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Development of *Stevia rebaudiana* Hybrids through *Trigona*-Assisted Pollination

(Pembangunan Hibrid *Stevia rebaudiana* melalui Pendebungaan Berbantukan *Trigona*)

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ABSTRACT

Hybridization is an important method to widen variations and to develop novel varieties in plants. The increasing interest in *Stevia rebaudiana* over the last decade soared by its potential as an alternative source of sugar. In this study, improvement in stevia has been conducted through hybridization using *Trigona* for the development of stevia hybrids. *Trigona*, which consists of small to medium-sized bees, is usually found in tropical and subtropical parts of the world. They are common visitors to flowering plants and pose an important function as crop pollinators. A field experiment was conducted using 17 stevia accessions (from Malaysia and Paraguay), and the F_1 hybrids were morphologically and chemically evaluated. Among the F_1 individuals, a wide range of variability with regards to qualitative and quantitative morphological parameters was observed. A selection of seven F_1 hybrids namely MS007HYB1, MS007HYB2, LangatHYB, EireteIIHYB, NilaiHYB1, NilaiHYB2, and NilaiHYB3 was made based on their promising features. Improvements were observed in total stevioside content for MS007HYB1 (45%) and MS007HYB2 (30%), rebaudioside content for MS007HYB2 (4.2%), NilaiHYB2 (3.8%), NilaiHYB3 (3.6%) and LangatHYB (14.4%), and delay in flowering for MS007HYB1 (46 days), MS007HYB2 (46 days), LangatHYB (51 days), EireteHYB (47 days), NilaiHYB1 (49 days), NIlaiHYB2 (46 days) and NilaiHYB3 (46 days) when compared to their respective mother plants.

Keywords: Hybridization; hybrids; *Stevia rebaudiana*; *Trigona*

ABSTRAK

Hibridisasi adalah satu kaedah yang penting bagi meningkatkan variasi dan membangunkan varieti tanaman baharu. Tumpuan pada *Stevia rebaudiana* semenjak beberapa dekad yang lalu dicetuskan oleh potensinya sebagai sumber gantian kepada gula. Dalam kajian ini, penambahbaikan stevia telah dijalankan melalui hibridisasi menggunakan *Trigona* bagi membangunkan hibrid stevia. *Trigona* yang terdiri daripada lebah berukuran kecil ke sederhana biasanya terdapat di kawasan tropika dan subtropika di dunia. *Trigona* merupakan pengunjung biasa bagi tanaman berbunga dan turut berperanan penting sebagai pendebunga. Uji kaji lapangan telah dijalankan menggunakan 17 aksesi stevia (dari Malaysia dan Paraguay) dan hibrid F_1 telah dinilai secara morfologi serta analisis kimia. Dalam kalangan individu F_1, pelbagai variasi merujuk kepada parameter morfologi kualitatif serta kuantitatif dapat diperhatikan. Pemilihan tujuh hibrid F_1 iaitu MS007HYB1, MS007HYB2, LangatHYB, EireteIIHYB, NilaiHYB1, NilaiHYB2 dan NilaiHYB3 telah dibuat berdasarkan kepada ciri-ciri yang berpotensi. Penambahbaikan diperhatikan pada keseluruhan kandungan steviosida bagi MS007HYB1 (45%) dan MS007HYB2 (30%), kandungan rebaudiosida untuk MS007HYB2 (4.2%), NilaiHYB2 (3.8%), NilaiHYB3 (3.6%) dan LangatHYB (14.4%), dan kelewatan berbunga untuk MS007HYB1 (46 hari), MS007HYB2 (46 hari), LangatHYB (51 hari), EireteHYB (47 hari), NilaiHYB1 (49 hari), NIlaiHYB2 (46 hari) dan NilaiHYB3 (46 hari) jika dibandingkan dengan induk masing-masing.

Kata kunci: Hibrid; hibridisasi; *Stevia rebaudiana*; *Trigona*

INTRODUCTION

Stevia rebaudiana Bertoni is well-known for its sweet properties abundant in the leaves (Uçar et al. 2018). The country of origin is Paraguay, but due to the global interest in this crop for natural sweetener production, stevia cultivation has spread to countries in Southeast Asia

and Asia. Stevia was first introduced into Malaysia in the 1970s (Tan et al. 2008). Stevia has gained its popularity as one of the main sources of non-caloric sugar due to being 300 times sweeter than sugarcane sugar (Ramesh et al. 2006). Among the 180 species in the genus *Stevia*, only *Stevia rebaudiana* produces the sweetest extract due to the sweet diterpenoid glycosides. The leaves also contain other compounds such as phenols, flavonoids, vitamins and alkaloids (Lemus-Mondaca et al. 2012; Tavarini & Angelini 2013; Wölwer -Rieck 2012). These properties make *Stevia rebaudiana* as a foremost species towards the development of functional foods characterized by a low caloric contribution and potent antioxidant properties appropriate for diabetics, dieters and health-conscious people (Kumuda 2006).

Stevia is a diploid with 2n=22 chromosomes (Yadav et al. 2011). It is an obligate short-day plant and under natural environments, it can grow up to 1 m or more in height (Satpathy & Das 2010). The plant has an extensive root system and brittle stems that produce small, lanceolate, oblong, serrate, and sweet leaves (Cimpeanu et al. 2006). The tiny white and purple disc florets (Cimpeanu et al. 2006) are perfect and borne in small corymbs of 2–6 florets (Yadav et al. 2011). Florets develop achene fruits about 3 mm in length, and a seed is contained in a slender achene that has about 20 persistent pappus bristles (Yadav et al. 2011). Reproductively, stevia is hermaphroditic, highly cross-pollinated and photoperiodically sensitive species (Abdullateef et al. 2015; Macchia et al. 2007; Yadav et al. 2011) with sporophytic self-incompatibility (Maiti & Purohit 2008; Raina et al. 2013) and probably insect-pollinated (Yadav et al. 2011). Plant reproduction is majorly affected by factors influencing the biological cycle such as photoperiod. The main constraint for stevia under short-day tropical environments is the fact that it is premature or flowers early. Under the short-day conditions, after the plant has attained a minimum of four leaves, the plant is set to go into the flowering phase although it has not accumulated adequate vegetative biomass (Yadav & Guleria 2012). Subsequently, the onset of bud formation or flowering will decrease the accumulation of glycosides in stevia (Yadav & Guleria 2012). In a study by Ceunen and Geuns (2013), the photoperiod effect on steviol glycoside content was investigated between two treatments (16 and 8 h). Long-day conditions prolonged vegetative growth and significantly increased total steviol glycosides.

Despite all the economic potentials in stevia, the availability of practical and suitable stevia varieties in Malaysia is still lacking. Therefore, suitable production technologies and varieties are critical. Thus, it is essential to broaden the genetic diversity in stevia and one of the approaches is through hybridization. However, in stevia, manual pollination is hard to be achieved due to the small inflorescence (Cimpeanu et al. 2006). In self-incompatible plant species like stevia, a pollinator is required for seed production purposes (Paalhaar et al. 2008). Generally, there are five modes of plant pollination mechanisms (Isagi 2011), but insect pollination is the most common pollination method reported (Cerana 2004). Stingless bees are reported to be an important pollinator for most crops in tropical and subtropical regions of the world (Slaa et al. 2006). *Trigona* is one of the two major genera of stingless bees besides *Melipona*, with *Trigona* being the largest and most widely distributed genus accounting for 130 species in 10 subgenera (Slaa et al. 2006). Thus, this study is initiated to develop F_1 stevia hybrids through *Trigona*-assisted pollination.

Materials and Methods

Study location

The hybridization experiment was conducted at the field plot of Kulliyyah of Science, International Islamic University Malaysia, Kuantan, Pahang, Malaysia from December 2013 to December 2015. The experimental site was situated in the tropical climate zone at 3 °N altitude and 103 °E longitude with heavy rainfall from October to February and scant rainfall during the rest of the year.

Plant materials

Thirty-one stevia samples corresponding to 17 stevia accessions (14 collected from across Malaysia and three was introduced from Paraguay) are listed in Table 1. Shoot micro-cutting practices were employed as the mode for propagation. To obtain micro-cuttings from Paraguay accessions and F_1 hybrids, seeds were first germinated on the seedling tray with peat moss as medium with irradiance under red light in a confined area as per standard protocol developed by Abdullateef and Osman (2011). After a month, the seedlings were further raised under shades in the nursery. At 2 months of age, micro-cuttings were transplanted into polythene bags in the field. The experiment was laid out in Randomized Complete Block Design (RCBD) with 17 treatments (accessions) in 5 replications arranged randomly. The distance between replication was 1 m, between rows was 0.5 m and between plants was 0.15 m.

TABLE 1. Detail list of stevia used in the study

Accession/ Variety	Origin	Area of Collection/ Source	Details
MS007	Malaysia	MARDI	Seedlings
MS012	Malaysia	MARDI	Seedlings
Bangi	Malaysia	UKM	Seedlings
Rawang	Malaysia	Rawang	Seedlings
Nilai	Malaysia	Nilai	Seedlings
Bertam	Malaysia	Cameron Highlands	Seedlings
Taman Pertanian	Malaysia	DOA, Kuantan	Seedlings
Souq Bukhori	Malaysia	Alor Setar	Seedlings
Langat	Malaysia	Batu 14, Hulu Langat	Seedlings
Mergong	Malaysia	Alor Setar	Seedlings
Rasa Sayang	Malaysia	Nursery, Kuantan	Seedlings
MNQ	Malaysia	Nursery, Kuantan	Seedlings
Exotic	Malaysia	Nursery, Kuantan	Seedlings
Kelantan	Malaysia	Wakaf Bharu	Seedlings
Eirete II	Paraguay	Lambare	From seeds
Morita III	Paraguay	Lambare	From seeds
Native	Paraguay	Lambare	From seeds

THE DEVELOPMENT OF *TRIGONA*-ASSISTED POLLINATION TECHNIQUE FOR PRODUCING AN INTRA-SPECIFIC F_1 HYBRID

For the development of hybrids, *Trigona itama* was used as the pollinator in the experiment. The stingless beehives were procured from MARDI Serdang, Selangor, Malaysia. The experiment was conducted under a large net house (1200 ft^3) constructed using a metal frame structure placed on an open field area. White nylon-netting (150 × 150 mesh/square inch) was placed surrounding the constructed net house to the ground level and a double door was created to maintain the stingless bees inside the net house at all times. In order to reduce any environmental stress on the stingless bees, black plastic with 70% shade-netting was placed on top of the net house as well as one-third of the sides, and a water sprinkler system was also placed at the top of the net house. *Trigona* hive was placed at the center of the net house on a concrete slab to avoid termite attacks via ground contact, and a small hut was put on top of the hive for shelter.

A total of 340 plants (20 replicates for every 17 individual accessions) were arranged in the net house at a random position. The experiment was allowed to run for a month to ensure pollination by *Trigona* was fully successful. Once the seeds had matured and ripened, all the seeds were harvested from individual mother plants and labelled. Mature seeds were considered ripened once they dehisced after gentle shaking to the plants. The seed harvesting process was done three times at biweekly intervals. Through this practice, the maximum number of seeds was obtained since the production of seeds process kept occurring in line with the occurring pollination.

From overall seeds harvested, only the black seeds were used for germination. The black seeds were sown under red light treatment as per standard protocol developed by Abdullateef and Osman (2011), in a confined area, and seedlings of a month old were transplanted in a polythene bag (12 × 12") filled with soil composition consisting of loam, sand and coconut husk in the ratio of (3:2:1) on the field. Liquid fertilizer namely Caviota 2000 was applied twice a month while organic fertilizer was applied once a month. The plants were irrigated twice daily through an automated overhead sprinkler system. The seedlings raised were tagged as stevia putative F_1 hybrids.

MORPHOLOGICAL EVALUATION OF F_1 HYBRIDS

All stevia accessions and F_1 plants were morphologically evaluated with regards to qualitative and quantitative

parameters. Qualitative morphological evaluation focussed on the leaf morphological variations (leaf shape, leaf tip, leaf base, and leaf margin) and was done as per the standard methods (Kumari et al. 2018) while quantitative morphological evaluations include parameters: (i) plant height, (ii) days to flowering, (iii) stem numbers, (iv) leaves number, (v) leaf size and (vi) stem girth (Abdullateef & Osman 2011). The data recording for these parameters was done in the duration of 3 months.

HPLC ANALYSIS

SAMPLE PREPARATION

Two hundred milligrams of dried stevia leaves were suspended with 20 mL HPLC-water at 70 °C using an overhead shaker for 24 h. The extract was filtered using a vacuum filter to separate the aqueous phase from leaves residues. The extract was filtered through a 1.2 µm nylon membrane filter (Merck Millipore, Darmstadt, Germany) to remove particulates prior to HPLC analysis.

HPLC PROCEDURE

The calibration with stevioside and rebaudioside A standards was performed with 5 concentration levels between 1 and 200 ppm. The analysis was performed on Perkin Elmer FLEXAR™ liquid chromatography system consisting of FLEXAR isocratic LC pump equipped with dual diode array detector and computerized data station using Chromera software. An NH-2 column (5 µm, 150 mm × 2 mm^2) from Knauer (Germany) was used as the stationary phase and the temperature was maintained at 30 °C. The mobile phase was a 20:80 (v/V) mixture of water and acetonitrile at a flow rate of 1 mL/min with a total run time of 10 min. The injection volume was 5 µL and the wavelength of the spectrophotometer was set to 205 nm (stevioside) and 210 nm (rebaudioside A). Peaks were assigned by spiking samples with standards, comparison of UV spectra and the retention times.

The concentration of stevioside in the sample was calculated based on the following formula from JECFA (2010):

$$C_{stv}(\%) = W_{stv\text{-}std} \times f_x \times A_{x\text{-}smp} \times 100\%$$

$$W_{smp} \times A_{stv\text{-}std}$$

where C_x (%) is the concentration of stevioside; $W_{stv\text{-}std}$ is the weight of the stevioside standard (dried basis); W_{smp} is the weight of the sample (dried basis); $A_{x\text{-}smp}$ is the peak area of the stevioside in the sample solution; $A_{stv\text{-}std}$ is the peak area of stevioside in the standard solution and f_x is the coefficient value for stevioside that represent the value of 1.

The concentration of rebaudioside A in the sample was calculated by the following formula:

$$C_{reA}(\%) = W_{reA\text{-}std} \times A_{x\text{-}smp} \times 100\%$$

$$W_{smp} \times A_{reA\text{-}std}$$

where C_x (%) is the concentration of rebaudioside A; $W_{reA\text{-}std}$ is the weight of the rebausioside A standard (dried basis); W_{smp} is the weight of the sample (dried basis); $A_{x\text{-}smp}$ is the peak area of the rebaudioside A in the sample solution; $A_{reA\text{-}std}$ is the peak area of rebaudioside A in the standard solution.

STATISTICAL ANALYSIS

Quantitative data was subjected to one-way ANOVA, where the individual mean for a particular parameter was compared to the population mean of such parameter.

RESULTS AND DISCUSSION

MORPHOLOGICAL EVALUATION OF F_1 HYBRIDS

The hybridization experiment using *Trigona* had resulted in the development of F_1 plants. The individual plants were extensively evaluated with regard to their morphology. Based on the results, a total of 7 putative F_1 hybrids were selected to be further evaluated in terms of their chemical content. These 7 putative F_1 hybrids were critically chosen based on the morphological significant differences observed and the comparisons were made with respective mother plants (Figure 1). Based on molecular analysis previously reported by Othman et al. (2016), two putative hybrids were derived from MS007 (tagged as MS007HYB1 and MS007HYB2), three hybrids derived from NILAI (tagged as NilaiHYB1, NilaiHYB2 and NilaiHYB3), a hybrid derived from Langat (tagged as LangatHYB) and a hybrid derived from Eirete II (tagged as EireteIIHYB). The detail qualitative and quantitative morphological results of the hybrids are presented in Table 2 and Figure 2, respectively.

Previous literature indicates various leaf shapes in stevia such as lanceolate (Cimpeanu et al. 2006), oblanceolate and serrated (Singh & Rao 2005) as well as elliptic (Yadav et al. 2011). Tan et al. (2008) introduced three promising stevia varieties from Canada into Malaysia and these varieties were reported to have leaf shapes ranging from ovate, oblanceolate and spatulate. With regards to leaf tips, it was noted that obtuse and acute leaf tips were more present compared to the rest of leaf tip types. This indicates the gene that is responsible for both obtuse and acute leaf tip is expressed more

phenotypically. The presence of these different leaf types in the population is indicative of considerable heterozygosity in its genes at least with regard to leaf tip and confirms the earlier reports of such variations in this species. Tateo et al. (1998) has reported morphological variability in a population of *Stevia rebaudiana* grouped into 13 categories based on growth habit. Moreover, the plants classified in the different groups were found

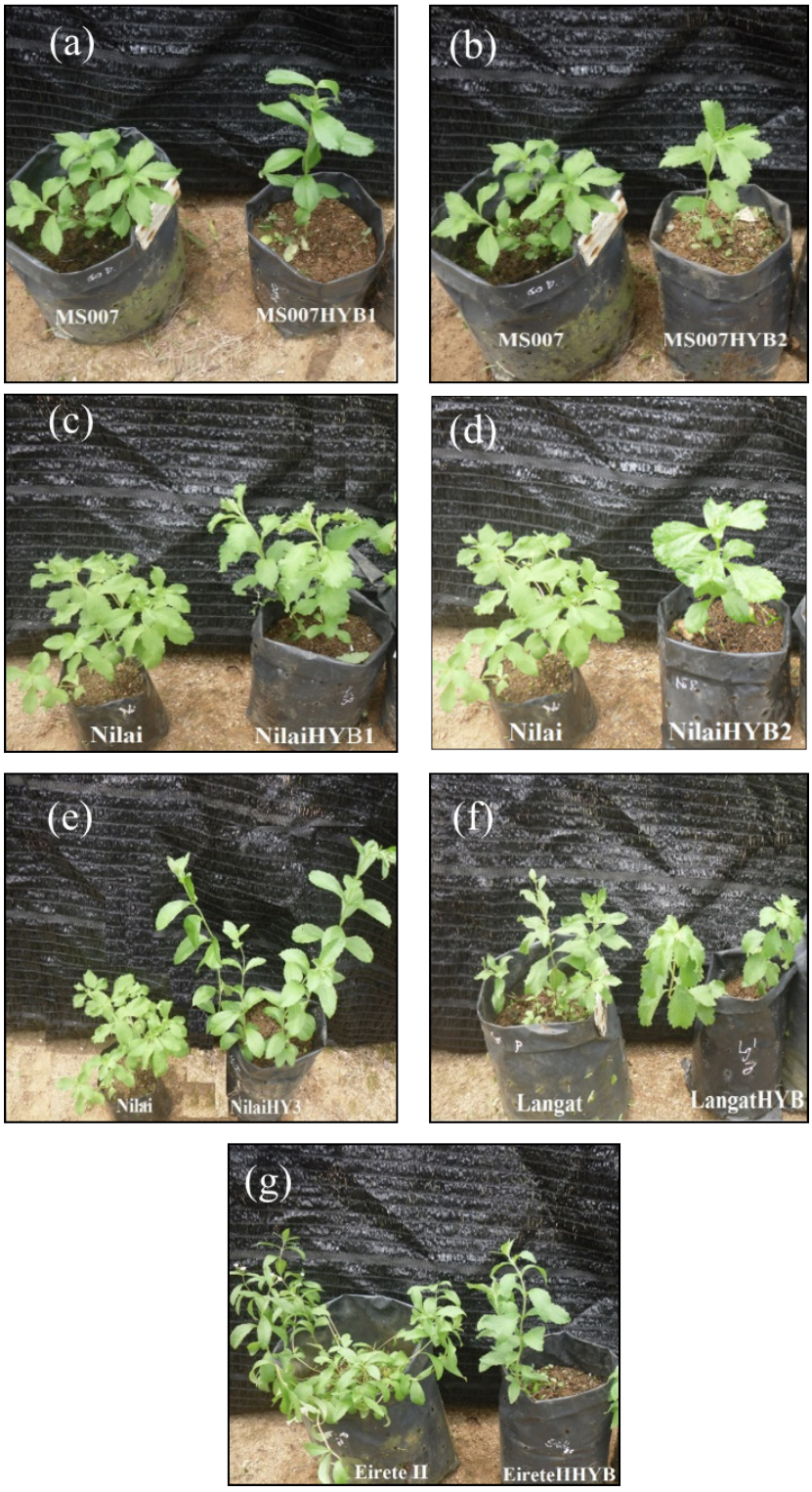

FIGURE 1. Stevia F1 hybrid plant (a) MS007HYB1 (b) MS007HYB2 (c) NILAIHYB1 (d) NILAIHYB2 (e) NILAIHYB3 (f) LANGATHYB (g) EIRETEIIHYB

to differ in other morphological characteristics (leaf length, leaf width, length/width leaf ratio, leaf surface area) even when the growth habits were the same. Overall, attenuate leaf base and lobate crenate margin are expressed more in the segregation pattern studied. This is indicative of the control from heterozygous alleles for both characters. Adaptation of a certain species in its growth habitat can also result in leaf character variations (Pandey & Nagar 2002). Variations in leaf size and leaf shape can take place at various levels especially in geographical origin and population (Viscosi & Cardini 2011). However, based on the molecular result previously done by Othman et al. (2016), the variations observed on the phenotypic characters indicated in this study are due to the genetics among the hybrids.

It was noted that most hybrids were exhibiting improved characteristics particularly concerning the plant height, days have taken to start flowering and the size of the leaves when compared to their mother plants (Figure 2). A significantly improved characteristic in plant height was shown in all hybrids derived from MS007, Nilai, Langat and Eirete II (Figure 2(a)). Typically, stevia in its natural habitat would grow to 50-60 cm (Sairkar et al. 2009) or as high as 100-120 cm (Cimpeanu et al. 2006). However, cultivated stevia was usually smaller, varied in the range of 60-80 cm compared to those in the natural habitat. Average plant height highly depended on the region where stevia cultivated in moderate climate was found to be shorter (Kakol et al. 2014).

Significant improvement of all hybrids was also observed in days to flowering (Figure 2(b)). LangatHYB was shown to exhibit a delay in flowering (51 days) when compared to the rest of the mother plants. Differences in the total number of flowering affect significantly the vegetative growth of the plant. The early commencement of flowering would decrease the plant's vegetative growth. Therefore, longer days taken for flowering would eventually result in higher total biomass as well as in the stevioside content of the leaves (Ceunen & Geuns 2013). Improvement in the late-flowering character is one of the breeder's focuses particularly in Malaysia (Tan et al. 2008). Rank and Midmore (2006) mentioned that early flowering is a commonly occurring condition in the tropics. Early-flowering hampers the accumulation of glycosides in the leaves; hence selection involving late-flowering individuals would later help towards plants with higher leaf biomass. Environmental factors such as temperature, photoperiod, and irradiation are linked in inducing flowering. Therefore, several cycles of selection are needed to achieve the aimed objective. Overall, the selected hybrids mostly exhibited better vigourosity from the mother plants. The most outstanding and prominent feature was the size of the leaves. All hybrids had bigger leaves compared to the mother plants (Figure 2(e)) and it was an added advantage towards higher leaf production (Figure 3).

A study done by Gaurav et al. (2008) proved that plant characters such as leaf yield, leaf width, leaf length as well as stevioside content were highly heritable characters and improvement can be aided through mass selection. Similarly, Brandle and Rosa (1992) who studied stevia lines from China reported that characters such as plant yield, leaf: stem ratio and stevioside content were highly heritable characters that could be exploited through selection.

TABLE 2. Qualitative morphological characteristics of 7 selected stevia F_1

Hybrid	Accession	Plant No.	Plant type	Leaf shape	Leaf tip	Leaf base	Leaf margin
MS007 HYB1	MS007	17	Compact	Obovate	Acute	Cuneate	Lobate crenate
MS007 HYB2	MS007	18	Compact	Lanceolate	Acute	Oblique	Incised
Nilai HYB1	Nilai	21	Compact	Ovate	Acute	Attenuate	Lobate crenate
Nilai HYB2	Nilai	22	Compact	Ovate	Acute	Cuneate	Incised
Nilai HYB3	Nilai	23	Compact	Ovate	Obtuse	Attenuate	Crenate
Langat HYB	Langat	26	Compact	Ovate	Obtuse	Rounded	Crenate
EireteII HYB	Eirete II	21	Compact	Lanceolate	Acuminate	Attenuate	Dentate

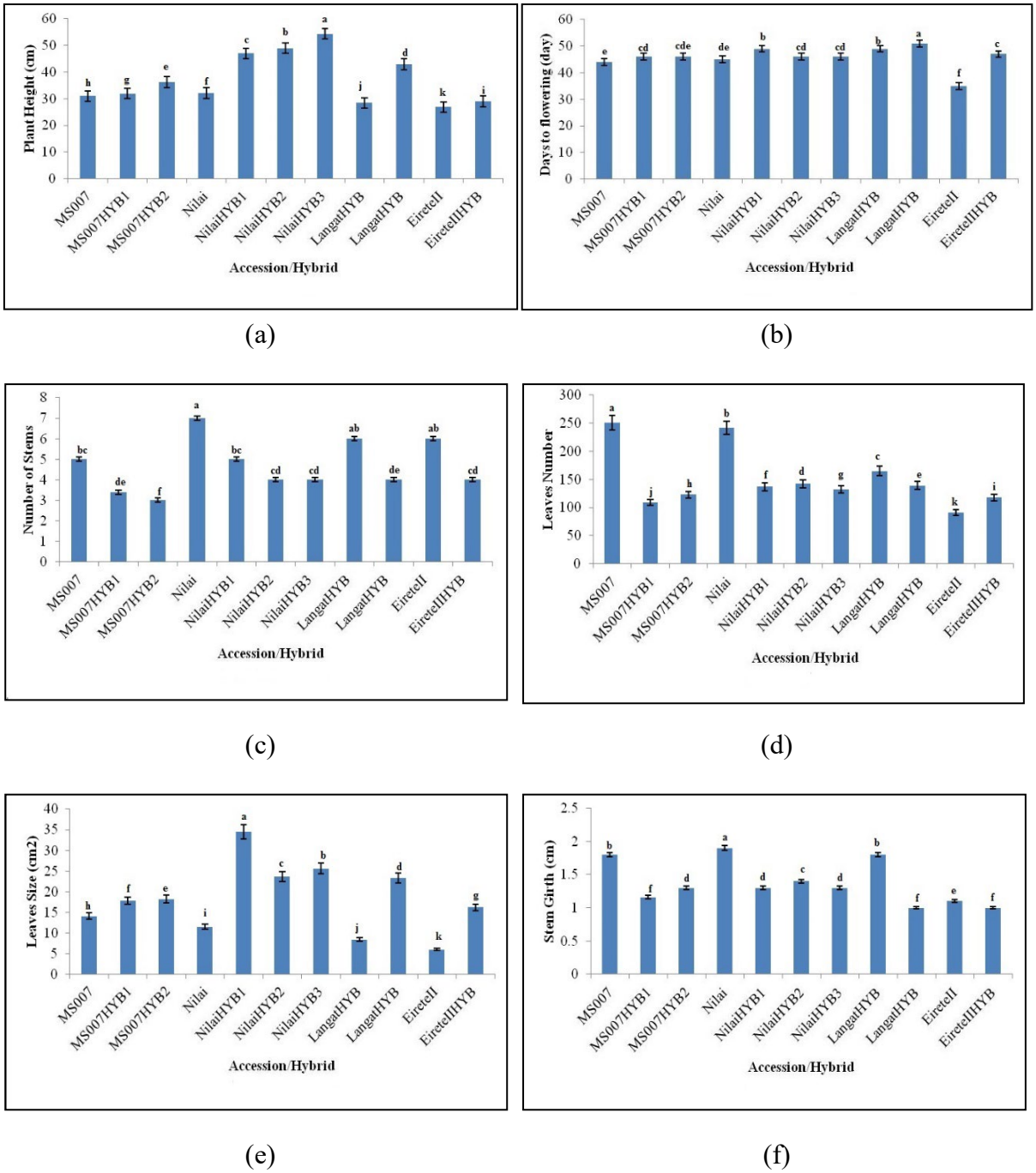

FIGURE 2. Morphological comparisons between F_1 hybrids and stevia mother plants (a) Plant height (b) Days to flowering (c) Number of stems (d) Leaves number (e) Leaves size (f) Stem girth

STEVIOL GLYCOSIDES EVALUATION OF F_1 HYBRIDS

The 7 selected hybrids were further evaluated for stevioside and rebaudioside A contents, and the results indicated that all hybrids were not statistically different among themselves except for rebaudioside A content. The stevioside content ranged from 20.46% (EireteIIHYB) to 43.54% (MS007HYB1) (Figure 4(a)). Hybrids derived from MS007 (MS007HYB1 and MS007HYB2) and

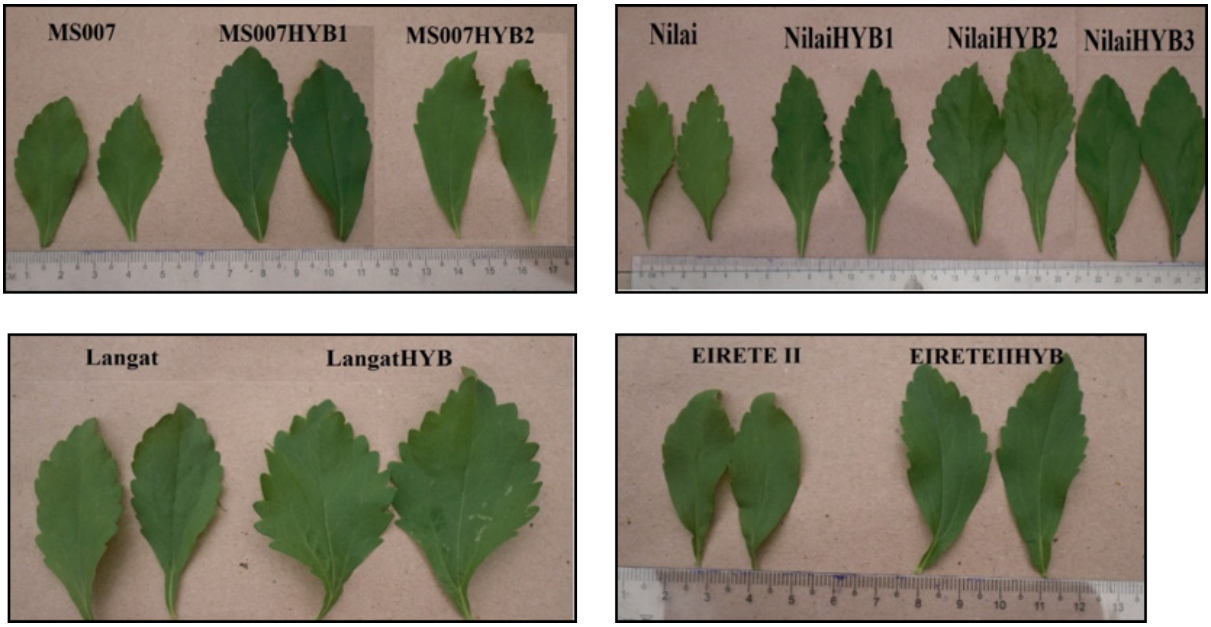

FIGURE 3. Leaves sizes comparisons between F_1 hybrids and respective mother plants

Langat (LangatHYB) were observed to have higher concentrations of stevioside compared to their respective mother plants. NilaiHYB1 had the lowest rebaudioside A content with 0.53% and the highest content was contained in LangatHYB with 14.07% (Figure 4(b)). All hybrids except MS007HYB1 and NILAIHYB1 showed an improved concentration of rebaudioside A compared to their respective mother plants. In fact, LangatHYB was shown to have the highest amount of rebaudioside A (14.07%) when compared to the rest of the mother plant accessions. Rebaudioside A is more preferred due to its better organoleptic and physicochemical profile (Barriocanal et al. 2008), development of new and improved varieties with higher rebaudioside A content become one of the main aims for stevia breeders (Tavarini et al. 2018).

With regards to total cumulative stevioside and rebaudioside A content, NilaiHYB1 recorded the lowest amount with merely 21.17% while LangatHYB had the most amount with 52.26% (Figure 4(c)). As far as total cumulative content was concerned, hybrids derived from MS007 (MS007HYB1 and MS007HYB2) and Langat (LangatHYB) were found to have a higher content of both chemical compounds. Rebaudioside A: stevioside ratio ranged from 0.02 (MS007HYB1) to 0.37 (LangatHYB) an indicator of a great variation (Figure 4(d)). With regards to rebaudioside A and stevioside ratio, all hybrids were observed to give higher ratios except for MS007HYB1 when compared to their mother plants. Most stevia breeding programs aimed at improvement in higher leaf yield with increase in total glycoside content and rebaudioside A: stevioside ratio (Tavarini et al. 2018). Various studies conducted have proven that significant genetic gains in leaf yield, rebaudioside A content and rebaudioside A: stevioside ratio were influenced by sufficient genetic variability in the stevia studied (Yadav et al. 2011). A stevia cultivar with rebaudioside A: stevioside ratio of 0.96:1 (0.36:1 starting material ratio) and total glycosides of 22.4% was developed (Brandle 2001). In a Japanese patent, a stevia cultivar exhibiting rebaudioside A: stevioside ratio of 9.1:1 and total steviol glycosides of 10.1% was developed. Britos (2012) patented a new stevia cultivar known as AKH L1 which was characterized as late-flowering, high yielding, containing stevioside of 1.3%, rebaudioside A of 11.5% and total stevioside and rebaudioside A content of 12.8%.

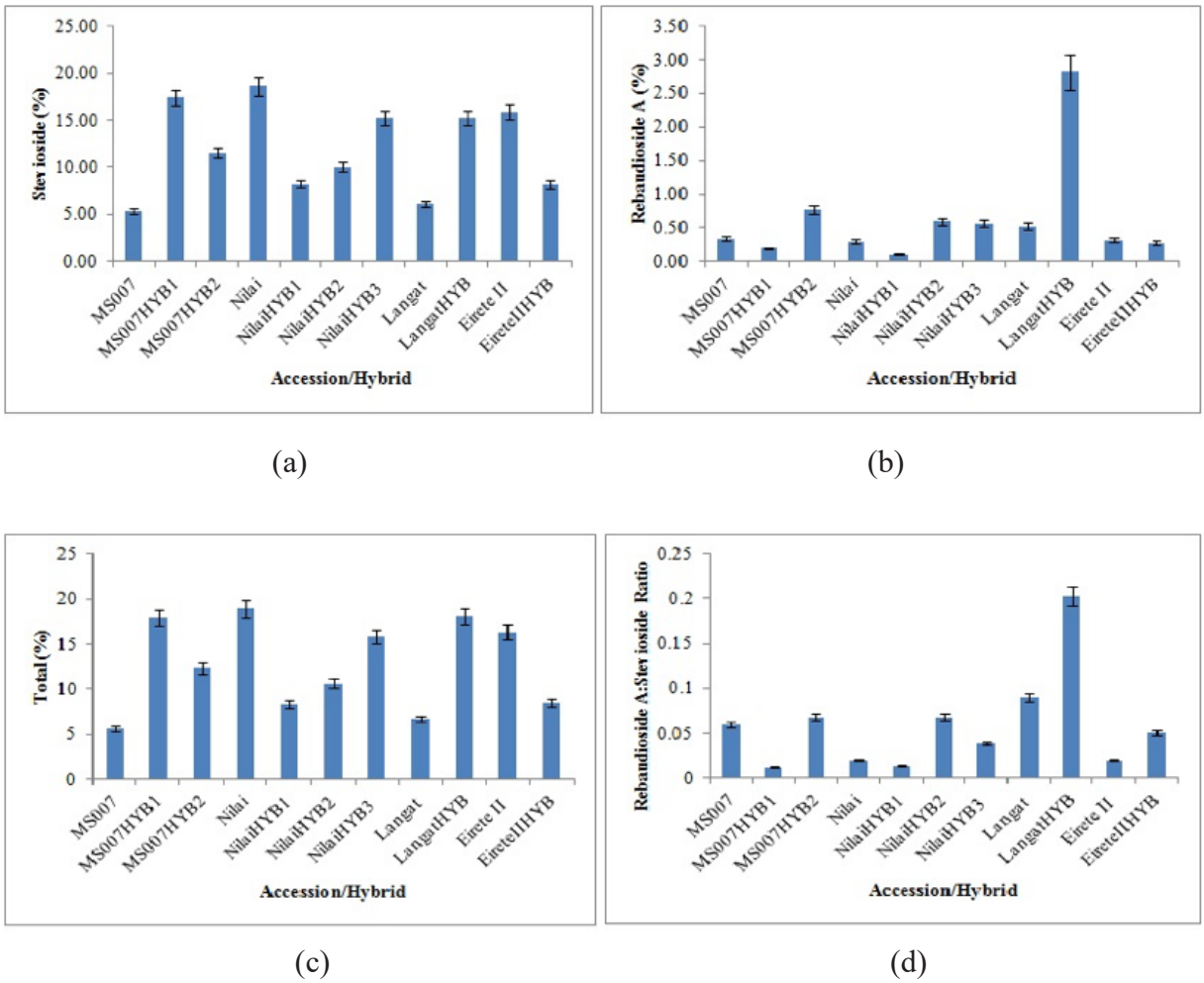

FIGURE 4. Steviol glycosides contents comparisons F_1 hybrids and stevia mother plants (a) Stevioside (b) Rebaudioside A (c) Total (d) Rebaudioside A: stevioside

CONCLUSION

In the present investigation, we have established the standard protocol technology in developing intra-specific F_1 stevia hybrids using *Trigona*. This study is among the pioneer studies regarding stevia breeding in Malaysia which have led to the development of seven F_1 stevia hybrids namely MS007HYB1, MS007HYB2, LangatHYB, EireteIIHYB, NilaiHYB1, NilaiHYB2 and NilaiHYB3. These hybrids have tremendous potential to delay flowering, providing higher leaf yield and improved content of stevioside and rebaudioside A. Conclusively, the information gathered from this study is a valuable tool towards proper breeding strategies in producing stevia with high sweetener content through selective crosses by using contrasting individuals with desired characteristics.

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