Introducing *Stevia rebaudiana*, a natural zero-calorie sweetener

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Two accessions of *Stevia rebaudiana* were successfully introduced in the experimental farm at the Institute of Himalayan Bioresource Technology, Palampur in 2000. Cultivation trial of these accessions was conducted during 2001–03. Overall crop performance was satisfactory for both the accessions and they were least affected by biotic and abiotic factors like high rainfall, frost, and infestation by insects and diseases. Quantitative differences were found in stevioside content of the two accessions, ranging between 6 and 8%. Accession 1 was superior in stevioside content and Accession 2 was superior in leaf biomass. Higher content of stevioside was found in the regenerated crop in January, during the second year of plant growth. With improved management practices, there is further scope for improvement in stevioside content. A laboratory-scale process was developed for the extraction of stevioside up to 63% purity. Although the crop is self-incompatible in its breeding behaviour, the prevalence of two diverse accessions has facilitated seed production under Palampur conditions. This has triggered the production of plant material for its introduction amongst interested growers in large numbers. The present communication outlines the growing pattern and stevioside yield from the North Indian region. Three cuts could be obtained during the two-year growing period of the crop.

STEVIA rebaudiana (Bert.) Bertoni is a herbaceous perennial plant of the Asteraceae family. It is native to Paraguay, where it grows wild in sandy soils[1]. The leaf extract of this plant has been used traditionally in the treatment of diabetes. The crop was first established in Japan[2] in 1968. By mid-1970s, standardized extract and pure stevioside was utilized commercially in Japan for sweetening and flavouring foods and beverage as a substitute for several synthetic sweeteners. The total market value of Stevia sweetener in Japan is estimated to be around 2–3 billion yen/yr. The crop has been introduced in other countries, including Brazil, Korea, Mexico, USA, Indonesia, Tanzania and Canada[3–9]. Leaves of this plant produce zero-calorie ent-kaurene diterpene glycosides (stevioside and rebaudiosides), a non-nutritive, high-potency sweetener, and substitute to sucrose, being 300 times sweeter than sucrose[10–12]. Presently, more than 1300 MT raw material is being cultivated in China, Taiwan and Malaysia for marketing in Japan[13]. It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects.

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Keeping in view the demand for natural products, two accessions of *S. rebaudiana* were introduced in the year 2000 in Palampur for standardizing its cultivation practices and processing technology for stevioside production.

Plant propagation and crop production studies were conducted in the Chandpur experimental field at the Institute of Himalayan Bioresource Technology (IHBT), Palampur located in the Kangra valley, Himachal Pradesh, India at an altitude of 1300 m msl and having sub-temperate humid climate. The soil of the experimental field is clay loam in texture, low in carbon (0.2%), high in total nitrogen (0.15%), medium in available P_2O_5 (0.18%) and available K_2O (1.48%), with a pH value of 5.6. The crop was grown under assured irrigation with efficient drainage system.

Two different planting materials designated as Accession 1 and Accession 2 of *S. rebaudiana* were used for conducting the present study.

Original collection of Accession 1 was in the form of plantlet and the seeds produced through the crop did not germinate. Therefore, further multiplication of the plants from this accession was achieved through stem-cuttings. Accession 2 was raised both through seeds as well as stem-cuttings. In 2001, both the populations were grown together to allow cross-pollination, in anticipation of viable seed production from Accession 1. At maturity stage, seeds were harvested and tested for germination. Seeds produced from both the accessions were F1 hybrids which successfully germinated.

In the first experiment (Experiment 1) the experimental crop of *S. rebaudiana* was raised during 2001 and 2002. During each year, nurseries were raised by sowing seeds of hybrid Accessions 1 and 2. Seeds were sown in July and 60-day-old seedlings were transplanted in September 2001 and 2002 at a density of 75,000 plants per hectare at spacing of 45 cm × 45 cm. Eight plots (2 accessions × 4 replications), each of 40.5 m^2 gross area were planted and observations were recorded from 9.9 m^2 net area per plot.

A second experiment (Experiment 2) was laid to evaluate the influence of harvest frequencies on *S. rebaudiana* hybrid. Accession 2 was studied extensively in terms of crop growth, productivity and stevioside content. The seedlings were raised in February 2002 and transplanted in May 2003, and this plot was treated as the first year plantation. The plot size was 40.5 m^2, with a spacing of 45 cm × 45 cm. Concurrently, one-year-old regenerated crop which was planted in May 2002, of the same accession was treated as the second year crop. The trial was planted according to randomized complete block design (RBD) with four replications. Harvests during September and January were taken as recommended by Columbus[14]. The growth and yield parameters recorded were plant height (cm), crop canopy in east to west and north to south direction (cm), fresh and dry weight of leaves (q/ha), fresh and dry weight of stem (q/ha), fresh and dry weight of herb (q/ha), moisture content in leaf and stem (%), stevioside content (%) and stevioside yield (kg/ha) (Table 1).

A brief detail of cultivation techniques followed for raising the experimental crop are described for Experiments 1 and 2.

Table 1. Herb yield of two accessions of *Stevia rebaudiana* recorded during crop cycle from September 2001 to January 2003 (data recorded September 2002 and January 2003)

Accession	Leaf weight (q/ha)		Stem weight (q/ha)		Leaf : stem ratio (w/w)		Herb yield (q/ha)		Stevioside content (%)	Stevioside yield of whole herb (kg/ha)
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry		
Accession 1	69.83	17.46	98.41	19.68	0.71	0.88	168.24	37.14	8.00	297.12
SEm ±	4.19	0.87	6.89	0.98	0.04	0.05	11.78	1.86	2.50	24.27
Accession 2	108.47	21.69	138.69	34.67	0.78	0.63	247.16	56.36	6.00	338.16
SEm ±	6.51	1.08	9.71	1.73	0.04	0.04	19.77	3.38	2.00	21.33

Seeds were sown in raised nursery beds of size 1.25 m × 10 m having a 1 : 1 : 1 mixture of sand, soil and organic manure. The sowing was done in July 2001 and 2002 for Experiment 1 and February 2002 and 2003 for Experiment 2. Seeds germinated within 7–10 days. The nursery was irrigated with sprinkler daily in the morning and evening during the first five days and once a day during the subsequent five days. Two-month-old seedlings of 5–7 leaf stage and 8–10 cm height were transplanted in the field in the September 2001 and 2002 for Experiment 1 and May 2002 and 2003 for Experiment 2.

The experimental field was irrigated prior to land preparation. Soil was thoroughly prepared by disc-ploughing, harrowing and planking. Well-rotted farmyard manure (FYM) @25 t/ha was mixed well into the soil ten days before transplanting of saplings. Transplanting was done manually in furrows at a spacing of 45 cm × 45 cm in September 2001 for Experiment 1 and May 2002 and 2003 for Experiment 2.

First irrigation was given immediately after transplanting and another after three days. Subsequent watering was done fortnightly up to a depth of 5 cm till winter rains.

Removal of weeds was done manually. Two hand weedings and hoeings were done to manage the weeds.

The crop was established successfully and an average plant height of 45 cm was attained by the end of October 2001 in Experiment 1 and in June 2002 and 2003 in Experiment 2. With the onset of winter in November, growth of the plants ceased. The crop was frost-susceptible and withered due to frost injury in January. Dead biomass was removed from all the plots during the end of February 2002. Vigorous crop regeneration was observed during onset of spring (first week of March) from the underground root crowns.

Harvesting was done manually leaving 8–10 cm stem height from the ground level. First harvest was taken at flower-bud initiation in September 2002 in Experiment 1 and September 2003 in Experiment 2. Subsequently, second harvest was taken after 90 days of the first harvest in January 2003 in Experiment 1 and January 2004 in Experiment 2. Flowering in the crop was avoided by pinching of the apical bud to enhance growth of side branches. After harvesting, different plant parts were separated, sun-dried, powdered and stored in airtight containers in a cool place for estimation of stevioside content.

At IHBT, a laboratory-scale processing technique was developed to produce 63% pure stevioside. The process involves water extraction of the dried powdered leaves, followed by filtration and crystallization to facilitate production of stevioside. For sample analysis of leaves and stem, dried powdered material of *S. rebaudiana* (0.5 g) was extracted with methanol (cold extraction). After 24 h, the solvent was decanted and the process was further repeated four times. Methanol extract was dried under reduced pressure at 40°C. Dried extract was washed four times with hexane (5 ml) and then with ethyl acetate (four times) to remove chlorophyll. The remaining material was subjected to HPLC analysis.

The standard concept was followed to work out the cost of cultivation. Cost of various inputs and operations were accounted for, on the basis of prevailing current market rates. Life span of the crop is reported to be 7–8 years and herb yield increases up to four years[14]. Therefore, economics of *S. rebaudiana* cultivation was calculated on the basis of four year crop life on an average annual basis. The cost of cultivation has been divided into establishment cost and variable cost. Establishment cost is the initial cost or one time investment for the establishment of crop during the first year and the cost incurred for the maintenance of the crop is the variable cost, which is required yearly. Fixed costs include rate of interest on establishment and variable costs @ 9% and rental value of land @ Rs 15,000/annum. An average net return per annum was worked out by subtracting the average annual total cost from the gross returns per annum. Gross returns are the product of dry leaf yield × price per unit.

Cost of cultivation for producing an average leaf yield of 17, 20, 23 and 25 q/ha for first, second, third and fourth years respectively, was worked out to be Rs 4.74 lakhs/ha during four years. Net returns for four years were calculated as Rs 3.75 lakhs, accounting for an average annual income of Rs 0.93 lakhs/ha at a sale price of Rs 100/kg dried leaves. It was, therefore, concluded that Stevia cultivation is a remunerative venture with a cost–benefit ratio at 1.89. Net profit of Rs 4.61 lakhs/acre during the third year has been reported[15], keeping the sale price of Rs 200/kg dried leaf. Through this analysis, reduction in the cost of production has been achieved by raising seedlings at cheaper rate (Table 2).

Germination of hybrid seeds of Accessions 1 and 2 was observed within 7–10 days. Germination percentage was higher for Accession 2 (35–40%) compared to Accession 1 (25–30%). Poor seed germination (less than 50%)[16],

Table 2. Economics of *Stevia rebaudiana* cultivation

Particulars		1st year	2nd year	3rd year	4th year
A	Establishment cost (Rs)	60,500	–	–	–
B	Variable costs (Rs)	73,500	82,100	82,200	82,200
C	Fixed costs (Rs)	27,060	22,389	22,398	22,398
D	Total production cost (A + B + C) (Rs)	161,060	104,489	104,598	104,598
E	Gross income (Rs) (Sale price of dried leaf @ Rs 100/kg dried leaf) yield 1700 kg in 1st, 2000 kg in 2nd, 2300 kg in 3rd and 2500 kg in 4th year	170,000	200,000	230,000	250,000
F	Net income (Rs)	8940	95,511	125,402	145,402
G	Benefit cost ratio (BCR) (Gross income/total production cost)	1.055	1.914	2.198	2.390

Table 3. Yield parameters of Accession 2 during different months of harvest

Month of harvest	Year*	Leaf weight (q/ha)		Stem weight (q/ha)		Herb yield (q/ha)		Leaf : stem ratio (w/w)	Stevioside content (%)		Stevioside yield (kg/ha)	
		Fresh	Dry	Fresh	Dry	Fresh	Dry		Leaf	Stem	Leaf	Stem
September	First year	40.50	7.53	35.22	9.58	75.72	17.11	0.79	6.09	1.54	45.86	14.75
	SEm ±	5.67	1.05	4.93	1.34	10.60	2.40	0.11	0.24	0.46	1.83	4.43
	Second year	90.33	18.00	57.90	15.77	148.23	33.77	1.14	7.63	3.85	137.34	60.71
	SEm ±	11.74	2.34	7.53	2.05	19.27	4.39	0.15	0.31	1.16	5.49	18.21
January	First year	33.50	8.75	31.62	9.47	65.12	18.22	0.92	3.17	2.03	27.74	19.22
	SEm ±	4.36	1.14	4.11	1.23	8.47	2.37	0.12	0.10	0.81	0.83	7.69
	Second year	66.66	14.22	48.20	14.55	114.86	28.77	0.98	9.94	3.30	141.35	48.02
	SEm ±	8.00	1.71	5.78	1.75	13.78	3.45	0.12	0.30	1.32	4.24	19.21
Total of September and January harvest	First year	74.00	16.28	66.84	19.05	140.84	35.33	–	–	–	–	–
	Second year	156.99	32.22	106.10	30.32	263.09	62.54	–	–	–	–	–

*First year of the crop planted in 2003 and second year of the crop planted in 2002.

lack of sterile seeds and low viable seeds have already been reported[5,14,17]. Odone[18] has reported self-incompatibility in *S. rebaudiana* seeds.

In Accession 1, fresh and dry leaf weight was recorded as 69.83 and 17.46 q/ha, fresh and dry stem weight was 98.41 and 19.68 q/ha, and fresh and dry herb yield was 168.24 and 37.14 q/ha respectively. In Accession 2, fresh and dry leaf yield per hectare was 108.47 and 21.69 q, fresh and dry stem weight was 138.69 and 34.67 q/ha, and fresh and dry herb yield was 247.16 and 56.36 q/ha respectively (Table 1). Leaf yield of 28.50 q/ha with stevioside content of 10.5% is reported in Canada[19].

Stevioside content was higher in Accession 1 (8%) compared to Accession 2 (6%) (Table 1).There are reports of stevioside content ranging between 4 and 20% on dry weight basis, depending on the cultivar and growing conditions[20,21]. However, a harvest management experiment was designed to see the variation in crop growth, productivity and the stevioside content during different periods of harvesting.

Performance of the crop in terms of growth and yield parameters was evaluated for September 2003 and January 2004 harvests (Table 3).

Fresh and dry leaf yields were maximum in regenerated crop in the September (90.33 and 18.00 q/ha) followed by January harvest in the second year of the crop (66.66 and 14.22 q/ha). Higher dry leaf yield from the regenerated crop has been reported earlier[22]. Maximum fresh and dry stem weight was also recorded for September harvest of the regenerated crop (57.90 and 15.77 q/ha) followed by January harvest of the ratoon crop (48.20 and 14.55 q/ha). Therefore, an increase in fresh and dry herb yield in September harvest of the regenerated crop was recorded (148.23 and 33.77 q/ha). Maximum leaf : stem ratio was recorded for September harvest of the regenerated crop (1.14) and minimum for September harvest in the first year of the crop (0.79).

Overall, fresh and dry leaf yields were higher in regenerated (ratoon) crop irrespective of the month of harvest (156.99 and 32.22 q/ha) compared to those from the crops in the first year of growth (74.00 and 16.28 q/ha). Total fresh herb yield was 140.84 q/ha in the first year of the crop and 263.09 q/ha in case of the regenerated crops whereas dry herb yield was 35.33 and 62.54 q/ha respectively.

Stevioside content in leaf varied from 3.17 to 9.94% and that in stem varied from 1.54 to 3.85%. Stevioside content

of leaf and stem was higher in regenerated crops, irrespective of the month of harvest (September and January). Maximum stevioside content in leaf was estimated in ratoon crop harvested in January (9.94%); however, it was low in January harvest in the first year of the crop (3.17). In stem, stevioside was higher in ratoon crop harvested in September (3.85%) and low in the September harvest in the first year of the crop (1.54%; Table 3).

In leaf, the overall yield of stevioside was maximum from January harvest of second year crop (regenerated) (141.35 kg/ha) and minimum in case of January harvest in the first year (27.74 kg/ha). In stem, stevioside yield was maximum in the regenerated crop harvested in September (60.71 kg/ha) followed by the regenerated crop harvested in January (48.02 kg/ha). Lowest stevioside yield in stem was obtained in the September harvest in the first year of the crop (14.75 kg/ha; Table 3).

Stem rot disease was recorded through visual observation, although incidences of powdery mildew (*Erysiphe cichoracearum* DC), damping-off (*Rhizoctonia solani* Kuehn.) and stem rot (*Sclerotium dephinii* Welch.) have also been reported by other workers[23]. Two fungal diseases caused by *Septoria steviae* and *Sclerotinia sclerotiorum* are also reported in *S. rebaudiana* grown in Canada[24,25].

Insects like aphids and white flies were observed in the experimental field. Attack by insects like aphids, mealy bugs, red spider, mites and white flies was also reported by Thomas[25], without much harm to the crop.

The above findings reveal that *S. rebaudiana* cultivation was successful under Palampur agroclimatic conditions. It was possible to have high biomass or high stevioside content depending upon the requirements. A minimum of two harvests were possible during the year from the regenerated (ratoon) crop.

According to the above studies, harvesting of the regenerated crop of Accession 2 in September is suitable for higher dry herb yield and harvesting in January is suitable for higher stevioside content. For higher dry leaf yield and stevioside content, the regenerated crop of Accession 2 should be harvested in September. Overall, regenerated crop (in the second year) gives higher yield of dry leaf and stevioside content compared to harvest from growth in the first year of the crop.

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