Flowering and seed production in a model pot-grown specimen of the sweet herb *Stevia rebaudiana* Bertoni (Asteraceae).

R.C. Southward[1], K.L. Kitchen[1], and D.W. Fountain[2]

[1]Centre for Plant Reproduction and Seed Technology, Institute of Natural Resources, Massey University, Private Bag 11222, Palmerston North, New Zealand.
[2]Centre for Plant Reproduction and Seed Technology, Institute of Molecular BioSciences, Massey University, Private Bag 11222, Palmerston North, New Zealand.
Email: R.C.Southward@massey.ac.nz

Abstract

A single representative plant from a larger pot trial of *Stevia rebaudiana* is used as a model to illustrate issues in assessing and achieving optimal seed yield from this agronomically-significant species. The plant was optimised for initial vegetative growth, local growth conditions and pollinator access and when flowering commenced, was assessed weekly for inflorescence, floret and seed production. Flowering and seeding phenology are described. Seed harvest was commenced as individual inflorescences began to exhibit seed shatter. Fecundity measurements of seed yield per floret, per inflorescence and per plant are reported. Seed quality was assessed by estimating weekly full: total seed proportion (range 0-80 %, average 35 %) and in standard germination tests at 25 °C, light (69 %), 25 °C, light, prechill (70 %), 25 °C, dark (48 %), and alternating 20-30 °C, light-dark (68 %). Germination of the initial seed lots from Paraguay were 62 % and 37 %. The results are discussed in the context of issues important to optimising seed production in this species.

Additional key words: germination, empty seed, fecundity

Introduction

Stevia rebaudiana (Asteraceae), an herbaceous small shrub originating from the Rio Monday Valley in the highlands of Paraguay, South America, contains several 'stevioside' substances that are 200-300 times sweeter than sucrose (sugar). Stevia has been used for hundreds of years in South America and more recently in Russia (from 1930s), and in parts of Asia, particular Japan for about 30 years. Japan is now the largest consumer of steviosides extracted from stevia leaves. It is used primarily as a food or beverage sweetener in Japan, for example in diet Coke, where chemical sweeteners such as aspartame and saccharin were banned in the 1970s (Midmore and Rank, 2002).

Stevia is claimed to be ideal in diabetic and low calorie diets as it has no calorific value, not being absorbed by the human body. Norina *et al.*(2003) argue that Stevia has particular relevancy to New Zealand with its high levels of Type II diabetes, particularly among the Maori and Pacific Island peoples. There remains, however, the need for appropriate toxicological studies to satisfy the relevant food authorities in New Zealand. Currently, Food Standards Australia New Zealand (FSANZ formerly ANZFA) does not permit steviosides to be used as a food additive or sweetener. FSANZ has ruled that stevia (whole or crushed leaf) is a novel food and it therefore can only be legally sold in Australia and New Zealand as a dietary supplement (Midmore and Rank, 2002; Peter Keegan, FSANZ, 2004 pers. comm.).

Stevia has been grown widely, particularly through South America, Russia & Asia, ranging in latitudes from 7 °S to 60 °N (Palmerston North is ~40 °S). The natural habitat is 200-400 m above sea level, 1500-1800 mm annual rainfall with temperature extremes of 43 to -6 °C and poor sandy acidic soils adjacent to swamps (Midmore & Rank, 2002).

Stevia is propagated both vegetatively and by seed. Brandle, Starratt and Gijzen (1997) and Midmore and Rank (2002) indicate that the most cost effective method is by seed. However, seed germination is commonly reported to be poor (Brandle, Starratt and Gijzen, 1997) probably through a combination of self-incompatibility mechanisms, insufficient pollinator activity, premature harvest and/or poor seed cleaning (retention of light non-viable seeds). Also, viable seed yields are uncertain with variation between 8 – 67 kg/ha being reported from China (Midmore & Rank, 2002). In addition, seeds are very small (1000 seeds weigh 0.3 -1.0g (Midmore & Rank, 2002)) so handling is difficult and pelleting would be expected to facilitate the sowing of seeds, whether direct seeded in the field or for mechanical seeding into cell trays for initial greenhouse germination, and early growth. In cooler non-tropical climates, such as in New Zealand, the shorter growing season is expected to necessitate seedling establishment in a glasshouse prior to the growing season to allow completion of reproductive phase development within one season.

Data reported here are taken from a model plant from a larger pot trial designed to assess variability within a population of plants grown from Paraguayan-sourced seed. The plant was selected to constitute a model phenological system for reproductive development in stevia and the data obtained track seed produced from its origins in florets of the inflorescences. Issues related to seed set, abortion, seed quality and yield are identified in the model and discussed in the context of optimising seed production.

Materials and Methods

Stevia seeds were germinated on Anchor blue blotter paper in June 2003 and seedlings were carefully transferred to cell trays with potting mix and placed in a greenhouse, approximately 16 °C heating, 25 °C venting and finally potted up into Planter Bags size 3 (PB3 – 100 mm x 107 mm x 125 mm) or plastic pots of a similar size in August. These seedlings were placed outside under a shade cloth area in mid-December 2003, and were transferred to full daylight on the 11 February 2004, plants were irrigated (garden sprinkler) for approximately 60 minutes each day over the summer period. Once flowering commenced for the model plant selected for this study (15 April), weekly flowering counts and/or seed harvesting were conducted as appropriate until 5 August when all seed heads had been harvested. Specifically, individual floret counts, total inflorescences with a minimum of 1 floret open, brown coloured inflorescences, (floret petals and other whorls senescent or lost, the bractate structure enclosing the florets green), and number of ripe seed heads harvested were recorded. On each date where seeds were harvested an assessment of the percentage of full versus empty seed from ten seed heads was undertaken after inflorescences (seed heads) had dried in a 25 °C temperature-controlled room. Average full-seed weights were calculated from nine replicates of 100 seeds spread over the total harvest period. Seed moisture contents were also assessed using the cool constant oven method at 103 ± °C for 17 hours (ISTA, 2004).

Full seeds were dusted with thiram fungicide (1 seed volume: 100 seeds) and germinated under a range of conditions on top of a germination blotter in plastic boxes, namely: 25 °C constant, light (4.0 μmole.m^{-2}. s $^{-2}$; 25 °C constant, dark (containers were enclosed in clipped black polythene photo-bags); prechill 5 °C, 3 days then 25 °C constant, light; and alternating temperature (20-30 °C, 16-8 hr, dark-light each 24 hr period). Germination results were compared with the results obtained from the original seed lot sourced from Paraguay. These seeds had been subjected to a range of germination conditions, namely: 25 °C constant, light (4.0 µmole.m^{-2}. s $^{-2}$); 25 °C constant, dark (containers were enclosed in clipped black polythene photo-bags); prechill 5 °C, 8 weeks then 25 °C constant, light; germination blotters soaked in % KNO3 solution & grown at 25 °C constant, light; 20 °C constant, light (4.0 µmole.m^{-2}. s $^{-2}$); and alternating temperature (20-30 °C, 16-8 hr each 24hr period).

Results and Discussion

Flowering & seed production

Flowering, for the plant reported here, began on 15 April and continued until 15 July (Figure 1). Stevia inflorescences were observed to usually produce five florets, which commonly opened together. Floret counts peaked on the week of 27 May (2050) after a smaller peak two weeks prior that seemed closely related to temperature (Figure 2). The number of inflorescences that had at least one floret open also peaked on the same week (623). There was a three week delay to when senesced inflorescences peaked (1209 on the week of 17 June) and a further two week delay (week of 1 July) to when seed harvesting peaked.

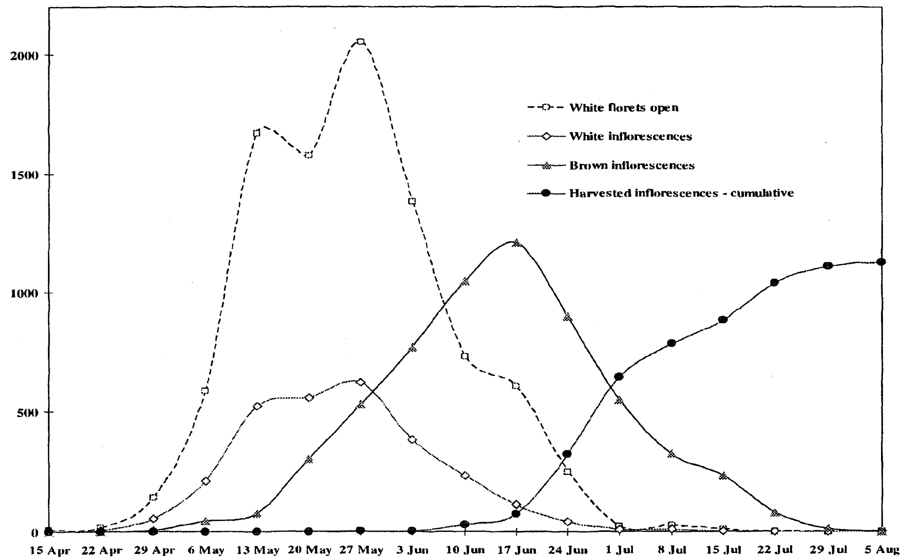

Figure 1. Weekly counts of flowering information and harvested seed from a model *Stevia* plant

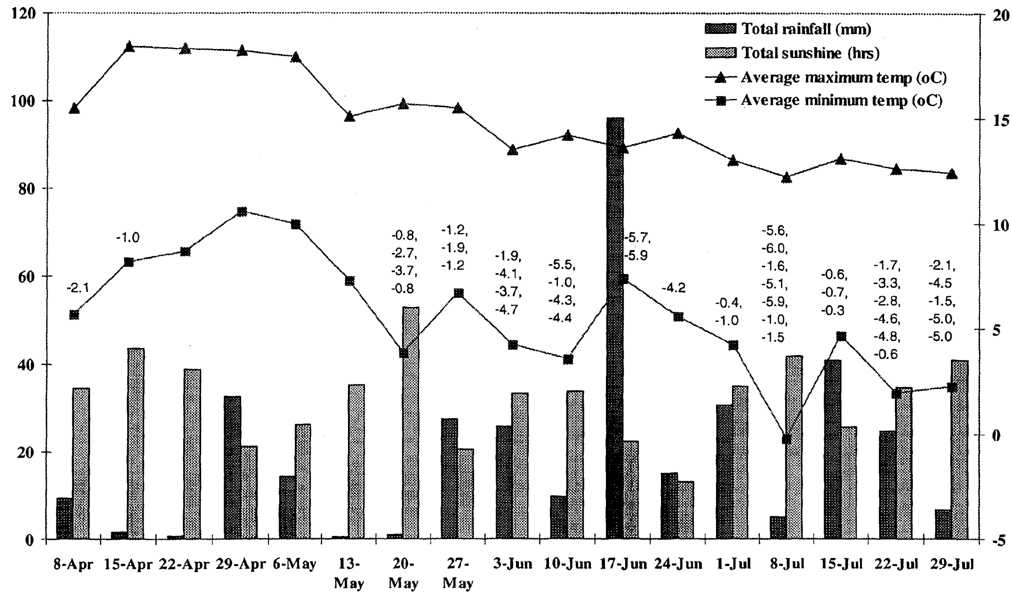

Figure 2. Weekly climate data summaries for the flowering & seed production season
Data averaged from AgResearch Grasslands, Palmerston North Meteorological data April, May, June, July and August 2004, approximately 1.2 km from the trial site. The number and intensity of ground frosts are indicated as numerals each week immediately above the average minimum air-temperature.

Flowering and seed production continued to occur through May, June and July despite cool winter conditions. However, the percentage of full to total seeds (full + empty) produced tended to decline through the season as cool temperatures declined or persisted (Figure 3). Some viable seeds were still being produced during July, the coolest month of the year in the Manawatu. Although number of inflorescences (seedheads) harvested peaked on the week beginning 1 July, the lower percentage of full seed produced this week compared to the previous week resulted in more full seed being harvested in the week beginning 24 June (670) compared with 1 July (486). Total seeds harvested from this model plant were 5621 from 1124 inflorescences (seed heads). Total full seeds harvested were 1969, 35 % of the total, weighing 0.744 g.

The flowering pattern of this plant mainly followed a slightly positively-skewed normal distribution (Figure 1), although there was a lower than expected number of florets and, perhaps, inflorescences opening on the week beginning 20 May. This appeared to be related to lower minimum temperatures including four ground frosts for that week, the first such series of frosts for the autumn-winter period (Figure 2). When minimum temperatures increased the following week both floret and open (white) inflorescence numbers increased to their respective peaks. Floret and white inflorescence numbers then declined steadily over the next month to be close to zero by the 1 July. Although no hives were introduced deliberately nearby, honeybees (*Apis mellifera* L.), bumblebees (*Bombus* spp.) and drone flies (*Eristalis tenax* L.) were all observed visiting receptive florets similar to pollinator behaviour reported by Southward *et al.* (1999) for another Asteraceae seed crop – Dahlia. The model stevia plant was one of a collection of about 90 plants, a requirement because self-incompatibility mechanisms would have resulted in no seed being produced if other flowering plants were not available for cross pollination (Midmore and Rank, 2002).

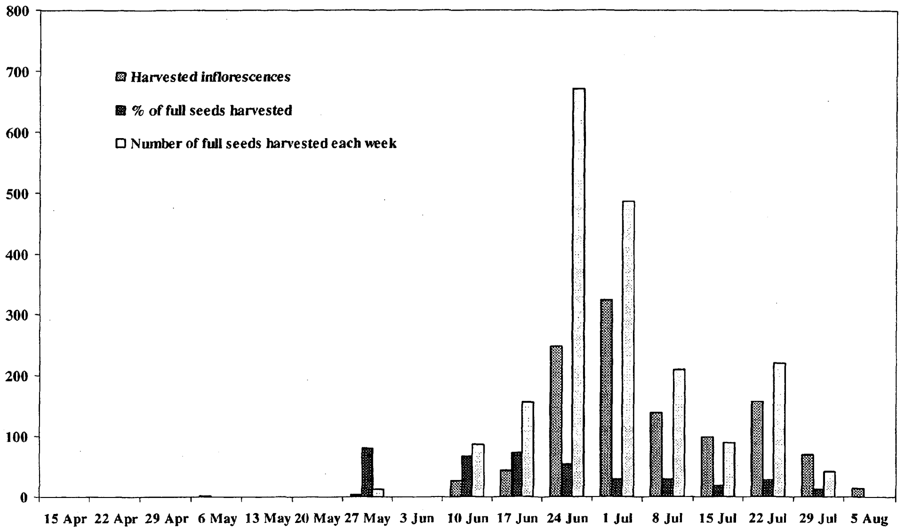

Figure 3. Production of full seed from a model *Stevia* plant at weekly harvest intervals

The number of maturing brown inflorescences steadily increased through May and June to reach a peak on the 17 June from which time significant numbers of ripe seedheads were harvested. The number of harvested inflorescences (seedheads) peaked (324) on the week beginning 1 July (324) indicating that it had taken approximately five weeks from when inflorescences were receptive through to mature seed development. However, this was during a cooler time of the year than would normally be expected with average maximum temperatures of 14.2 °C and average minimum temperatures of 5.5 °C being recorded. Seed development would be anticipated to occur more rapidly over a more ideal late summer/early-mid autumn harvest period.

Germination

Initial germination of the original Paraguayan seed (Table 1) was conducted over a range of germination conditions and methods and for a longer time period than the model plant study. The two seed lots reported reveal a mean germination result of 59 % and 33 %, respectively, after selection for full seed (not including data for germination in the dark). Germination under light at 25 °C gave optimum germination conditions.

Seed germination for the model plant, for seed judged to be full, averaged 69 % for three of the four germination regimes (Table 2) with only the absence of light leading to a lower germination result (48 %, with a further 19 % remaining as fresh ungerminated or dormant). Interim germination results showed that both germination regimes at 25 °C, light (mean 56 %) had a significantly faster germination than the 20-30 °C alternating temperature (27 %) and the 25 °C, dark regime (14 %), which combined the finding by Midmore and Rank (2002) that optimum germination conditions were 25 C with light.

Table 1. Germination results for original Paraguayan *Stevia* seed[1].

Seed lot 1	7-day interim germination[2]	14-day germination[3]	49-day final germination	Fresh ungerminated[4]	Dead
20 °C constant, light	16 ± 4.2	30 ± 3.9	62 ± 5.8	4 ± 2.1	31 ± 4.7
25 °C constant, light	26 ± 1.3	53 ± 3.0	60 ± 2.2	7 ± 1.9	34 ± 2.2
20-30 °C, dark-light, 16-8hrs alternating	9 ± 1.3	22 ± 2.4	56 ± 2.8	4 ± 1.0	38 ± 1.7
Seed lot 2					
25 °C constant, light	19 ± 4.1	26 ± 5.8	37 ± 4.9	4 ± 0.8	56 ± 4.7
25 °C constant, light, prechill[5]	26 ± 2.1	26 ± 2.1	29 ± 1.3	4 ± 1.0	64 ± 3.2
25 °C constant, light, KNO_3	10 ± 1.3	24 ± 2.1	33 ± 2.2	2 ± 0.5	66 ± 2.2
25 °C constant, dark	5 ± 1.0	5 ± 1.0	5 ± 1.0	33 ± 1.0	57 ± 2.1

Table 2. Germination results for seed produced from a model *Stevia* plant[1].

	6-day interim germination[2]	14-day final germination[3]	Fresh ungerminated[4]	Dead
25 °C constant, light	51 ± 4.5	69 ± 4.0	2 ± 0.5	30 ± 4.3
25 °C constant, light, prechill[5]	61 ± 6.5	70 ± 5.4	0	30 ± 5.5
25 °C constant, dark	14 ± 2.6	48 ± 3.8	19 ± 2.1	33 ± 2.9
20-30 °C, dark-light, 16-8hrs alternating	27 ± 2.4	68 ± 1.3	5 ± 1.7	25 ± 1.7

[1] Percentage abnormal seedlings are not reported due to their minor contribution & may be calculated by subtracting normal germination, fresh ungerminated & remainder seed from 100.
[2] Interim germination is the percentage of germinants assessed to be normal seedlings after 6 or 7 days.
[3] Final germination is the percentage of germinants assessed to be normal seedlings after 14 days.
[4] Fresh ungerminated is seed that is plump and firm but ungerminated at the end of the germination test period.
[5] Prechill is placing imbibing seeds at 5°C for 8 weeks (Paraguayan seed or 3 days for the model stevia plant.

Fecundity measurements

The assessment of stevia seed heads to ascertain the proportion of full seed produced during the harvest period revealed a general trend downwards from 80 % on only 3 seed heads on 27 May, to 72 % on 43 seed heads on 17 June, to 54 % on 248 seed heads on 24 June, to 30 % on 324 and 139 seed heads on 1 and 8 July respectively, to 12 % on 70 seed heads on 29 July and finally, 0 % on 14 seed heads on 5 August. This decline in seed set resulted in the largest harvest of seed heads on 1 July yielding less full seed than the second largest harvest on 24 June (Figure 3). Earlier harvests, even prior to mid-June, therefore should provide relatively good seed yields.

The decline of seed production appears to be related to declining and persistent cooler temperatures (Figure 2) that likely resulted in lower levels of pollinator activity, non-seed set through a possible lack of pollination, pollen germination or fertilisation, and possible abortion of seed development. This was compounded by the development of a disease on some branchlets that appeared to cause a cessation to seed development with the resultant seed heads suddenly opening, forcing a harvest or else the result of losing the seed through wind dispersal. Development of brown lesions on the stems may indicate that *Sclerotinia sclerotiorum* (Brandle *et al.,* 1997) infects stevia in New Zealand. However, while some localised mild wilting was observed, complete collapse of the plant as was recorded by Brandle *et al.*, (1997) was not.

Arguably, however, the most surprising aspect overall was that there were still significant amounts of full, viable seed being produced throughout much of the winter period. The plants themselves were not damaged by ground frosts down to -6 °C, which confirms other reports of stevia tolerating temperatures down to -6 °C (Midmore & Rank, 2002), although growth was slowed and finally stopped as the winter progressed.

The germination result from the model plant was from seed judged to be full (35% of all seed produced) but if all seed had been included then the germination result would have been only about 24 %, a very low result. This large amount of empty or very immature seed possibly explains reports of very low germination results (Midmore & Rank, 2002). Whether such seed lots have been subjected to seed cleaning is unknown, but removing light seed so that the TSW of remaining seed is at least 0.3 - 0.35 g (our seed was 0.378 g at 8.7 % seed moisture content) is recommended. To do this adequately the pappus hairs would also need to be rubbed off, to facilitate cleaning, subsequent storage and sowing, in a way that minimises any mechanical damage. Development of seed processing protocols is therefore recommended.

Conclusions

Full and viable stevia seed was harvested as late as 29 July, past mid-winter, although higher fecundity levels and seed yield were harvested during early winter. Higher seed yields are anticipated if flowering and seed development can be manipulated to occur earlier in the season and possibly with the introduction of more pollinating insects. Optimum germination conditions were confirmed to be with light at 25 °C. Retention of empty non-viable seed is reported to be at least part of the reason for generally low germination reports in the literature. Appropriate seed production techniques including providing adequate bee-hives and removing empty and immature seed during seed cleaning is recommended for improving germination levels.

Acknowledgments

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References

Brandle, J.E., Starratt, A.N. and Gijzen, M. 1997. *Stevia rebaudiana*: Its biological, chemical and agricultural properties. Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, Ontario N5V 4T3. http://res2.agr.gc.ca/london/faq/stevia_rev_e.htm

Brandle, J. 1998. *Stevia rebaudiana*: its agricultural, biological and chemical

properties. *Canadian Journal of Plant Science* 78:527-536.
ISTA 2004. International Rules for Seed Testing Edition 2004. Bassersdorf, Switzerland: The International Seed Testing Association.
Midmore, D.J. and Rank, A.H. 2002. A new rural industry – Stevia – to replace imported chemical sweeteners. Australia Capital Territory: Rural Industries Research & Development Corporation Web Publication No W02/022. RIRDC Project No UCQ-16A. http://www.rirdc.gov.au/reports/NPP/w02-022.pdf
Norina, L.V., Bailey, W.C. and Timcke, K. 2003. Stevia (*Stevia rebaudiana* Bertoni): Its potential for New Zealand. *Food New Zealand* 3:29-35.
Southward, R.C., Hampton, J.G. and Hill, M.J. 1999. Clonal dahlia seed production. *In* Current Research on Seeds in New Zealand (eds. M.T. McManus, H.A. Outred, and K.M. Pollock), pp. 69-76. Agronomy Society of New Zealand Special Publication No. 12.