such as stevia, mainly due to the poor uptake and low penetration of the chemical compounds into the plant tissues. Physical mutagen, especially for gamma rays, provides accurate dosimetry which allocates sufficient reproducibility and a high and uniform

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penetration capability to plant tissues (Jain, 2005). Besides, physical mutagens are cost effective, bring about fast responses as well as produces rare mutant events with specific doses (Janick, 2015). Nevertheless, mutagenesis is still a random event and high percentage of sterility had also been observed in treated plants. Thus, optimisation of dose rate is required for each genotype treatment.

Applications of Gamma Rays in Stevia

Among all the physical mutagenic agents, the most frequently used mutagens in mutation breeding are gamma rays and x-rays (Mba, 2013). As reported by Joint FAO/IAEA, the usage of gamma rays to induce mutation has become extensive over the last 40 years, while the usage of x-rays had significantly declined. Gamma rays are the most energetic type of electromagnetic radiation, having energy level from around 10 kiloelectronvolt (keV) to several hundreds keV (Maamoun, El-Mahrouk, Dewir, & Omran, 2014). Gamma rays are ionising radiation with short wavelength but high penetration capacity, interacting with atoms or molecules to create free radicals in the cells where these free radicals can destroy or alter plant cells components (Maamoun et al., 2014). Forster et al. (2012) explained that the ionisation process can take place through three major mechanisms, including the photoelectric effect, Compton scattering and pair production as gamma rays pass through the plant tissues. Depending on the irradiation level, they influence germination of seed, morphology, anatomy, biochemistry and physiology of plants (Wi et al., 2007). Gamma rays cause noticeable morphological alterations in plant tissues and other biochemical responses at cellular level (Hasbullah, Taha, Saleh, & Mohamed, 2012; Hussein et al., 2012).

The usage of gamma facilities for irradiation is preferred due to its wide availability and versatility of use (Forster et al., 2012). In plant mutation induction, gamma rays are emitted by the disintegration of the radioisotopes cobalt-60 ($_{60}$Co), cesium-137 ($_{137}$Cs) and to a lesser extent, plutonium-239 ($_{239}$Pu). Generally, all the gamma irradiators are lead shielded for protection against unintended irradiation. Only well-trained, certified personnel can manage the sources with special precautions enforced to avoid accidents (Mba, 2013). A gamma cell irradiator, containing one of the radioisotopes mentioned above as gamma source, is commonly used for acute irradiation (i.e. exposure of plants to high dose rate for short durations). In contrast, chronic irradiation (i.e. exposure of plants to relatively low dose over extended durations) is carried out in gamma rooms, gamma greenhouse or gamma field with gamma irradiators (Forster et al., 2012).

Development of a high number of useful mutants can be possible by application of gamma irradiation in plants, and it showed increasing potential in vegetatively propagated plants, especially in stevia (Predieri, 2001). Several researches had been carried out on exposing stevia plants to gamma irradiation. Nurhidayah, Norazlina, & Rusli, (2014) performed the radiosensitivity test for stevia in which the effective doses chosen (10 Gy, 20 Gy and 30 Gy) were applied for *in vitro* mutagenesis of stevia shoots. Snehal and Madhukar (2011) reported that high doses of gamma irradiation (25 kilorad (kR) and 30 kR) caused necrosis in explants and delayed callus induction, and low doses of gamma irradiation (5 kR to 15 kR) were more effective and practical in creating variabilities in stevia plants. Further research also revealed that gamma rays (15 kR to 30 kR) significantly reduced stevia seeds germination and seedlings survival rates (Pande and Khetmalas, 2011).

It is warranted crucial to determine the effective dosage of mutagen applied that is able to induce desirable alterations with minimal unwanted effects to ensure the success of mutational induction (Kangarasu, Ganeshram, & Joel, 2014). Higher doses inevitably bring mortality and sterility, while lower doses allow plant’s recovery after treatment. Therefore, in order to obtain effective dosage of mutagen used, fixation of lethal dose (LD_{50}) is crucial before massive irradiation of similar materials is performed (Rajarajan, Saraswathi, & Sassikumar, 2016). Lethal dose, LD_{50}, is referred to a dosage at which the highest frequency of mutation occurred with half of the treated plants still surviving, while the other half died (Kangarasu et al., 2014). The value of this LD_{50} varied according to plants, genera and cultivars (Roslin and Fiatin, 2015). For instance, the LD_{50} of *Stevia rebaudiana* was reported to be 29 Gy, *Manihot esculenta* L. as 27.5 Gy (Kangarashu et al., 2014), *Jatropha crucas* L. as 600 Gy (Songsri et al., 2011), and *Pisum sativum* L. as 200 Gy (Aney, 2013).

Exposure of stevia to gamma radiation is useful in developing new varieties of stevia with enhancement in SGs contents. It was reported that higher stevioside contents (2.808±0.070 mg/g-DW) were obtained from *in vitro* shoots generated from irradiated seeds of stevia (Khalil et al., 2014). They proposed that the best organ for stevioside and rebaudioside A accumulation was the *in vitro* shoots obtained through tissue culture. Chromatographic data also showed that there was slight increment in the stevioside content in 15 Gy treated callus cultures. This revealed that 15 Gy slightly intensified biomass and bioactive compounds whereas other irradiation doses presented negative effects on biomass accumulation and metabolites production during stevia callus proliferation (Khalil et al., 2015). There were reports on combining somaclonal variations and gamma radiation in inducing mutations in stevia. Ali et al. (2015) showed that combined effects of gamma rays and salinity critically affected the number of shoots formed in treated stevia plants. As the dose of gamma radiation increased, the stevioside contents declined significantly. RAPD analysis performed detected somaclonal variations in the regenerated stevia plants. Despite the various studies discussed above, stevia remains a species investigated to a rather limited extent. There is still room for further works on the development of new stevia varieties for better quality and quantity of steviol glycosides yield.

Conclusion and Future Prospects

Stevia rebaudiana shows immense potential as an agricultural crop for the development of a high potency sweetener. The steviol glycosides, particularly rebaudioside A, extracted from the leaves of stevia received great attention currently as a possible sugar substitute due to its most desirable sweetness and safety profile. Safety studies conducted indicated the absence of any negative side effects. High purity stevia extracts are approved for use as a sweetener worldwide. Through previous researches on stevia, it can be summarised that producing new stevia plants with improved features is possible from mutation induction using gamma irradiation. This paves the way for the development of a new stevia variety enriched with higher steviol glycosides and suitable for local cultivation in Malaysia.

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