Some studies on the physiology of *Stevia rebaudiana* (Bertoni)

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Abstract

Stevia, a zero calorie natural sweetener, is a new crop for Australia. It was approved in 2008 for use as a food ingredient by Food Standard Australia and New Zealand (FSANZ). Steviol Glycoside (SG) found in the leaves is responsible for the sweetness of stevia. As a new crop to Australia, commercial cultivation of stevia is yet to commence. The agronomic requirements of the crop in Australian conditions are yet to be determined. Therefore, the current study aims to lay the foundation for developing both agronomic practice and varietal selection for stevia cultivation in Australia. Stevia seeds were imported from China and grown under controlled Australian conditions. Flowering and biomass of three stevia varieties were studied in the first and ratoon crops. There was no variation in the time of commencement of flowering between stevia varieties; they all flowered quite soon after transplanting. However, they did differ in the average number of days from transplanting to flowering both in first crop and ratoon crop. Early transplanting age (4 weeks) increase biomass and delay flowering than did later transplanting age (7 weeks). The long vegetative period of young seedlings enhanced high biomass yield. The SG concentration was higher in plants transplanted at the age of 5 weeks. The ratoon crop harvested after 94 days from the first harvest yielded high biomass compared to early (87 days) or later (108 days) harvest. Selecting for lateness and crossing those plants to select in the F1 for added lateness was successful, but only marginally so further selections cycles will be necessary to affect a marked delay in flowering time.

Flowering and biomass yield of stevia were strongly associated with photoperiod. In a controlled photoperiod experiment, the percent of flowering plants was highest at 12 hours photoperiod, confirming stevia a short day plant. However, there was the

varietal difference in response to photoperiod. Variety Fengtian was less sensitive to short photoperiod as compared to 99-8 and Shoutain.

Stevia growth and yield was related to nutrient availability. Deficiency of single elements decreased yield and SG concentration, the exceptions were treatments without Zn, P, N, Cl, Mn, S, Fe, B and Mo. The SG content (mg/plant) was higher in treatments without Mo, Zn, Cl, Mn, K, and B but did not differ from that with the complete nutrient. The total SG content (product of % SG concentration and dry weight of leaf) was dependent on the total leaf yield. Micro nutrients were essential for enhancing leaf yield and SG concentration.

Biomass yield of stevia was also related to the available soil water. Yields under water stressed conditions, both at low and very high moisture content, were low. Highest leaf yield was obtained in plants grown under field capacity and 80% of FC. The total SG concentration was highly significant between the treatments, higher concentration was observed in plants grown under water-logged conditions.

Leaf yield is the most important aspect for stevia cultivation. One study explored the growth and SG concentration of stevia under different pH, ranging from highly acidic to alkali conditions. Growth and leaf yield was maximum at pH ranging from 4-6. High pH levels from neutral to alkali reduced plant growth and leaf yield. The SG concentration did not vary between the treatments. Australian agricultural soils are highly to moderately acidic in nature. An acid tolerant species such as stevia would adapt well and contribute to the rural economy.

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Declaration

I declare that I am the sole author of this thesis. Information derived from other sources has been acknowledged in the text and list of references is given.

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List of Abbreviations

A	Photosynthetic rate
BOM	Bureau of Meteorology
CPWS	Centre for Plant & Water Sciences
CQ	Central Queensland
E	Transpiration rate
EC	Electric conductivity
EFSA	European Food Safety Authority
FC	Field capacity
FSANZ	Food Standard Australia and New Zealand
Gs	Stomatal conductance
HPLC	High performance liquid chromatography
IRGA	Infra-Red Gas Analyser
LSD	Least significant difference
QDPI	Queensland Department of Primary Industries
Reb A	Rebaudioside A
RIRDC	Rural Industries Research and Development Corporation
RO	Reverse osmosis water
SE	Standard Error (of the means)
SG	Steviol glycoside
SPAD	Soil-Plant Analyses Development (SPAD) unit of Minolta camera
Stev	Stevioside

Chapter One: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Stevia (*Stevia rebaudiana* (Bert.)), a perennial herb, has been known for centuries by the native Guarani Indians of Eastern Paraguay for the sweet compounds found in the leaves. They are used in sweetening herbal teas. The importance of this plant was re-discovered by M.S. Bertoni in the eighteenth century (Kulasekaran, Singh & Megeji 2006). The herb produces a high potency, zero calorie sweetener in its leaf tissue with the steviol glycosides[1] (SG), stevioside and rebaudioside A, reported to be 200-300 times sweeter than cane sugar (Kulasekaran, Singh & Megeji 2006). Thus stevia may be used to replace the high calorie sugar sources in food products. It is recommended for use in the management of diabetes and has been extensively tested in animals and has been used by human with no side-effects (Brandle, Starratt & Gijzen 1998; Megeji et al. 2005). It is used for medical purposes such as in the treatment of hypertension and obesity, dental care and also used in cosmetics to cure acne. The product is licensed for human consumption in Japan, China, Taiwan, Korea, Mexico, Thailand, Indonesia and India (Kulasekaran, Singh & Megeji 2006). Recently, the Australian food authority FSANZ (Food Standard Australia and New Zealand) approved the use of stevia as a food ingredient in Australia and New Zealand (FSANZ 2008). In the United States of America it is approved as a dietary supplement. On 14 April 2010, the European Food Safety Authority (EFSA) published a positive opinion on the use of steviol glycosides as a food additive. SG is

[1] The term steviol glycosides (SG) has only been used since 2004 as the collective name for the diterpene glycosides of stevia. Previous to 2004 the word "stevioside(s)" was used both as the collective name for all the glycosides as well as the name of the most common glycoside. Hence, in papers and articles written prior to about 2004 the name "stevioside" frequently referred to the group of glycosides in total. When it is considered likely that "stevioside" was used in the collective sense, (SG) has been added.

underway to be approved by the European Commission (EFSA 2010). Japan has been one of the major growers and markets for the sweetener.

Stevia has been formally studied in Australia since 1999. A review made by Midmore & Rank (2002) concluded that stevia is a potential crop for commercial cultivation in Australia. A later report by the same authors Rank & Midmore (2006) recommends potential areas for cultivation of stevia in Australia, in positions along the eastern coast of Australia from Mareeba to Tasmania. Issues such as site selection, seed and seedlings, irrigation, pest diseases and harvesting were covered in their publications. During the site selection process, performance of two varieties, 99-8 and Fengtian was documented. As leaf is the valuable part of the crop for SG production, early flowering is regarded as an undesirable trait for commercial stevia production. Therefore, it is important to understand the flowering response of stevia to environmental variables and to explore the possibilities of selecting late flowering plants from existing varieties/populations of stevia. Similarly, response of stevia plants to different macro and micro nutrients has not been documented under Australian conditions, and also not adequately elsewhere, except for a few countries in their local languages (Lima Filho & Malavolta 1997a; Sheu, Tamai & Motoda 1987; Utumi et al. 1999). The effects of nutrient deficiencies on stevia growth and SGs are investigated in this thesis. Stevia in its native place is found growing in marshy lands and is well adapted to acid, infertile sandy soils with ample supply of water (Megeji et al. 2005; Shock 1982). The soil types along the eastern coast of Australia vary considerably in their pH, fertility and water holding capacities. For optimum production of stevia, the water requirement greatly depends on the types of

soil on which the crop is being grown. This study will look at water requirements and their effects on growth and SG concentrations.

1.2 Literature Review

1.2.1 Species description and morphology

Stevia belongs to the Asteraceae family. There are more than 200 species in the genus Stevia but *Stevia rebaudiana* has proved to be the only species which has significant steviol glycosides concentrations (Soejarto et al. 1983). Stevia is a small perennial bushy herb growing up to 50-60 cm height (Brandle & Rosa 1992). It has sessile, oppositely arranged lanceolate (a leaf tapering towards the apex) to oblanceolate (a leaf having rounded apex) leaves, with margins serrated in the upper part of the leaf (Brandle, Starratt & Gijzen 1998). The flowers are arranged in a cyme of corymbs with five white tubular flowers. Corymbs are arranged in loose panicles (Goettemoeller & Ching 1999). It is observed that at full blooming stage, stevia can produce more than 500 inflorescences in one plant (Southward, Kitchen & Fountain 2004). The fruit is an achene, one-seeded with a feathery pappus (Brandle, Starratt & Gijzen 1998). Harvesting of seeds is done gradually i.e. seeds mature at different times and they have to be collected progressively. The growth pattern of stevia is broadly divided into four stages; germination, grand growth period, flowering and seed maturity. The first stage includes germination and establishment, the second vegetative growth, the third floral bud initiation to pollination and fertilization, and the fourth seed growth (Kulasekaran, Singh & Megeji 2006).

Stevia is considered to be a self-incompatible insect-pollinated crop (Oddone 1997). Goetemoeller & Ching (1999) reported that pollination treatment (by introducing bee

hives and by hand) increased viability and seed germination over the control, suggesting that some active manipulation of the blossoms is necessary to improve pollination. Stevia is a diploid having 11 pairs of chromosomes (Brandle, Starratt & Gijzen 1998). There are about 90 varieties of *Stevia rebaudiana* developed throughout the world (Kulasekaran, Singh & Megeji 2006). These varieties were developed for different climatic requirements. As the varietal diversity in stevia is reportedly quite high, selection of appropriate varieties for specific areas is essential. Brandle & Rosa (1992) found that there is high heritability for leaf yield (75%) and leaf-stem ratio (83%), and noted that leaf yield or the leaf-stem ratio were not related to stevioside (SG) concentration in the landrace cultivar (imported from China) grown in Delhi Research Station, Ontario Canada. They also found that heritable variation in the stevioside (SG) concentration was also high.

1.2.2 Distribution, climate, soil and water requirements

Stevia originates from the highlands of Paraguay within the latitudes 21^{o}S and 24^{o}S (Shock, 1982) at an altitude 500-1500 metre above sea level (masl), with average annual temperature of 25^{o} C and average rainfall of 1400 mm/year. Stevia in its native place is found to be grown in marshy land and is well adapted to acid, infertile sandy soils with ample supply of water. It has been successfully grown in different geographic locations around the world. It is grown as a perennial crop in subtropical regions and as an annual in mid-high latitude regions as it is frost sensitive (Goettemoeller & Ching 1999; Kulasekaran, Singh & Megeji 2006; Megeji et al. 2005). In addition, it can be grown in saline soils (Shock 1982) but for better yield the pH should range from 5-7. The plant prefers lightly textured and well-drained soil which needs ample water so that the soil is moist, but not wet throughout the year. As reported by Megeji et al. (2005) stevia cannot tolerate drought. But once it

is established it can tolerate drought to some extent as reported by Tonello, DeFaveri & Weeden (2006). It requires frequent irrigation for commercial leaf production. The crop water requirement reported by Goenadi (1983) is 2.33 mm/plant/day on oxic trofudalf soils (latosols). The growth of stevia was optimum when the soil content was 47% and the authors also state that below 30% soil water content the plant reached permanent wilting point (-1.5 MPa). Total consumptive water requirement for this crop using a micro-lysimeter was 5.44 mm/day for the crop cycle (80 days) as reported in Brazil (Fronza & Folegatti 2002), double that of Goenadi (1983). Plant yield is affected by the amount of water use. Lavini et al. (2008) reported that leaf yield increased by up to 40% when soil moisture was increased from 33-100% of soil water consumption (based upon the soil water balance method), suggesting 100% soil water consumption is best for stevia cultivation.

1.2.3 Flowering in relation to photoperiod and temperature

Stevia is a photoperiod sensitive plant. It is a short day plant with a critical photoperiod somewhat between 12-13 hours day length (Midmore & Rank 2002; Valio & Rocha 1977). Percent of flowering was high at 12 hours day length (almost 80%) after more than 60 days of treatment application and time to flowering decreased with decrease in day length at 10 and 8 hours day length. As reported by Valio & Rocha (1997), plants kept at 14 and 16 hours photoperiod fail to induce flowering. Flowering and the growth of the plant is also affected by temperature. A temperature range from 6-43^{o}C with an average of 23^{o} C is ideal for the crop growth in semi-humid tropical climate (Brandle & Rosa 1992). A review made by Midmore and Rank (2002) when introducing stevia to Australia concluded that vegetative growth is reduced when the maximum day temperature is below 25^{o}C or over 35 ^{o}C.

When the plant starts to flower, nutrients accumulate in the reproductive organs and as a result vegetative growth declines. As leaf is the commercially important part of stevia, delay in flowering will enhance vegetative growth and economic yield. Most studies mentioned that stevia starts to flowering after 5-6 weeks of transplanting depending on light period. Harvesting is usually done before flowering which is generally after 7-8 weeks after transplanting or up to 10 weeks after ratooning (Tonello, DeFaveri & Weeden 2006). In order to achieve high leaf yield common practice in China is the pinching of buds before harvest, which enhances branching and increase leaf yield. Selection for late flowering has not yet been reported. As reported by Tonello, DeFaveri & Weeden (2006) clipping of stevia seedlings before transplanting delayed flowering time by two weeks during research trials conducted in Mareeba, Australia.

1.2.4 Propagation

Seed germination is commonly very poor in stevia and the growth of seedlings is slow (Brandle, Starratt & Gijzen 1998). To improve seed germination research recently conducted in India used growth regulators such as IAA to improve seed germination and biomass yield. Percent germination and biomass yield were positively correlated with the application of the growth regulator (Enkeshwer & Sandhya 2010). Propagation may also be done by tissue culture using nodal explants, axial buds or shoot tips (Hossain et al. 2008; Huda et al. 2007; Smitha et al. 2005), and by stem cuttings (Chalapathi et al. 2001). Tamura et al. (1984) also suggested that propagation by stem-tip culture is an effective method for obtaining a homogenous population of uniform plants for the production of SGs. However, for large scale production propagation with the use of seeds is easy and cost-effective.

For annual cropping with stevia growing plants from seed is considered to be more economically viable for stevia grown in Canada (Brandle, Starratt & Gijzen 1998). CQU experience suggests that with good fresh seed 80 – 90% germination can be expected (Andrew Rank, personal communication).

1.2.5 Steviol glycoside concentration

The sweet taste of stevia is derived from eight diterpene glycosides, of which the major agents are stevioside, rebaudioside A, rebaudioside C, and dulcoside A (Brandle & Rosa 1992). Stevioside content in dry leaves represents 5-10 % on average of dry weight depending on variety and growing conditions, and rebaudioside A 3-5 %. It has been reported that stevioside is 110-270 times sweeter than sucrose, while rebaudioside A is 150-320 times sweeter than sucrose (Yao, Ban & Brandle 1999). The highest amount of steviol glycosides is found to be in young actively growing shoots and leaves as compared to lower, senescent leaves (Bondarev et al. 2003). Steviol glycoside content of the plant varies according to plant organ, with leaves containing the highest concentration of SGs followed by flowers, stem and seeds (Bondarev et al. 2003; Zaidan, Deietrich & Felippe 1980). The stevioside content in the leaf is correlated with different phases of plant development. The SG is higher in the vegetative stage and levels decrease as the plants start flowering (Guzman 2010). Glycoside synthesis is reduced at or just before flowering (Brandle, Starratt & Gijzen 1998) and delay of flowering with long days allows more SG accumulation. Early flowering varieties would have a negative effect on economic returns so it is necessary to select late lines to prolong vegetative growth. The yield of SGs is related to photoperiod and also to irradiance levels (Zaidan, Deietrich & Felippe 1980). Plants grown in field conditions have higher SG

(% dry weight) content (375 percent higher) than those grown in the greenhouse provided with similar but lower irradiance conditions (under short days). The SG concentration in the leaves also varies with the propagation method. The variation in SG concentration is higher between plants grown from seeds than those propagated from stem-tip culture (Tamura et al. 1984). Nakamura and Tamura (1985) reported that variation is also observed at different stages of growth i.e. from the seedling stage to the harvesting stage and further added that SG levels at the seedling stage does not represent of that of mature leaves. The SG content (total content in the plant) in the leaves was compared between plant and ratoon crops and found that ratoon crops had higher content than the first crop as reported by (Megeji et al. 2005). Steviol glycosides content does not change with the water status of the plant and it is most likely genetically inherited (Lavini et al. 2008).

1.2.6 Stevia, its nutrient requirements

The nutrient requirement for this crop is low to moderate as it is adapted to poor quality soils in its natural habitat at Paraguay (Kulasekaran, Singh & Megeji 2006). Kawatani, Kaneki & Tanabe (1977) have reported that as the nitrogen levels increase from 20 to 60 kg/ha there is increase in growth, stem thickness, and the number of branches per plant. A similar effect was also obtained in response to potassium (Kawatani et al. 1980, cited in Kulasekaran, Singh & Megeji (2006). Brandle, Starratt & Gijzen (1998) have recommended 105 kg N, 23 kg P and 180 kg K/ha for a moderate biomass yield of 7500 kg/ha dry weight under Canadian conditions. Similarly in a field experiment in Bangalore, India, (Chalapathi, Shivaraj & Parama 1997) recommended a rate of 60 kg N, 30 kg P and 65 kg K/ ha, with the biomass yields noted to decrease when N rates were further increased.

Utumi et al. (1999) reported that deficiencies of secondary nutrients such as Ca, Mg, and S induced some morphological changes such as apical necrosis, chlorosis and inverted "V" shaped necrosis (leaves drying from the tip) and small pale green leaves. Some studies did report (in Portuguese) on the nutrient requirement in relation to growth, flowering, root weight and also stevioside (SG) content (Lima Filho & Malavolta 1997a; Sheu, Tamai & Motoda 1987) in different geographical conditions. Nutritional interactions have been studied showing synergistic effects on foliar content between N and P, P and Cu, and P and Fe; antagonistic effects between N and K, N and Zn, K and Mg, and K and S; or both between Zn and B, and Mn and Mg (Lima Filho & Malavolta 1997b). The requirements for micro-nutrients have also been mentioned by those authors but not clearly defined.

By growing plants in nutrient solutions with single element deficiencies, specific deficiency symptoms may be characterized. Through observation stevia growers may be able to identify macro and micro-nutrient requirements for this crop. Pictorial records for deficiency symptoms of stevia have not yet been published. Such records should be a useful resource for stevia growers.

1.2.7 Pests and diseases of stevia

Incidences of grasshoppers at their experimental site have been reported by Tonello, DeFaveri & Weeden (2006) during ongoing trials on stevia in Southedge Research Station, North Queensland, Australia. Attack by aphids, spider mites, white flies has also been reported, although the harm to the plants was minimal (Li 2000). Similarly, diseases such as powdery mildew, damping off and stem rot have been reported by other workers cited in Megeji et al. (2005). Two fungal diseases caused by *Septoria steviae* and *Sclerotinia sclerotiorum* have been also reported in stevia

grown in Canada (Lovering & Reeleder 1996) and in India (Bhandari & Harsh 2006).

These constraints to stevia cultivation require investigation, but were not studied during the course of the current research.

1.3 Purpose of the research

Based on the review of literature, the overall objective of the study is to provide information that allows for the optimising of agronomic practices and varieties for stevia in Australia for better steviol glycoside production.

The specific objectives are

- To determine whether it is possible to select late flowering lines from populations of stevia varieties through mass selection and to gain better understanding of the control of flowering
- To determine the effect of nutrient deficiencies on plant morphology, foliage symptoms, SG concentration and yields.
- To gain an understanding of plant response to soil water availability.
- To determine the effect of pH on growth and yield of stevia

A number of experiments were conducted at the CQUniversity Campus, Rockhampton to achieve these objectives.

Chapter Two: Flowering and biomass yield of stevia varieties

2.1 Introduction

Flowering is one of a number of the important factors that influence stevia cultivation. Production of high leaf biomass is a desired objective of stevia cultivation. Stevia leaf contains about 5-10 % stevioside and 3-5% rebaudioside A of leaf dry weight (Brandle & Rosa 1992). The SG concentration is highest in leaves, followed by inflorescence and stem (Zaidan, Deietrich & Felippe 1980). Generally plants are harvested before onset of flowering in order to achieve maximum leaf and SG yield. When the plant starts flowering, vegetative growth ceases and current photo-assimilates are transferred to the reproductive parts. The formation of SG decreases when the plant shifts from the vegetative to reproductive stage. To stop plants from early flowering, various practices have been employed, including pinching out of buds and clipping of seedlings before transplanting.

As reviewed by Bernier & Périlleux (2005), flowering time is control by a number of factors which were categorised as primary (e.g. photoperiod and temperature), secondary (ambient temperature, irradiance and water availability), or tertiary (less predictable influences such as mineral availability, light quality and adjacent vegetation). For example, high temperature and long photoperiod (day length) are known to delay flowering in plants such as chrysanthemum (Kim et al. 2009).

Stevia is said to be a short day plant with a critical photoperiod 13-14 hour day length for flowering. With day lengths >14 hours flowering is not profuse in stevia. Valio & Rocha (1977) reported that plants flower at 8 and 10 hour photoperiod and percent of flowering was highest at 12 hour day length. Thus exposure to more than 14 hour day length or short days with interruption of night maintains plants in a

vegetative stage. Valio & Rocha (1977) also noted that flowering was induced in plants with more than four pairs of leaves when exposed to just two short days (with less than 14 hours photoperiod), and that percentage of flowering increased as of the number of short days increased. Monteiro et al. (2001) also noted that stevia grown at 25^{o}C with 16 hours photoperiod remains in a vegetative stage. Zaidan, Deietrich & Felippe (1980) reported that stevia plants flower when exposed to between 8 and 14 hours photoperiod. In their studies they found that plants grown in natural conditions had higher stevioside (SG) content than those grown in the greenhouse, suggesting that total irradiation might have an effect on the content of stevioside (SG) in leaves rather than photoperiod.

Slamet &Tahardi (1988) reported that the time of flowering was delayed by a shading effect. In their studies, they found that 59.5% shade delayed flowering time, reduced percentage of flowering branches but also decreased the total biomass production. However, 38.4 % shade did not have an effect on these parameters.

For varietal improvement, phenotypic mass selection and cross breeding are the most common methods used so far in China, Canada, Korea and Russia (Brandle & Rosa 1992; Lee et al. 1979; Shu 1995; Zhuzhzhalova et al. 2004). Selections have been based on leaf yield, glycoside content, disease resistance and adaptation to different climates.

Brandle and Rosa (1992) reported on research conducted in Canada using cultivar imported from China, indicating that yield, leaf : stem ratio, and stevioside (SG) content were highly heritable and these characters can be used for further selection. Gaurav, Singh & Sirohi (2008) also reported that leaf yield, leaf width, leaf length, inflorescence number and the stevioside (SG) content are highly heritable.

As reported by Rank & Midmore (2006), premature and early flowering is prevalent in the tropics. Selection of late flowering populations from within a variety would help to increase leaf biomass, which would increase profits for the commercial growers and favour establishment of an industry.

The current study was conducted to study the relationships between flowering, biomass and SG content using available varieties. The varieties used for this research were imported from China. There is a lack of information about these varieties on their performance. However, two of the varieties (99-8 and Fengtian) were also used during the research reported by Tonello, DeFaveri & Weeden (2006) on biomass yield and flowering behaviour in north Queensland. It was reported that they flowered within 84 days and maximum flowering was attained 98 days after sowing.

Four experiments are presented in this Chapter. The first and second experiments document the flowering time, biomass and SG content of three stevia varieties (99-8, Fengtian 4(T4) and Shoutain) grown from seed and as a ratoon. The third experiment studied the flowering and biomass of F1 plants made by crossing some early, medium and late flowering selections from the first and second experiments. The fourth experiment evaluated the performance of the three varieties to different day length to assess their photoperiod sensitivity. The plants in experiment four were introduced to different day lengths (24, 16, 14 and 12 hours) in the growth cabinet using rooted cuttings as the planting material.

2.1.1 Experiment 1: Flowering and biomass yield of first crop

2.1.1.1 Materials and methods

Location details

All the experiments were conducted at CQUniversity, North Rockhampton (23 ^{o} 22', 0.345"S, 150^{o} 31' 0.53"E), Australia. All the experiments were conducted with potted plants.

Plant material

Seeds of three varieties (99-8, Fengtian 4 (T4) (referred to herein as Fengtian) and Shoutain-2 (referred to herein as Shoutain) of stevia were obtained from Andrew Rank of CQUniversity. These varieties were imported from Shandong, China. They are cross-pollinated and produced by specialized seed production farmers (Andrew Rank, 2008, personal communication). The seeds were 14 months old and had been stored in a refrigerator at 4^{o} C. Seeds were without hairs (pappus). The thousand seed weight for 99-8, Fengtian and Shoutain were 0.37, 0.34 and 0.30 g respectively. There are no published data for the photoperiod requirement of these varieties. Of the three varieties 99-8 and Fengtian were previously grown in a research trial in Mareeba, Australia. Shoutain is a newer Chinese variety.

Seed germination

Seeds were sown on 17/12/2008. Stevia seeds are very small in size and are very light in weight. Thus, for an even distribution of seeds, they were mixed with fine

sand and spread evenly on the surface of the soil mixture. The seeds apparently require some light for their germination as reported by Goettemoeller & Ching (1999). The soil mixture was sand, potting mix and coconut peat in the ratio of 4:3:3, respectively. The soil pH was 5.3. Seeds were germinated in the germination trays inside the screen house. Before sowing all plastic pots and trays were sterilised with a disinfectant sanitizer (5 ml sodium hypochlorite of 10% concentration in 5 litres of water), and placed in the sun to dry. The germination trays were manually watered daily.

Transplanting of seedlings

Seedlings were transplanted at weekly intervals starting from four weeks (19/01/2009) to 7 weeks (09/02/2009) after sowing. Seedlings had approximately 4-5 pairs of leaves at the time of transplanting, and were 8-10 cm in height. Individual seedlings were transplanted to 15 cm diameter plastic pots containing the potting media (same composition as for seed germination). The seedlings were selected according to size, with the largest remaining seedlings being selected on each occasion. The four transplanting dates were regarded as four treatments (T1, T2, T3 and T4). Two hundred plants of each variety (50/variety/transplant date) were grown in pots outside in a semi-controlled environment. Pots for each transplanting date were randomly placed on the bench (300 cm x 120 cm) as per completely randomised design. There were 19 rows altogether with 8 pots/row for each bench.

Growing conditions

To facilitate irrigation, six sprinklers were fitted on each bench and a watering regime was set to irrigate three times a day, each for a period of 15 minutes.

Nitrophoska (Brunnings Ltd.), a complete slow release fertilizer (with a nutrient composition of 16% total N, 3% of P, 12.5% of K, 7.2% of S, 1.2% Mg, 0.5% Fe, 0.1% of B, 0.1% Mn and 0.002% of Zn) was applied one week after transplanting. Fertilizer was applied at the rate of 2 g/plant.

Day length during the period of the experiment is shown in Tables 1 and 2. Data were obtained from the time and date website (Time and Date 2011) . Monthly maximum and minimum temperature was obtained from the Bureau of Meteorology (BOM), Rockhampton (Table 3).

Table 1: Day length (h) not including civil twilight for whole months at weekly intervals at Rockhampton (23° 23' S)

	Day lengths (hrs)				
Month	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
January	13.55	13.50	13.43	13.22	
February	13.15	12.89	12.74	12.58	
March	12.56	12.42	12.26	12.10	11.95
April	11.86	11.72	11.57	11.43	11.29
May	11.23	11.13	11.01	10.91	
June	10.79	10.74	10.70	10.71	
July	10.72	10.75	10.81	10.89	10.99
August	11.05	11.15	11.28	11.41	
September	11.64	11.77	11.92	12.08	
October	12.30	12.44	12.59	12.75	12.89
November	12.98	13.09	13.22	13.33	13.43
December	13.46	13.52	13.56	13.57	13.58

Table 2: Day length (h) associated with growth stages of stevia in Experiments 1, 2 & 3 at Rockhampton.

Activities	**Month**	**Year**	**Average day length (h)**
Sowing to seedling transplantation	Dec-Jan	2008	13.48
Flowering of three varieties of stevia	March-April	2009	11.91
Flowering of first ratoon	June-July	2009	10.76
Flowering of F1 generation	Nov-Dec	2010	13.28

Table 3: Average monthly maximum and minimum air temperatures (°C) during Experiments 1, 2 and 3 at Rockhampton.

Months	2009 Temperature		2010 Temperature	
	Maximum	Minimum	Maximum	Minimum
Jan	32.0	23.0	32.0	23.1
Feb	31.1	22.9	30.1	23.2
Mar	30.0	18.4	29.6	22.1
Apr	30.8	20.4	29.4	20.5
May	28.2	16.3	28.1	17.2
Jun	26.0	10.9	23.9	11.5
Jul	25.0	9.3	24.1	13.2
Aug	29.1	13.1	24.7	11.8
Sep	30.4	15.1	26.9	17.8
Oct	31.4	17.1	27.6	17.9
Nov	33.1	20.1	27.5	19.8
Dec	33.4	22.1	29.9	22.7

Flowering

Plants were observed daily for flowering. The day of the appearance of the first flower bud and the first day when the bud opened, revealing white petals was recorded for each individual plant.

Harvest

Plants were harvested after 12 weeks from each transplanting date (at which time more than 95% of the plants had flowered). Plant height (from the base of the stem to the topmost node of the apical stem) was measured before harvesting. Plants were harvested 6-8 cm above the base of the stem, as Tonello, DeFaveri & Weeden (2006) has been reported that harvesting close to the soil surface leads to plant mortality as well as to a decrease in steviol glycoside content of the ratoon crop.

From the population of 50 plants per variety, 15 plants of each variety of each treatment (four transplanting ages) were randomly selected and measured for plant

height, leaf fresh weight, leaf dry weight, stem fresh weight and dry weight and total biomass. Leaves were stripped from the stem and weighed separately. The plant samples were dried in an oven at 60^0 C for 48 hours.

Determination of leaf steviol glycoside concentration

Steviol glycoside (SG) concentration was determined using a HPLC (High–Performance Liquid Chromatography) based technique. Leaves were sampled at random from the sampled plants when most of the plants were at the flowering stage. For this analysis leaf samples from two treatments (T1 and T2) were taken. Depending upon the size of the leaves, two or three fully expanded mature leaves were taken from the mid portion of the plant. Leaves were oven-dried at 60^0 C for 48 h, and then ground to a fine powder with a mortar and pestle. The ground powder was stored in an air tight plastic container.

Approximately 0.1 g of stevia powder was used for the HPLC analysis. Analysis was carried out at CQUniversity, using a modified procedure of Hearn & Subedi (2009). For the extraction, 0.1 g of stevia powder was diluted in 5 ml of Milli-Q water and the sample tube was placed in the hot water bath at 70^oC for half an hour. The sample tube was centrifuged at 3500 rpm for 5 min. The supernatant was decanted and the extraction repeated. Solutions were combined from the two extractions. A 2 ml aliquot from the combined 10 ml water extract was filtered through a 0.45 µm filter (syringe driven filter unit, Millex®) before transferring to a 2 ml HPLC vial for the HPLC analysis.

An Agilent 1100 Series HPLC with multiple UV wavelength and auto-injector was used in conjunction with a Zorbax column (250*4.6 mm, 5µm) and an Agilent Zorbax High Pressure Reliance Cartridge guard column (12.5* 4.6 mm, 5 µm). The

mobile phase was 80% acetonitrile (pH 5), buffered with 100 ml of 0.02 M glacial acetic and 200 µL of 0.1 M sodium hydroxide (aq.). A flow rate of 1 $ml.min^{-1}$ and an injection volume of 5 µL were used. The UV detector was set at 210 nm with 360 nm as reference, and slit width was set to 4 nm. The HPLC was calibrated with stevioside and rebaudioside A standards (0.125, 0.25, 0.5, 0.75, and 1.0 g L^{-1}). Standards for stevioside and rebaudioside A were obtained from Wako Pure Chemical, Japan Pty. Ltd. Note: The level of other glycosides, though present, was not measured. Stevioside + rebaudioside-A generally make up approximately 90% of the total steviol glycosides.

Statistical analysis

Data were analysed using the statistical package for ANOVA (analysis of variance) for a completely randomized design through GenStat version 11.1 Least significant differences between means were calculated by Fisher's Protected LSD test ($P<0.05$).

2.1.1.2 Results

Effect of transplanting age on flowering time

The photoperiod from the date of transplanting to flowering decreased from 13 to 12 h day length (January - March). First transplanted seedlings (32 days old seedlings at transplanting) started flowering from 47 days after transplanting for Fengtian and 99-8, and 49 days for Shoutain. Flowering data (days after transplanting) for all treatments are presented in Table 4. Some plants did not flower by the time of harvest, but the number of such plants was minimal (varying from 1-4 per treatment and variety combination). There was a significant difference ($P<0.001$) in the average number of days to flowering between the three varieties of stevia, (Table 5).

The transplanting age had significant effect (P<0.001) on flowering of stevia. Similarly, the interaction between the transplanting age and variety was also significant (P<0.003). Time to average flowering date was least affected by transplant age in Fengtian. There was more difference in the number of days to flower within than between the varieties. The flowering duration (i.e. the time period between the date of flowering of the first and the last plant within a variety) for all the varieties was greatest in the fourth transplanting date (Table 4).

Table 4: Days to flower and duration of flowering for three stevia varieties in Experiment 1(n=50)

Age of the seedlings (Days)	Variety	First flowering (DAT)[1]	Flowering duration[2] (Days)
T1 (32)	99-8	49-56	7
	Fengtian	47-63	16
	Shoutain	47-60	13
T2 (39)	99-8	42-56	14
	Fengtian	44-57	13
	Shoutain	42-59	17
T3 (45)	99-8	39-62	23
	Fengtian	41-62	21
	Shoutain	41-55	14
T4 (52)	99-8	34-66	32
	Fengtian	40-74	34
	Shoutain	38-66	28

[1] DAT-Days after transplanting ; [2] For plants that flowered

Table 5: Average number of days from transplant to flower of three stevia varieties in Experiment 1 (n=15)

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	52.7	52.1	54.4	53.1
T2 (39d)	48.0	50.5	49.7	49.4
T3 (45d)	47.8	49.6	48.4	48.6
T4 (52d)	42.7	50.7	47.6	47.0
Mean	47.8	50.7	50.0	

Between variety P= <.001; lsd (154 df) = 1.427
Between treatment P= <0.001; lsd (154 df) = 1.648
Interaction between treatment and variety P= 0.003; lsd (154 df) = 2.854 (calculated at 5%)

Effect of transplanting age on yield attributes

There was no significant difference between the three stevia varieties in biomass (Table 6), but for plant height (Table 7) differences were significant (P<0.05). Transplanting age had a significant effect (P<0.001) on biomass at harvest, plant height, leaf yield and stem yield (Table 6, 7, 8 and 9). Seedlings transplanted earlier had higher biomass and greater plant height at harvest (12 weeks after transplanting) than the later transplanted ones. The interaction between transplanting age and variety for biomass was significant (P<0.007). Differences between varieties were greater at the earlier transplant age. Later transplanting led to lower leaf and stem yield per plant (Table 8 and 9) and height (Table 7).

Table 6: Effect of seedling age on above ground biomass (dry weight, g/plant) at 12 weeks from transplant of three stevia varieties in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	6.2	5.6	7.2	6.3
T2 (39d)	5.2	3.9	4.8	4.7
T3 (45d)	4.4	3.9	3.1	3.8
T4 (52d)	2.1	2.7	2.8	2.5
Mean	4.5	4.0	4.5	4.3

Between variety P= 0.140
Between treatment P= <0.001; lsd (154 df) = 0.611
Interaction between treatment and variety P= 0.007; lsd (154 df) = 1.058 (calculated at 5%)

Table 7: Effect of seedling age on plant height (cm) at 12 weeks from transplant of three stevia varieties in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	55.0	51.4	58.0	54.8
T2 (39d)	41.6	36.2	41.2	39.6
T3 (45d)	34.9	31.7	31.4	32.6
T4 (52d)	23.5	24.9	24.6	24.3
Mean	38.7	36.0	38.8	

Between variety P= 0.054; lsd (154 df) = 2.543
Between treatment P= <0.001; lsd (154 df) = 2.937 (calculated at 5%)
Interaction between treatment and variety P= 0.197

Table 8: Effect of seedling age on leaf yield (dry weight, g/plant) at 12 weeks from transplant of three stevia varieties in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	1.7	1.8	2.0	1.8
T2 (39d)	1.5	0.8	1.4	1.2
T3 (45d)	1.4	1.3	0.7	1.1
T4 (52d)	0.6	0.9	1.0	0.8
Mean	1.3	1.2	1.3	

Between variety P= 0.641; lsd (154 df) = 0.214
Between treatment P= <0.001; lsd (154 df) = 0.247 (calculated at 5%)
Interaction between treatment and variety P= 0.197

Table 9: Effect of seedling age on stem yield (dry weight, g/plant) at 12 weeks from transplant of three stevia varieties in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	4.4	3.7	5.1	4.4
T2 (39d)	3.7	3.1	3.4	3.4
T3 (45d)	3	2.5	2.3	2.6
T4 (52d)	1.5	1.7	1.7	1.6
Mean	3.1	2.8	3.1	

Between variety P= 0.116
Between treatment P= <0.001; lsd (154 df) = 0.472
Interaction between treatment and variety P= 0.058; lsd (154 df) = 0.818 (calculated at 5%)

Effect of transplanting age on SG concentration

Stevioside concentration in leaves differed between the varieties of stevia (P<0.04). Of the three varieties, 99-8 has less stevioside concentration compared to other two varieties of stevia (Table 10). Plants of the later transplanting age (39 days) had significantly higher stevioside concentration in leaves of two varieties (P<0.019), but not for Shoutain. The rebaudioside A concentration did not differ between the varieties but there was a significant difference between the treatments (Table 11). From the data it is clear that total percent dry weight of stevioside and rebaudioside A for Fengtian and Shoutain was significantly (P<0.005) greater than that of 99-8 (Table 12). The total SG concentration in leaves was significantly greater when the older seedlings were transplanted irrespective of the varieties. The interaction between treatment and variety was non-significant. The average SG content

(mg/plant) for the two transplanting ages was highest for Shoutain followed by 99-8 (Table 13).

Table 10: Average stevioside concentration (% dry weight) in leaves of three varieties of stevia in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	5.0	6.6	6.6	6.0
T2 (39d)	6.5	8.6	7.3	7.5
Mean	5.7	7.6	6.9	

Between variety P= 0.019; lsd (35 df) = 1.430
Between treatment P= 0.040; lsd (35 df) = 1.167 (calculated at 5%)
Interaction between treatment and variety P= 0.728

Table 11: Average rebaudioside A concentration (% dry weight) in leaves of three varieties of stevia in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	1.1	1.9	1.6	1.5
T2 (39d)	2.4	2.2	2.4	2.3
Mean	1.7	2.1	2	

Between variety P= 0.760; lsd (35 df) = 1.068
Between treatment P= 0.093; lsd (35 df) = 0.872
Interaction between treatment and variety P= 0.676

Table 12: Average total stevioside + rebaudioside A concentration (% dry weight) in leaves of three varieties of stevia in Experiment 1.

Transplanting age	99-8	Fengtian	Shoutain	Mean
T1 (32d)	6.1	8.5	8.2	7.6
T2 (39d)	8.9	10.9	9.7	9.8
Mean	7.5	9.7	9.0	

Between variety P= 0.024; lsd (35 df) = 1.755
Between treatment P= 0.008; lsd (35 df) = 1.433
Interaction between treatment and variety P= 0.692

Table 13: Average SG content from leaves (mg/plant) of three varieties of stevia grown at different transplanting ages in Experiment 1.

	Varieties		
Treatment	99-8	Fengtian	Shoutain
	SG content (mg/plant)	SG content (mg/plant)	SG content (mg/plant)
T1 (32)	103.7	154.2	164.0
T2 (39)	133.5	87.2	135.8
Mean	118.1	70.4	149.9

2.1.1.3 Discussion

Effect of transplanting age on flowering time of three stevia varieties

The time of first flowering for the three genotypes was not statistically different, with flowering commencing 80 days after sowing (sown on second week of December, 2008) (Figure 1) over a period in which day length shortened from 12.5 h to 11.2 h. Zaidan, Deietrich & Felippe (1980) reported the commencement of stevia flowering occurs 50-60 days after sowing when the crop was grown under 12 hours constant day length. As both studies involved photoperiods less than the critical period of 14 h, the differences in time to flower must represent other growth conditions.

The effect of seedling age and time of transplanting on flowering has been reported for species such as rice (Viraktamath et al. 1998) and German chamomile (*Matricaria chamomilla*) (Rafieiolhossaini et al. 2010). An effect of the transplanting age on flowering was evident in stevia. When 4 weeks old seedlings were transplanted, the flowering commenced after 52 days, while earlier flowering occurred in plants transplanted as 5, 6 and 7 weeks old seedlings. The response in time to flower at different transplanting age varied among the varieties of stevia (Fig 1).

Maximum flowering occurred for all three varieties (99-8, Fengtian and Shoutain) between 84 and 89 days from sowing (Figure 1.). The flowering duration within a population of stevia varieties was from one week to five weeks from the days of transplanting as indicated in Table 4, and shown in Figure 1.

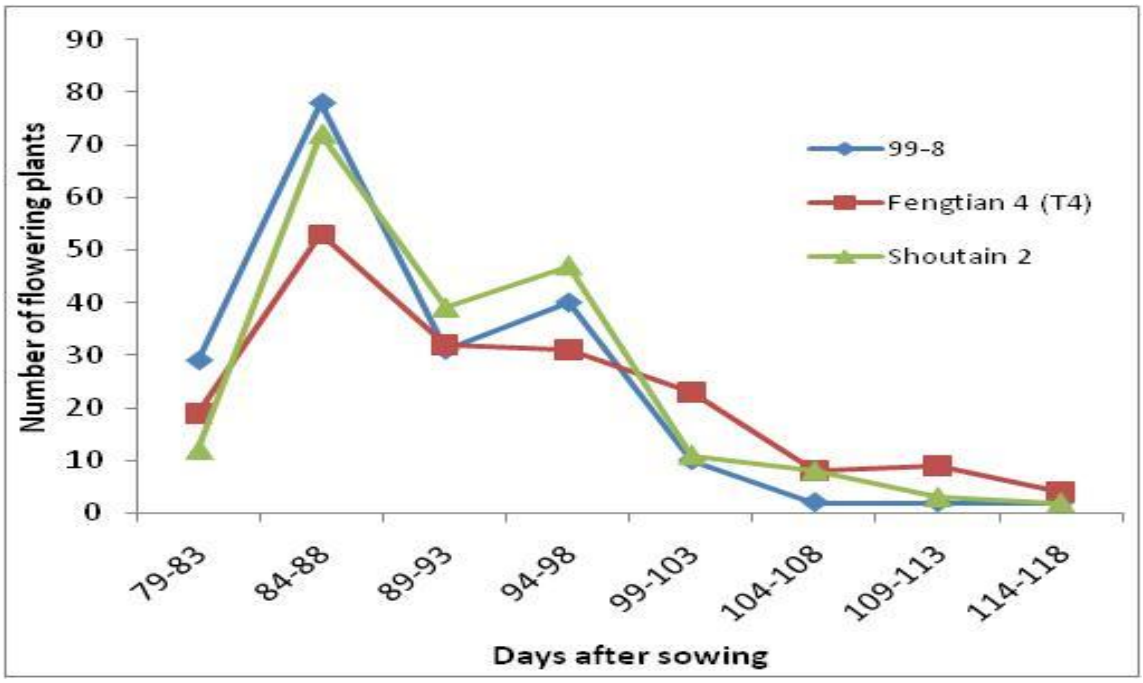

Figure 1: Flowering of three varieties of stevia as days from the date of sowing (totals from four transplant dates).

The wide range of the flowering time in different plants within a variety offers an opportunity to select potentially later flowering individuals, and to cross these individuals in an attempt to move the resulting population towards a later flowering mean.

German chamomile, a long day plant grown in Belgium, commencement of flowering was delayed from 74 DAS to 130 when the age of seedlings was increased from 30 days to 90 days (Rafieiolhossaini et al. 2010). The opposite result was obtained in the current study, with flowering time decreased as seedling age at

transplant was increased. This result is ascribed to stevia being a short day plant and the later transplanting being in shorter days.

Relationship between time of flowering and biomass in three varieties of stevia

There was no significant difference in biomass yield between the varieties. In all varieties, early transplanted seedlings had higher biomass at harvest, compared to later transplanted seedlings. A delay in flowering increased partitioning of assimilates in the vegetative parts such as leaves and stem.

However, the different varieties responded differently. The effect on leaf yield of transplanting seedlings of different ages was not significant for all varieties, but leaf yield was significantly decreased when older aged seedlings were transplanted for the varieties Fengtian and Shoutain. The early transplanted seedlings produced high biomass and leaf yield and plant height increased compared to later transplanted seedlings. In this case, variety 99-8 seems to be slightly less sensitive to the transplanting age of the seedling as compared to rest of the other varieties. The correlation between the time of commencement of flowering and the biomass yield of the variety 99-8 (r^2= 0.93) and Shoutain (r^2=0.96) ($p<0.05$) was very strong and positive as compared to that of Fengtian (r^2 = 0.40). Leaf yield is the most important component of stevia from economic point of view, so the impact of transplanting age is of agronomic interest.

Effect of seedling age on SG concentration

There was no significant difference in the SG concentration in the leaves of the three varieties of stevia. The SG concentration in the leaves of the three varieties (Figure 2) was similar to those mentioned in Fronza & Folegatti (2003). Percent dry weight of

stevioside and rebaudioside A was slightly higher (6-9%) in this study compared to that (5-6%) reported by Tonello, DeFaveri & Weeden (2006) for the same varieties (99-8 and Fengtian). SG concentration significantly increased with older transplanted seedlings irrespective of the variety.

Total SG content is the product of SG concentration and the leaf yield. The three varieties of stevia responded to seedling age differently. For variety 99-8, leaf yield was less affected by the age of seedling during transplantation as compared to the varieties Fengtian and Shoutain. Therefore, a delay in transplanting may be beneficial for variety 99-8 as it increases the SG concentration and did not decrease the leaf yield significantly. As the leaf yield of varieties Fengtian and Shoutain significantly decreased with the increased age of seedlings, despite the increased SG concentration, the total SG content per plant still decreased (Table 13). Therefore, the optimum seedling age for transplanting stevia depends on the variety. The concentration of stevioside also depends on genotype and growing conditions. Under long day conditions, plant accumulate more biomass in their photosynthetic tissues whereas in short day conditions plants tends to flower and most of the energy is accumulated to the reproductive organs.

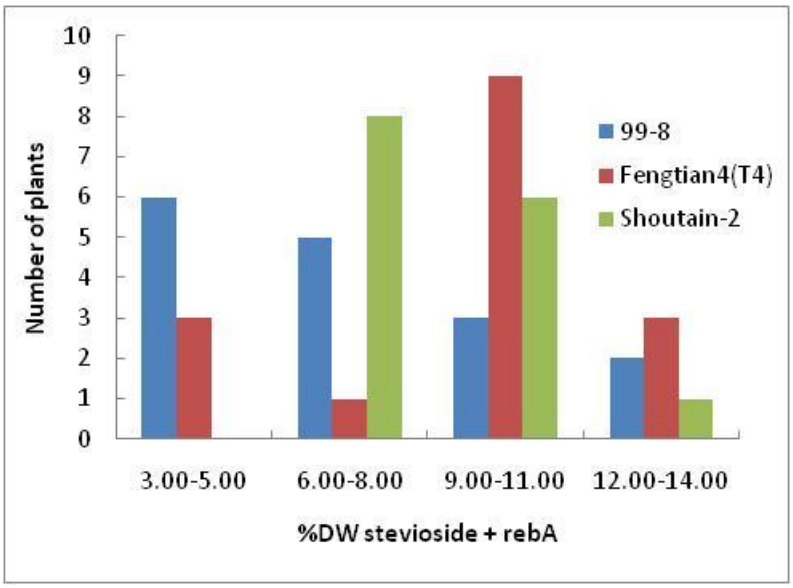

Figure 2: Total percentage dry weight of stevioside + rebaudioside concentration within three stevia varieties in Experiment 1.

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2.1.2 Experiment 2: Flowering and biomass yield of ratoon crop

2.1.2.1 Materials and Methods

Plants of Experiment 1 were harvested between April and May 2009. Harvesting was done at weekly intervals, T1 first and T4 last. New shoots appeared within one week of harvest. Nitrophoska slow release complete fertilizer was applied on the second day after harvest at the same rate as in Experiment 1. Ratoon crop (new shoots develop from the nodes at the base of the main stem) was harvested on the 3rd August 2009. Fifteen plants of each variety/treatment were randomly selected and growth parameters such as plant height, leaf and stem fresh weight and dry weight were measured following the same procedure as in Experiment 1. Flowering time for each treatment was recorded as in Experiment 1. Average photoperiod during the experiment ranged from 11- 10 hours as shown in Table 1. Statistical analysis was as in Experiment 1.

2.1.2.2 Results

Flowering time

Flowering of plants in the ratoon crop started between 29 to 51 days after ratooning. One plant from the first, seven from the second, six from the third and 25 plants from the fourth treatment did not flower by the time of harvest (Table 14). For stevia harvested on the 3rd week (T1) and the 4th week (T2) of April, days from first flowering to 50% flowering ranged from 2-3 weeks for the three varieties. Those plants that were harvested later at the end of April and first week of May, the time from first flowering to 50% flowering increased to 4-5 weeks (Table 15). There was a significant difference for average number of days to flower for ratoons between

varieties (P<0.004) and treatment (P<0.001). The average number of days to flower for Fengtian was 56 days whereas for 99-8 and Shoutain the average numbers of days to flowering were 60 and 61 days, respectively. There was no difference in time of flowering between the later two varieties (Table 15). The treatment with the later transplanting and therefore ratoons also flowered later as expressed by an increase in the number of days to flowering.

Table 14: Days to first flower and 50% flowering of three stevia varieties in Experiment 2 expressed as days after ratooning (DAR) (n=50).

Days after ratooning	Variety	First flowering (DAR)	Days to 50 % flowering	Flowering duration to 50% flowering*	Flowering duration (Days)	Non-flowering plants (#)
T1 (108)	99-8	39- 74	54	15	35	1
	Fengtian	28 -77	50	22	49	0
	Shoutain	33 -77	54	21	44	0
T2 (101)	99-8	37-100	60	23	63	3
	Fengtian	32-100	53	21	68	2
	Shoutain	32 -101	53	21	69	2
T3 (94)	99-8	33 – 94	63	30	61	3
	Fengtian	36 – 94	63	27	58	1
	Shoutain	33- 94	60	27	61	2
T4 (87)	99-8	33 – 87	68	35	54	8
	Fengtian	39 – 84	67	28	45	5
	Shoutain	51 – 87	76	25	36	12

***** flowering duration to 50% is the time from first flowering to 50% flowering

Table 15: Average number of days to flowering expressed as the number of days after ratooning of three stevia varieties in Experiment 2.

Days after ratooning	99-8	Fengtian	Shoutain	Mean
T1 (108)	57.0	48.6	57.47	54.4
T2 (101)	56.2	47.7	54.0	52.6
T3 (94)	62.7	60.0	61.5	61.4
T4 (87)	69.0	67.1	70.3	68.8
Mean	61.2	55.8	60.8	

Between variety P= 0.004; lsd (124 df) = 3.468
Between treatment P= <0.001; lsd (124 df) = 4.00
Interaction between treatment and variety P= 0.651; lsd (124df) = 6.93

Growth parameters

The ratoon crop was harvested when >90% plants were in flowering. There was no significant difference in total dry biomass yield, leaf weight (Table 16 and Table 17), stem weight and plant height (Table 18 and Table 19) between the varieties. However, the treatment, now a difference in days after ratooning, has a significant effect on biomass yield (P<0.003), leaf weight (P<0.001) and stem weight (P<0.001). The interactions between the treatment and variety for all the parameters were non-significant. The biomass and leaf yield in the third treatment (T3) for all varieties was higher than those harvested earlier or later. Plants in T3 were also taller than those in T1 or T2 (Table 19)

Table 16: Average biomass dry yield/plant (g) of three varieties of stevia (ratoons) in Experiment 2.

Days after ratooning	99-8	Fengtian	Shoutain	Mean
T1 (108)	7.5	8.2	7.9	7.9
T2 (101)	6.4	6.3	6.3	6.3
T3 (94)	9.7	7.6	8.3	8.5
T4 (87)	6.9	5.9	7.5	6.7
Mean	7.6	7.0	7.5	

Between variety P=0.527
Between treatment P= 0.003; lsd (154 df) = 1.311
Interaction between treatment and variety P= 0.604

Table 17: Average leaf dry weight/plant (g) of three varieties of stevia (ratoons) in Experiment 2.

Days after ratooning	99-8	Fengtian	Shoutain	Mean
T1 (108)	2.9	3.4	2.9	3.0
T2 (101)	3.7	3.4	3.4	3.5
T3 (94)	5.6	4.1	4.4	4.7
T4 (87)	3.6	3.4	4.4	3.8
Mean	3.9	3.6	3.8	

Between variety P=0.537
Between treatment P= <0.001; lsd (154 df) = 0.772
Interaction between treatment and variety P= 0.314

Table 18: Average stem dry weight/plant (g) of three varieties of stevia (ratoons) in Experiment 2.

Days after ratooning	99-8	Fengtian	Shoutain	Mean
T1 (108)	4.6	4.9	5.0	4.9
T2 (101)	2.7	2.9	2.8	2.9
T3 (94)	4.2	3.5	2.5	3.6
T4 (87)	3.2	2.5	3.1	2.9
Mean	3.6	3.4	3.7	

Between variety P=0.618
Between treatment P= <0.001; lsd (154 df) = 0.716
Interaction between treatment and variety P= 0.891

Table 19: Average plant height (cm) of three varieties of stevia (ratoons).

Days after ratooning	99-8	Fengtian	Shoutain	Mean
T1 (108)	35.6	33.4	33.3	34.1
T2 (101)	35.4	32.6	34.6	34.2
T3 (94)	40.3	35.2	39.5	38.4
T4 (87)	34.9	35.4	39.3	36.5
Mean	36.5	34.1	36.7	

Between variety P=0.099; lsd (154 df) = 2.62
Between treatment P= 0.017; lsd (154 df) = 3.03
Interaction between treatment and variety P= 0.525

2.1.2.3 Discussion

Effect of time of harvest on flowering of three varieties of stevia

Flowering in all the three stevia varieties started 4-5 weeks after the first harvest. Tonello, DeFaveri & Weeden (2006) also reported that flowering commenced after four weeks of first harvest of stevia in unclipped seedlings. They also used two of the varieties (99-8 and Fengtian) used in this study. Commencement of flowering varied greatly (32 to 100 days) within a variety suggesting considerable genetic variation within a variety for time to flower. The early flowering plants are presumably more

responsive to short days, those flowering later being less sensitive. This possibility was tested in Experiment 4.

Effect of time of harvesting on growth parameters

Stevia has the ability to ratoon. Higher biomass was obtained for ratoon crops compared to first crop due to the increased number of branches and leaf yield. Chalapathi et al. (1999) also reported that total biomass, plant height and leaf yield increased in ratoon crop as compared to the first crop. Time of harvest for the ratoon crop is important because of its leaf yield. In our study we found that yield was low when plants were harvested after 108 days compared to those harvested earlier. The highest yield for the three varieties was obtained when they were harvested after 94 days from the first crop. The low biomass yield at first harvest was because most of the plants were in flowering stage (90%). They had flowered in a shorter period after ratooning than plants in T3 and T4, and therefore, their biomass yields were more likely constrained. There was no variation between varieties in total biomass yield.

2.1.3 Experiment 3: Flowering and biomass yield of F1plants

2.1.3.1 Materials and Methods

Location and duration of experiment

The experiment was conducted in the same location as for Experiment 1. The duration of the experiment was 14 weeks from the date of sowing until date of harvest (20th September – 24th December, 2010).

Crossing and seed collection

Plants of three varieties of stevia grown in Experiments 1 and 2 were selected based on their flowering time in the first crop and the ratoon crop. They were categorised as early flowering, medium flowering and late flowering. There were altogether nine groups based on variety and flowering time, and there were 8 plants in each group. An isolation distance was maintained between each group, in order to avoid cross pollination. Before flowering, every individual group was covered by the fly screen of about 80 cm x 80 cm x 100 cm (widths x height) to protect from insects. At two day intervals plants within each group were rearranged and plants were lightly shaken for the cross-pollination based on the method used by Brandle & Rosa (1992). These groups were grown inside the CQUniversity compound, Rockhampton, for 5-6 weeks for seed production. Seeds from each group were collected separately during February and March (2010). Seeds were stored in an air tight container at 4^{o}C inside the cold room.

Seed germination and transplanting

Seeds collected from each flowering group (early, medium and late) were sown on 20th September, 2010 in speedling trays using vermiculite as a growing media.

Seeds were sown without removing pappus (hairy structure) from the seeds. Seeds were covered with a thin layer of vermiculite to stop them from blowing away. Seed germination was done inside the screen house with 67% light intensity. Seedlings were hand-watered and after three weeks they were watered with half strength of Manutec hydroponic solution (Manutec Pty. Ltd.). Seedlings were transplanted after four weeks into 150 ml plastic pots (one plant/pot) using the same potting media as in Experiment 1.

The experimental design used was completely randomized with three varieties and three flowering groups (early, medium and late), with 9 plants in each group. There were altogether 27 plants per variety. Nitrophoska slow release fertilizer was applied after one week of transplanting, at the rate of 2 g/plant. Data on day length and temperature (maximum and minimum) were obtained from the Bureau of Meteorology (BOM), Rockhampton (Table 3). Harvesting was done on 24th December, 2010. Day length during the start of experiment and at harvesting was 11.9 h and 13.5 h respectively.

Data collection for flowering and growth attributes

Data were collected following the same method used in Experiment 1.

2.1.3.2 Results

Flowering

Flowering for early groups started after 116, 130 and 111 days after sowing for 99-8, Fengtian and Shoutain, respectively (Table 20). Plants in the medium group for the same varieties started flowering 101, 122 and 136 days after sowing. Late flowering group for the same varieties started flowering 132, 146 and 151 days after sowing.

Percentage of flowering in the early groups was highest in Fengtian and Shoutain (56%), whereas 99-8 had the lowest flowering plants. Flowering for the medium groups was highest in 99-8 (56%) followed by Fengtian and Shoutain. Flowering for the late groups was highest in Shoutain (33%) followed by Fengtian and 99-8. Plant mortality was observed in early and late groups of 99-8 (44% and 11%). Flowering of medium groups was earlier (15 and 8 days) than early groups of both 99-8 and Fengtian. However, flowering for Shoutain flower early (111 DAS) and medium groups started flowering 25 days after the early group and the late group started flowering 15 days later after than the medium group.

Table 20: Flowering of F1 plants for stevia variety selections for early, medium or late flowering (days after sowing).

	99-8		Fengtian		Shoutain	
Flowering group	**Days to first flowering**	**Flowering plants at harvest (%)**	**Days to first flowering**	**Flowering plants at harvest (%)**	**Days to first flowering**	**Flowering plants at harvest (%)**
Early	116	20	130	56	111	56
Medium	101	56	122	44	136	22
Late	132	12	146	22	151	33

Yield attributes

Plants were harvested after 14 weeks from the date of sowing. There was no significant difference in plant height between the varieties (P=0.638) and also with plants from early medium and late flowering groups (Table 21). The interaction between variety and flowering group was also non-significant.

Total biomass yield difference between the three varieties was non-significant (Table 22). There was no significant difference in leaf (Table 23) and stem yield for the

three varieties and flowering groups of stevia. No table has been presented on stem yield.

Table 21: Average plant height (cm) in early, medium and late populations from stevia varieties in their F1 generation.

Flowering groups	99-8	Fengtian	Shoutain
Early	62.8	56.7	48.3
Medium	64.4	60.9	58.0
Late	54.1	57.9	63.9

Table 22: Average above ground dry biomass (g/plant) in early, medium and late populations from stevia varieties in their F1 generation.

Flowering groups	99-8	Fengtian	Shoutain
Early	5.3	5.9	3.7
Medium	5.8	5.9	6.1
Late	5.4	5.2	5.1

Table 23: Average leaf dry weight (g/plant) in early, medium and late populations from stevia varieties in their F1 generation.

Flowering groups	99-8	Fengtian	Shoutain
Early	2.1	2.7	1.4
Medium	1.9	2.6	2.7
Late	2.7	2.6	2.3

2.1.3.3 Discussion

Plants transplanted in the month of January-February started to flower after 81-83 DAS in all the varieties (99-8, Fengtian and Shoutain) (Experiment-1). Plants selected (in experiment one and two) from these groups after crossing started to flower after 110 DAS for early groups. The number of days to flowering was delayed (3 weeks) for F1 generation plants compared to parent plants, but the day length also differed, that for Experiment 1 went from 13.2 h to 11.2 h while for the F1 Experiment it went for 11.9 h to 13.5 h. The number of days to flowering for different groups (early, medium and late) was not consistent with the selection. In this study medium flowering groups flowered earlier (99-8 and Fengtian) than early flowering timing. However, for Shoutain there was consistency with the flowering.

The difference in days to flowering between early group and late group was high (40 days) for Shoutain compared to other varieties (99-8 and Fengtian). The medium flowering groups flowered early for the two varieties (99-8 and Fengtian). As was expected, if flowering was hastened, then the proportion of plants that flowered by harvest would be higher in the selections for earliness. The percentage of early flowering was highest for early groups of Fengtian and Shoutain.

Brandle and Rosa (1992) reported that through phenotypic mass selection for leaf yield, leaf : stem ratio, and stevioside (SG) content can be improved. Gaurav, Singh & Sirohi (2008) also reported that leaf yield, leaf width, leaf length, inflorescence number and the stevioside (SG) content are highly heritable, thus could be improved through mass selection. However, flowering is strongly associated with environmental conditions such as photoperiod, irradiation and temperature. Therefore, one selection cycle was not enough to decide whether this method was appropriate or not. In this study there is some trend in time of flowering between early, medium and late groups. However, further selection cycles are required to achieve the desired objectives.

2.1.4 Experiment 4: Effects of photoperiods on flowering and biomass

2.1.4.1 Materials and Methods

Preparation of plants

Two node cuttings were taken from growing shoots of early, medium and late flowering plants of the three stevia varieties, dipped in rooting hormone, Clonex (a.i. IBA) with the concentration of 3 g L^{-1}, and placed into vermiculite media in speedling trays in a growth cabinet with 14 hours day length, 25/20^{o} C day / night temperature, with relative humidity of 70% and full light intensity (350-400 µmoles $m^{-2}sec^{-1}$) for eight weeks. Rooted cuttings were clipped and then transplanted to 150 ml pots with the same media and fertilizer as in Experiment 1. Plants were irrigated using an over-head sprinkler, with a watering regime of 10 minutes run time, three times a day.

Experimental treatments

A complete randomised design with five replications was used, with each single potted plant representing an experimental unit. There were five plants from each group (early, medium and late flowering plants) of each variety (99-8, Fengtian and Shoutain), or 45 plants in total, selected as for the parents in Experiment 1.

The experiment was conducted inside growth cabinets, running from 6th of May, 2010 to 12th March, 2011. Four constant photoperiods were imposed: 24, 16, 14 and 12 hours light in a 24 h diurnal cycle for 15, 12, 10 and 7 weeks respectively. The 24 hour photoperiod was divided into 10 hours with full intensity light (350-400 µmoles $m^{-2}sec^{-1}$) and 14 hours with low intensity (31-40 µmoles $m^{-2}sec^{-1}$). Day and night

temperature was 25°C/20°C and relative humidity was maintained at 70%. All treatments were imposed in the same growth cabinet on the same plants.

On 6th May 2010, plants were clipped 6 cm from the ground surface and introduced into 24 hours of continuous photoperiod. The number of days to flower was recorded from the date of treatment imposition. Plants were harvested after 15 weeks, with above ground biomass cut 6-7 cm above the ground surface. Plant height, leaf and stem dry weight were measured.

After the first harvest plants were subjected to a 16 hour photoperiod (full light intensity of 350-400 µmoles $m^{-2}sec^{-1}$) treatment, with parameters such as day/night temperature, relative humidity, watering regime and full light intensity as in the 24 hours treatment. The number of days to flower was again recorded. After 12 weeks, aboveground biomass was harvested as before. The same procedure was repeated with full light intensity day lengths of 14 hours and 12 hours, with harvests after 10 and 7 weeks, respectively. For every harvest days to flower, plant height and total biomass were measured. The time to the first flowering of any plant in a given treatment (of 5 plants) was recorded.

2.1.4.2 Results

Response of stevia to 24 hour photoperiod

Flowering

Under continuous light, 40%, 20% and 20 % of early flowering plants of the three varieties (99-8, Fengtian, Shoutain) flowered (Table 24). For medium flowering plants of Fengtian and Shoutain, 20% and 60%, respectively, flowered. However, there was no flowering for 99-8. Of the late flowering group, 20% of 99-8 flowered,

whereas none of the plants of the other two varieties flowered. Early flowering plants started flowering 79, 48 and 87 days after treatment application for 99-8, Fengtian and Shoutain, respectively. For medium flowering plants first flowering started at 103 and 27 days for Fengtian and Shoutain, respectively. First flowering for late flowering plants of 99-8 was at 103 days.

Table 24: Flowering of stevia varieties and flowering groups under 24 hour photoperiod.

Flowering groups	99-8		Fengtian		Shoutain	
	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)
Early	79	40	48	20	87	20
Medium	0	0	103	20	27	60
Late	103	20	0	0	0	0

Yield attributes

Plants were harvested 15 weeks after clipping. There was neither significant difference in plant height between the varieties, nor between early, medium and late flowering groups (Table 25). The interaction between variety and flowering groups was also non-significant.

There was no significant difference in total above ground biomass, leaf and stem yield between the three varieties of stevia, or between early, medium and late flowering groups (Tables 26, 27 and 28). However, leaf yield was higher for Shoutain late and for Fengtian late groups than their respective early and medium groups.

Table 25: Average plant height (cm) of stevia varieties and flowering groups under 24 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	52.0	40.2	41.8	44.7
Medium	45.0	44.6	35.4	41.7
Late	33.2	50.6	34.8	39.5
Mean	43.4	45.1	37.3	

Between variety P=0.468; between flowering group P=0.737;
Interaction between flowering group and variety P= 0.493

Table 26: Average above-ground dry biomass (g/plant) of stevia varieties and flowering groups under 24 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	3.7	1.3	1.5	2.2
Medium	3.3	1.1	2.9	2.4
Late	3.3	1.2	4.5	2.9
Mean	3.1	1.5	3.0	

Between variety P=0.209; between flowering group P=0.742;
Interaction between flowering group and variety P= 0.529

Table 27: Average leaf dry weight (g/plant) of stevia varieties and flowering groups under 24 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	1.9	0.9	0.9	1.2
Medium	1.9	0.7	1.5	1.3
Late	1.3	1.3	3.4	2.0
Mean	1.7	1.0	1.9	

Between variety P=0.256; between flowering group P=0.375;
Interaction between flowering group and variety P= 0.230

Table 28: Average stem dry weight (g/plant) of stevia varieties and flowering groups under 24 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	1.8	0.5	0.7	0.9
Medium	1.4	0.4	1.4	1.0
Late	1.1	0.7	1.1	0.9
Mean	1.4	0.5	1.1	

Between variety P=0.127; between flowering group P=0.966;
Interaction between flowering group and variety P= 0.766

Response of stevia varieties to 16 hour photoperiod

Flowering

Total percentages of flowering for 99-8 and Shoutain in the early groups were 20 and 20% respectively (Table 29). None of the early group plants flowered in Fengtian. For medium flowering groups, percentages of flowering for Fengtian and Shoutain were 50% and 60%, respectively. There was no flowering for the medium flowering group of 99-8. None of the plants flowered in late flowering groups in any of the three varieties. First flowering for the early groups of 99-8 and Shoutain was 48 and 79 days, respectively. Similarly for medium flowering groups for Fengtian and Shoutain varieties, plants started to flower at 59 and 72 days, respectively.

Table 29: Flowering of stevia varieties and flowering groups under 16 hour photoperiod

Flowering groups	99-8		Fengtian		Shoutain	
	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)
Early	48	20	0	0	79	20
Medium	0	0	59	50	72	60
Late	0	0	0	0	0	0

Yield attributes

Plants were harvested after 12 weeks. There was no significant difference in plant height between the varieties or between plants from early, medium and late flowering groups. The interaction between variety and flowering groups was non-significant (Table 30).

Total biomass yield differences between varieties were non-significant (Figure 3a). Biomass yield was low for late group compared to early and medium groups of 99-8.

For Fengtian, biomass was high for medium group compared to early and late groups. For Shoutain, high biomass was obtained for the late group compared to early and medium group. Interaction between variety and flowering group was statistically significant (P=0.029) (Figure 3a).

There was no significant differences in leaf and stem yield between the three varieties. There was also no difference between early, medium and late flowering groups. Interaction between variety and leaf yield was significant (P=0.01) (Figure 3b). Leaf yield was highest for Shoutain late group followed by 99-8 medium group. Stem yield (Table 31) was highest for Fengtian medium group followed by 99-8 early group.

Table 30: Average plant height (cm) of stevia varieties and flowering groups under16 hour photoperiod.

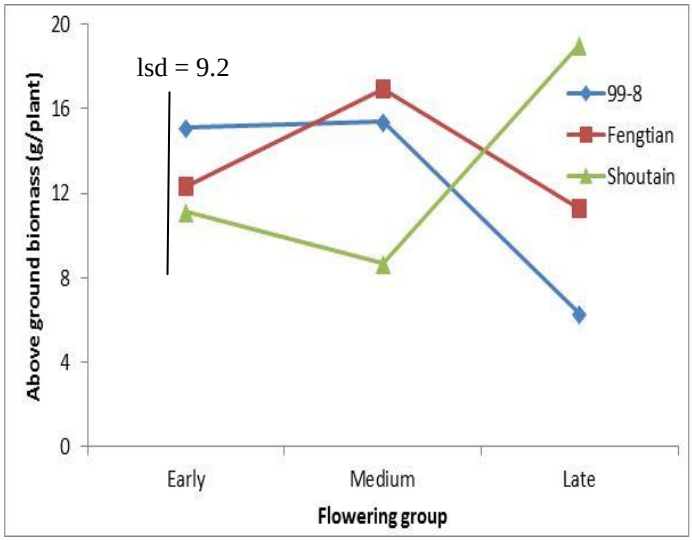
Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	42.2	28.6	35.0	35.3
Medium	38.0	42.6	28.8	36.5
Late	29.6	29.1	25.2	28
Mean	36.6	33.4	29.7	

Between variety P=0.356; between flowering group P=0.172;
Interaction between flowering group and variety P= 0.444

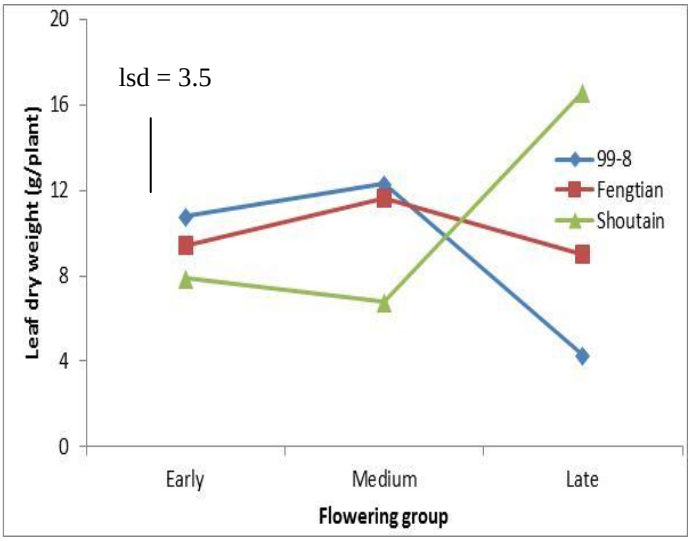
Table 31: Average stem dry weight (g/plant) of stevia varieties and flowering groups under 16 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	4.3	2.9	3.2	3.4
Medium	3.1	5.3	1.9	3.4
Late	2.0	1.9	2.4	2.2
Mean	3.1	3.5	2.5	

Between variety P=0.383; between flowering group P=0.172;
Interaction between flowering group and variety P= 0.163



(a)



(b)

Figure 3: Total above ground biomass (a) and leaf yield (b) of three stevia varieties across three flowering groups under 16 hours photoperiod. Lsd refers to the interaction between flowering groups and varieties.

Response of stevia varieties to 14 hour photoperiod

Flowering

Total percent of flowering plants for the 99-8 early group was 40% (Table 32). There was no flowering for Fengtian and Shoutain varieties in the early group. For the medium flowering group, the percentage of flowering for 99-8, Fengtian and Shoutain was 40%, 40% and 80%, respectively. Flowering percentage for 99-8, Fengtian and Shoutain late groups were 20%, 22% and 20%, respectively.

Table 32: Flowering of stevia varieties and flowering groups under 14 hour photoperiod.

Flowering groups	99-8		Fengtian		Shoutain	
	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)
Early	59	40	0	0	0	0
Medium	52	40	59	40	46	80
Late	70	20	59	20	52	20

Yield attributes

Plants were harvested after 10 weeks. There was neither difference in plant height between the varieties nor between plants from the early, medium and late flowering groups (Table 33). The interaction between variety and flowering groups was also non-significant. Differences in total biomass yield between the three varieties were non-significant (Table 34). Biomass yield was low for late group of 99-8 compared to early and medium groups. Fengtian biomass was high for medium groups compared to early and late groups. For Shoutain biomass was same for all the three groups.

There was no significant difference in leaf and stem yield between the three varieties. There was also no difference between early, medium and late flowering groups. Leaf yield was highest for 99-8 early group followed by medium and late groups. Leaf yield was highest for Fengtian in the medium group whereas the late group of Shoutain had higher biomass compared to early and medium groups (Table 35 and 36).

Table 33: Average plant height (cm) of stevia varieties and flowering groups under 14 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	65.0	42.8	51.5	53.1
Medium	47.2	53.3	46.4	49.0
Late	46.6	47.1	27.4	40.4
Mean	52.9	47.7	41.8	

Between variety P=0.081; lsd (29 df) = 8.11(calculated at 10%)
Flowering group P=0.037; lsd (29 df) = 9.76 (calculated at 5%)
Interaction between flowering group and variety P= 0.063; lsd (29 df) =14.04 (calculated at 10%)

Table 34: Average above-ground dry biomass (g/plant) of stevia varieties and flowering groups under 14 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	26.2	14.7	15.0	18.6
Medium	18.5	20.2	15.2	17.9
Late	11.7	13.7	15.6	13.6
Mean	18.8	16.2	15.3	

Flowering group P=0.094; lsd (29 df) = 4.03 (calculated at 10%)
Interaction between flowering group and variety P= 0.061; lsd (29 df) = 6.98 (calculated at 10%)

Table 35: Average leaf dry weight (g/plant) of stevia varieties and flowering groups under 14 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	12.9	8.6	8.5	9.9
Medium	10.5	11.0	6.3	9.2
Late	6.7	7.8	11.8	8.7
Mean	10.0	9.1	8.9	

Interaction between flowering group and variety P= 0.014; lsd (29 df) = 4.57 (calculated at 5%)

Table 36: Average stem dry weight (g/plant) of stevia varieties and flowering groups under 14 hour photoperiod

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	13.4	6.1	6.6	8.6
Medium	8.1	9.2	8.9	8.7
Late	5.0	6.0	3.8	4.9
Mean	8.8	7.1	6.4	

Flowering group P=0.008; lsd (29 df) = 3.5 (calculated at 1%)
Interaction between flowering group and variety P= 0.048; lsd (29 df) = 2.6 (calculated at 5%)

Response of stevia varieties to 12 hour photoperiod

Flowering

Days to first flowering was compared between varieties and flowering groups, ranged from 38-42 days after ratooning (Table 37). 100% flowering was recorded in all three varieties and flowering groups. Under 12 hour photoperiod, all plants had flowered by the time of harvest (after 6 weeks) except for few that were in budding

stage. There was no difference in number of days to flowering between varieties or with flowering groups within variety (Table 38).

Table 37: Days to first flowering and percent of flowering at the time of harvest (6 weeks) of stevia varieties and flowering groups under 12 hour photoperiod.

Flowering groups	99-8		Fengtian		Shoutain	
	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)	Days to first flowering	Flowering plants at harvest (%)
Early	38	100	39	100	42	100[a]
Medium	38	100	39	100	40	100
Late	40	100[a]	41	100[a]	38	100

[a] only at bud stage

Table 38: Average number of days to flower of three stevia varieties and flowering groups under 12 hour photoperiod

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	40.8	41.0	42.6	41.4
Medium	39.8	40.7	41.4	40.6
Late	42.0	40.9	39.8	40.9
Mean	40.8	40.9	41.2	

Interaction between flowering group and variety P=0.095; lsd (24 df)= 2.29 calculated at 10%

Yield attributes

Plants were harvested after 6 weeks. There was significant difference in plant height between the varieties (P<0.009) but there was no difference in plants from the early, medium and late flowering groups. The interaction between variety and flowering groups was also non-significant. However, average plant height was higher for 99-8 than Fengtian and Shoutain (Table 39).

Difference in total biomass yield between the three varieties was non-significant. Biomass yield was low for late group of 99-8 compared to early and medium groups. In contrast, the biomass for Shoutain was higher for late group compared to early

group. Whereas in Fengtian, the biomass was higher for the medium group compared to early and late groups (Table 40).

There was no significant difference in leaf and stem yield neither between the varieties nor between early, medium and late flowering groups. Leaf yield was highest for 99-8 early group followed by medium and late groups. Leaf yield was highest for Fengtian in medium group and Shoutain late group, compared to early and medium groups (Table 41 and 42).

Table 39: Average plant height (cm) of stevia varieties and flowering groups under 12 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	35.8	31.8	27.4	31.6
Medium	39.4	33.5	32.4	35.1
Late	35.6	29.8	27.0	30.8
Mean	36.9	31.7	28.9	

Between variety P= 0.009; lsd (27 df)= 2.4 (calculated at 5%)

Table 40: Average above-ground dry biomass (g/plant) of stevia varieties and flowering groups under 12 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	5.0	4.3	2.0	3.8
Medium	5.5	5.8	5.8	5.7
Late	3.4	2.8	6.3	4.2
Mean	4.6	4.3	4.7	

Between variety P= 0.907; lsd (27 df) = 1.7
Flowering group P=0.086; lsd (27 df) = 1.4 (calculated at 10%)
Interaction between flowering group and variety P= 0.06; lsd (27 df) = 2.57 (calculated at 10%)

Table 41: Average leaf dry weight (g/plant) of stevia varieties and flowering groups under 12 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	2.3	1.6	1.0	1.6
Medium	1.9	2.0	2.3	2.1
Late	1.6	1.3	2.9	1.9
Mean	1.9	1.6	2.1	

Interaction between flowering group and variety P = 0.027; lsd (27 df) = 1.17

Table 42: Average stem dry weight (g/plant) of stevia varieties and flowering groups under 12 hour photoperiod.

Flowering groups	99-8	Fengtian	Shoutain	Mean
Early	3.2	2.7	1.0	2.3
Medium	3.5	3.8	3.4	3.6
Late	1.9	1.5	3.3	2.2
Mean	2.9	2.6	2.5	

Flowering group P=0.040; lsd (27 df) = 1.16
Interaction between flowering group and variety P= 0.072; lsd (27 df) = 1.68 (calculated at 10%)

2.1.4.3 Discussion

Effect of photoperiod on flowering

In all three varieties considered, 100% flowering was evident within seven weeks of ratooning under the 12 hour photoperiod. Increasing the photoperiod from 12 h to 14 h and above (up to 24 h) significantly reduced the percentage of flowering plants in all the three varieties (Figure 4). This result confirms that all three varieties tested are photoperiod sensitive and are short day varieties, consistent with the classification of stevia as a short day plant (Kudo & Koga 1977; Valio & Rocha 1977; Zaidan, Deietrich & Felippe 1980) with a critical day length of 12-14 hours.

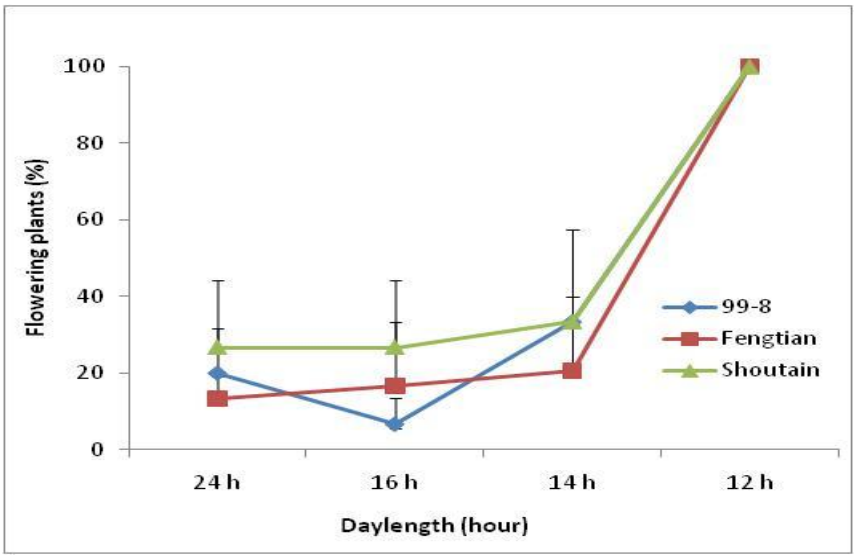


Figure 4: Effect of different photoperiods on flowering of three stevia varieties. Error bars represent SE.

However, the number of days to flower under different constant photoperiods did not consistently relate to the early, medium and late flowering groups as identified in this experiment. The group of plants believed to be medium in terms of flowering had a greater percentage of flowering plants across all treatments then did the early or late groups (Figure 5).

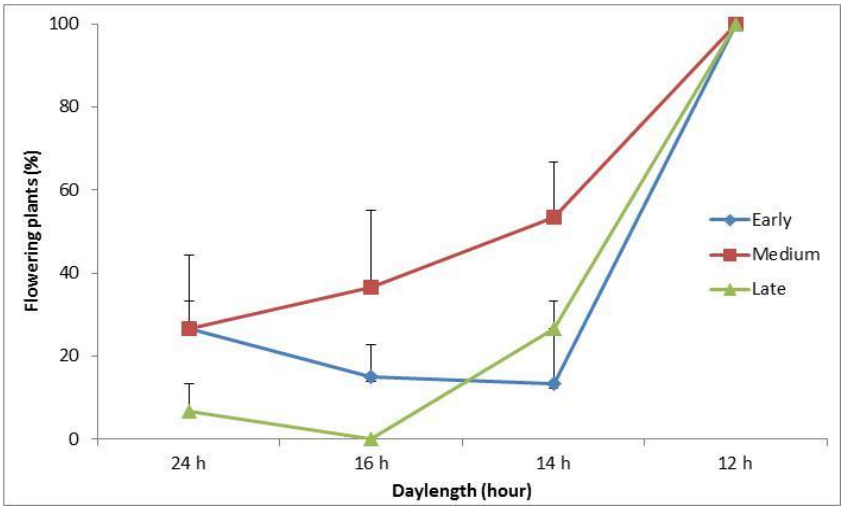


Figure 5: Effect of different photoperiods on flowering of three stevia varieties, of planting lines selected for early, medium and late flowering. Error bars represent SE.

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Kudo & Koga (1977) reported that flowering commenced 38 days after clipping (ratooning) when the stevia was grown under a 12 hour photoperiod. Similar to their findings, plants were observed to start flowering after 38 days of ratooning under 12 hour photoperiod in the current experiment as well. The average number of day to first flower was much greater under a 24 h photoperiod than a 12 h photoperiod, ranging from 103 to 41 days between early, medium and late flowering groups (Figure 6). Under long day conditions (above 14 hour day length), a greater variation in time to flowering was observed. Kudo & Koga (1977) also noted variation in time to flowering when the plants were subjected to long day photoperiod, but not in plants grown under the critical day length.

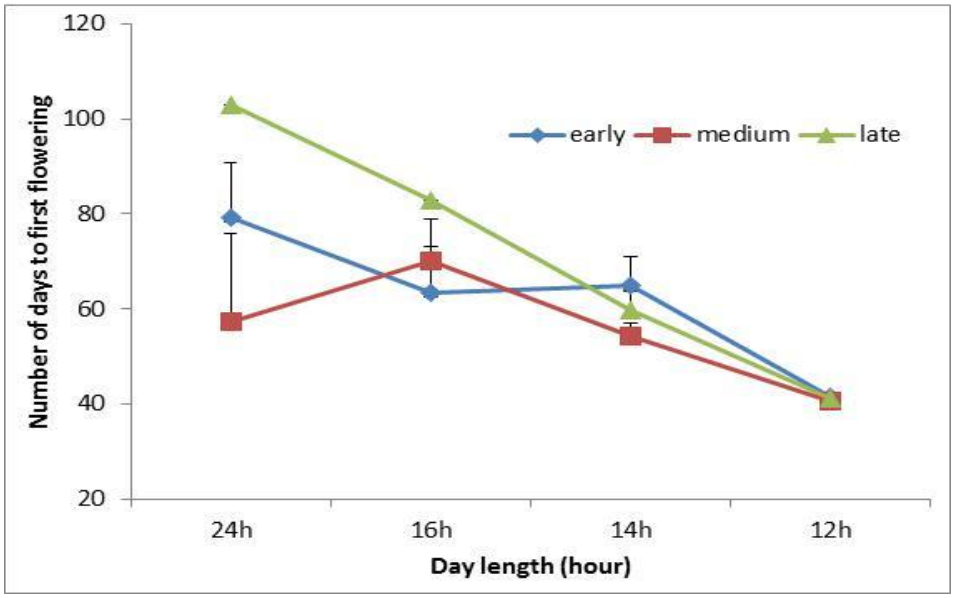

Figure 6: Effect of different photoperiods on number of days to first flowering of three flowering groups of stevia varieties. Error bars represents the SE.

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Effect of photoperiod on biomass

A photoperiod of 14-16 hours seems to be ideal for optimum growth of biomass of stevia (Figure 7). When the plants were subjected to the short day light period (12 hour), flowering commenced early. The early commencement of flower decreased the vegetative growth of the plant. Therefore, the total biomass of stevia decreased significantly, irrespective of variety. However, under a 24 hour photoperiod leaf and total above ground biomass was also reduced, relative to that observed in plants under 16 and 14 h photoperiods. This result is ascribed to the much reduced total daily energy for photosynthesis.

Ermakov & Kochetov (1994) studied the effect of light intensity and photoperiod on growth and yield of stevia and found maximum biomass yield under 16 hour photoperiod and with optimum light intensity of 414-506 µmol m^{-2} s^{-1}. Valio & Rocha (1977) reported a higher SG content in stevia when grown under long day

photoperiod (more than 14 hour). Similarly, Shock (1982) recommended growing stevia under long day photoperiods for higher stevioside (SG) yield.

Stevia grown under long day condition yields higher biomass and higher SG content, thus, increasing profitability of SG production. However, if the stevia is grown for seed production, growing plants under long day condition will delay or prevent flowering.

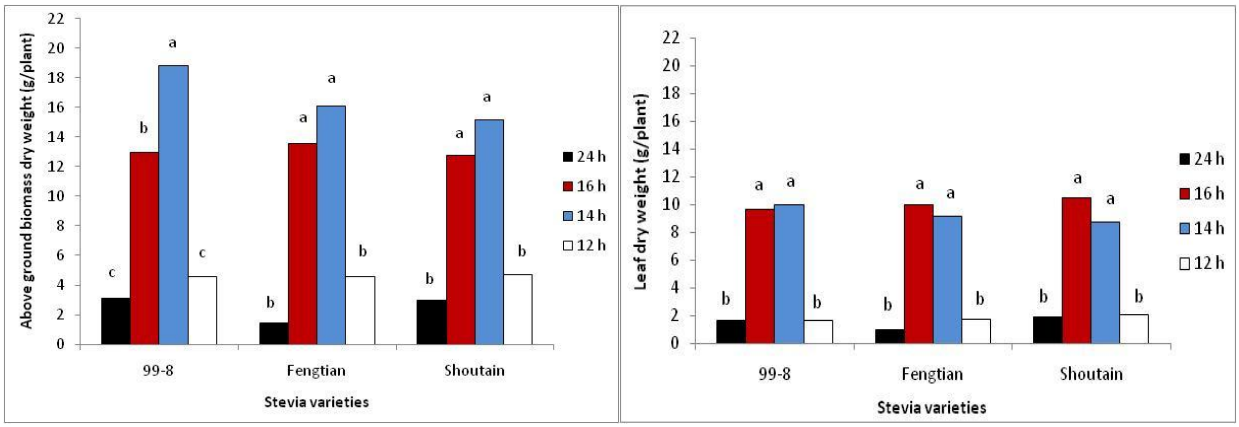


Figure 7: Effect of different photoperiods on above ground biomass and leaf yield (dry weight) of three stevia varieties (g/plant). Same letter indicates no significant differences for the varieties.

2.1.5 Overall discussion

Age of the seedlings during transplantation is a key variable influencing flowering, along with factors such as photoperiod, temperature and growing conditions. Older plants of *Lolium temulentum* (Evans 1960) and stevia (Valio & Rocha 1977) have been reported as more sensitive to photoperiod. For example, Valio & Rocha (1977) found that 70 days old stevia seedlings were more sensitive to photoperiod than younger seedlings when exposed to less than a 13 hour photoperiod. The long vegetative period of the young seedlings transplanted in our studies further supports the findings of Valio & Rocha (1977) and Evans (1960).

Therefore, the timing of transplanting of seedlings is an important agronomic consideration. Under Queensland conditions, appropriate time for transplanting for the photoperiod sensitive varieties should be after September (onset of spring) when the day lengths start to increase. However, for the seed production purpose, the ideal time for transplanting the seedlings of stevia would be from January (day length shortening).

Stevia can be harvested at least two to three times a year depending upon the growing and climatic conditions. Ratoon crops have been reported to be early flowering. In order to achieve maximum yield it is therefore important to understand the timing of ratooning after the first seedling crop. Ratoon biomass yields were higher than first crop have been reported (ChalapathiThimmegowda et al. 1999; Megeji et al. 2005). Tonello, DeFaveri & Weeden (2006) reported that to achieve maximum yield (at latitude 16^{o} 58' S), stevia should be harvested after 7-8 weeks as plant start flowering after this period. In the current study (at latitude 23^{o} 22' S) harvesting of ratoon crop after 87 days gave the highest yield for all three varieties.

Stevia has been reported as a short day plant with commencement of flowering under 12 hours photoperiod or less (Valio & Rocha 1977). In the current studies, no major variation in flowering between the three stevia varieties was found. The highest percentage of flowering was found with 12 hours photoperiod. This result is similar to the findings of Valio & Rocha (1977) and Zaidan, Deietrich & Felippe (1980). The total biomass for the three varieties was highest between 14-16 hour photoperiod.

To improve production prospects, genotypes of stevia are required that are photoperiod insensitive, or have a shorter critical photoperiod such that they do not flower under intended production conductions. Mass selection is one of the common

methods used in plant breeding to select for the desired traits of the crop based on the phenotypic character. The number of cycles of selection required to achieve a desired character will depend on the heritability of that character. In our experiment plants were selected for their date of first and ratoon flowering before crossing like flowering types, however, only one selection cycle was employed. Therefore, the results were not consistent for all flowering groups except for the late flowering group. Therefore, further studies with more selection cycles need to be undertaken in order to confirm the flowering patterns. Galabarreta & Alvarez (2008) reported that divergent mass selection was effective in maize to select early and late flowering population however; it required selection for 8 cycles to achieve a positive effect.

Chapter Three: Influence of soil water on stevia growth and yield

3.1 Introduction

Plants are classified as hydrophytes, mesophytes or xerophytes based on their ability to adapt to water stress, with hydrophytes adapted to conditions of water 'plenty', and xerophytes to dry conditions. For example, the water plant *Hydrilla* is a hydrophyte, while the tomato is mesophyte featuring tap roots and (weakly) woody stems and *Hakea* species are xerophytes with sunken stomata, leaves reduced to spines and waxy epidermal layers. The morphological features of *Stevia rebaudiana* are that of a mesophyte, however it is reported to grow naturally on shallow water tables usually in the edges of mashes and grassland communities (Lester 1999). In such a moist environment, or at least a fluctuating wet – dry environment, stevia is expected to be a hydrophyte or mesophyte.

Water deficit negatively affects cell wall synthesis, protein synthesis, nitrate reductase, activity, and indeed most biochemical processes within a plant. Water deficit also impacts physiological processes such as cell expansion, stomatal opening, transpiration rate and photosynthetic rate. However, sugar content, proline accumulation, and ABA accumulation may increase with increased water stress (Salisbury & Ross 1992). The accumulation of secondary metabolites may also be affected.

For example, Bettaieb et al. (2009) reported plant growth, water potential and fatty acid content to decrease, while essential oil content increased, in moderately drought stressed *Salvia officinalis*, the common sage, relative to a well-watered controls. Marchese et al. (2010) reported that a moderate stress (38 hours of water deficit

before harvesting) on *Artemisia annua* L. (wormwood) resulted in a higher leaf dry weight and artemisinin content compared to irrigated plants. Baher et al. (2002) reported an increase in essential components such as carvacrol with moderate stress, while y-terpinene content decreased with severe water stress in *Satureja hortensis*, an Iranian native savoury herb. Marchese et al. (2010) also reported that moderate water stress of 38 hours, increased artemisinin by 29 % compared to irrigated plant, whereas the content decreased when plants were subjected for a longer stress period from (38-86 hours).

Several studies on the response of stevia to water stress have been published, generally around the topic of determining the optimum water requirement based on the local environment. Geonadi (1983) reported on a glasshouse trial involving plants grown in a latosol, with optimum growth noted to occur at soil water potentials between -0.1 to -0.5 MPa, and wilting when the soil water potential was -1.5 MPa. Lavini et al. (2008) reported a field trial involving a sandy clay soil in southern Italy in which dry leaf yield was 40 % higher but a decreased harvest index in plants maintained on soil at field capacity, compared to on a soil of 33% of FC (no data on soil water potential was presented). They concluded that increasing soil water decreases leaf dry yield in relation to total biomass produced. Leaf steviol glycoside content was noted not to change with plant water status; and it was suggested that this trait is constitutive, with a genetic variation. Guzman (2010) also reported no change in the SG concentration, while plant water potential and leaf sap osmotic potential decreased, following a short term (4-8 days) water stress of stevia plants.

The present study will consider the effect of soil water status on stevia growth and leaf yield and SG content, to confirm the literature understanding of stevia water relations and SG accumulation.

3.2 Materials and Methods

Soil preparation

The soil used for this experiment was a ferrosol (a red soil). Soil was sterilized for 12 hours in a steam sterilizer and air-dried for one week inside a polyhouse. Soil was turned once a day to facilitate drying of the soil. Soil was then sieved with a 5 mm sieve and 4 kg of soil was filled in each 5 litre pot. For soil dry weight determinations, soil was oven dried at 105^{o}C.

Bulk density was assessed to be 1.27 g/cm^{3}. Soil field capacity was assessed to be 32% water by weight on a dry weight basis. Plant permanent wilting point was assessed at -1.9 MPa by measuring the water potential of a severely wilted shoot in a Scholander pressure bomb, and the corresponding soil water content was measured at 14.72 % (weight basis). The experiment was set up with treatments between 120% of field capacity and the permanent wilting point.

Location and experimental design

The experiment was conducted at CQUniversity, North Rockhampton (23 ^{o} 22', 0.345"S, 150^{o} 31' 0.53"E), Australia. The experiment was performed inside a screen house with 67 % of ambient light intensity. Weather data were collected from the nearby Bureau of Meteorology (BOM) station, Rockhampton. The mean ambient temperature during the experiment period ranged from 17-22^{o}C and the solar

radiation for the same period inside the screen house was 10-14 MJ m^{-2} day^{-1}. A completely randomised design with five treatments and eight replications were used. Treatments were five soil moisture levels (120, 100, 80, 60 and 50 % of field capacity; i.e. a gravimetric soil moisture content of 38.4, 32, 25.6, 19.2, and 16 % w/w, respectively), with the lowest soil water level set just above the assessed soil permanent wilting point (of 14.7 % w/w).

Seeds of stevia (variety 99-8) were sown into seedling trays as in Experiment 1 Chapter 2, on 20/07/2010. Seedlings were transplanted after four weeks to the 5 litre pots. Two seedlings per pot were transplanted. Pots were without drainage holes and were covered with 2 mm thick plastic bags to minimize soil evaporation loss. All the pots were equally fertilized with 2 g/pot of slow release fertilizer (Nitrophoska, Brunnings). Moisture content of the soil was measured gravimetrically. Pot weight was maintained at field capacity for seven weeks. Plants were then trimmed leaving 5 cm stem above soil the surface. Treatments were started one week after trimming (22/10/2010). Fertilizer was not applied during the treatment period.

Soil water content was allowed to decrease to the target level of each treatment, and then water was added to bring the weight of each pot to the required moisture content. Each pot was weighed on alternate days and water was added to re-establish initial weight. Weighing of the pot was done in the morning between 9:00-10:00 am.

Data collection

Growth parameters were measured starting two weeks after treatment application. Plant height and chlorophyll concentration were measured at fortnightly intervals.

Chlorophyll concentration was measured using a Minolta SPAD meter following the method used by Bhattarai et al. (2008). Chlorophyll content of the leaf was measured on three leaves and the mean value calculated. Plants were harvested after eight weeks (11/12/2010). Data for yield parameters such as plant height, leaf fresh and dry weight, stem fresh and dry weight were collected. Plant samples including roots were oven-dried at 60 ^{o}C for 48 hours. All the harvesting procedures were as in Chapter 2. For root dry weight, roots were washed thoroughly by soaking for 3-4 hours.

Gas exchange measurements

Photosynthetic rate, transpiration rate and stomatal conductance were measured using an Infrared Gas Analyser (IRGA) model LCA-4 from ADC-UK following the method of Bhattarai, Midmore & Pendergast (2008) . Photosynthetically active radiation (PAR) during the measurements for leaf gas exchange ranged from 672-1242 µmol $m^{-2}s^{-1}$. IRGA readings were taken between 11:30-2:00 PM four weeks after treatments began.

Stem water potential

Stem water potential was measured by using a Scholander Pressure bomb. Stem samples with 4-6 leaves were taken at dawn before 5:00 am and placed in a zipped locked bag stored on ice (in order to maintain that condition). The measurement was performed according to the method of Turner (1988).

Leaf samples of the same plant were used to measure their osmolality (mmol/kg). Samples were dipped in liquid nitrogen for one minute. Frozen leaves were squeezed into a 10 ml syringe and sap was collected. About 10 µl of the sap was used to

measure osmolality using a Wescor vapour pressure osmometer (Model 5520). The osmometer was calibrated using NaCl standards. The osmolality for leaf sap of stevia were presented based on the osmometer readings.

SG analysis

Most recently matured leaves were used for HPLC analysis after five weeks of treatment application. Leaf SG concentration was analysed as described in Chapter 2.

Statistical analysis

Data were analysed using the statistical package for ANOVA (analysis of variance) for a completely randomized design through GenStat version 11.1 Least significant differences between means were calculated by Fisher's Protected LSD test ($P<0.05$).

3.3 Results

Yield attributes

Plant height differed significantly ($P<0.001$) between the treatments (Table 43), although there was no significant difference between 100 and 80% nor between 120, 80, and 60% of FC soil moisture treatments. However, plant height decreased when moisture content exceeded FC, being 37 % less than that of the FC treatment. Similarly, plant height decreased by 53% in the 50% of FC soil moisture treatment, relative to the control.

Leaf dry weight differed significantly ($P< 0.01$) between the control and 120, 60 and 50% of FC soil moisture treatments. However, there was no significant difference in leaf weight for plants grown in 100 and 80 % of FC soil moisture. Increasing

moisture level increased leaf yield up to FC but it decreased at the higher soil moisture treatment (120% of FC). There was no significant difference in stem yield between 120, 80, 60 and 50 % of FC soil moisture, and all four treatments had significantly less (P<0.01) stem weight than that of 100% soil moisture. Stem yield in 50% of FC soil moisture was less than one fifth that at 100% soil moisture. Root dry weight was higher at 100% of FC soil moisture than in other treatments, but not significantly different to root dry weight at 80 and 60% of FC soil moisture (P<0.29). Root dry weight was significantly less at 120 and 50% of FC soil moisture. Root dry weight was one half of 100% of FC in 120% of FC, and one third at 50% of FC soil moisture.

Table 43: Mean height and dry weight of stem, leaf, root and total above ground biomass (g/plant) and leaf to stem ratio at harvest as affected by soil moisture. Data collected after eight weeks of treatment imposition. Mean values within a column with the different letters are significantly different at P<0.05.

Soil moisture (% of FC)	Dry weight (g/plant)				Plant height (cm)
	Stem	Leaf	Root	Biomass above ground	
120	1.4 a	1.3 a	1.4 a	2.6 a	23.8a b
100	2.8 b	3.0 b	2.9 b	5.8 b	37.8c
80	1.5 a	1.8 a b	2.0 a b	3.3 a	29.7b c
60	1.1a	1.4 a	1.8 a b	2.5 a	28.1b
50	0.5a	0.7 a	0.9 a	1.2 a	17.6a
lsd	1.2	1.3	1.2	2.3	8.6
P value (df=27)	0.010	0.02	0.03	0.006	<.001

Stem water potential and osmolality

There was no significant difference in stem water potential in the 120, 100 and 80 % of FC soil moisture treatments, while that at 50 and 60% of FC soil moisture was significantly more negative that of other treatments (Table 44).

Osmolality was calculated from the same plant used for stem water potential. There was no difference between the treatments in osmolality.

Table 44: Mean stem water potential and osmolality as affected by soil moisture. Values with different letters in a column are significantly different at P<0.05.

Soil moisture (% FC)	Stem water potential (MPa)	Osmolality (mmol/kg)
120	-0.71 a	329 a
100	-0.76 a	305 a
80	-0.65 a	375 a
60	-0.95 b	387 a
50	-0.94 b	342 a
lsd	1.125	58
P value (df)	<0.001 (12)	0.056 (19)

Gas exchange and chlorophyll content

There was no significant difference in photosynthetic rate, transpiration rate and stomatal conductance between the treatments (Table 45). A, E, and Gs were 39.5%, 30.3% and 50.4% less, at 60% compared to 100 % of FC respectively. There were no data for 50% of FC soil moisture as the leaf size were very small and did not fit in the chamber. There was no significant difference in chlorophyll content between the treatments (Table 45). However, at 100 % of FC soil moisture the chlorophyll content was highest (46.3 SPAD unit).

Table 45: Effect of soil moisture on photosynthetic rate, transpiration rate, stomatal conductance and chlorophyll content (* data not available).

Soil moisture (% of FC)	Photosynthetic rate (µmol $m^{-2}s^{-1}$)	Transpiration rate (mmol $m^{-2}s^{-1}$)	Stomatal conductance (mol $m^{-2}s^{-1}$)	Chlorophyll content (SPAD unit) at harvest
120	7.43 a	1.89 a	0.06 a	44.6 a
100	8.30 a	2.70 a	0.11 a	46.3 a
80	7.16 a	2.19 a	0.08 a	43.8 a
60	5.02 a	1.88 a	0.05 a	44.3 a
50	*	*	*	44.0 a
lsd	3.14	0.85	0.05	4.8
P value (df)	0.19 (15)	0.18 (15)	0.19 (15)	0.84 (27)

Steviol glycoside concentration and content

Stevioside as a percentage of dry weight significantly differed (P<.001) between treatments (Table 46). Percent dry weight of stevioside was highest (5.6 %) at 120% of FC soil moisture, whereas treatments with 60 and 50% of FC soil moisture had much lower concentration of 2.07% and 2.7 % respectively. There was no significant difference between treatments at 100, 80 and 50% of FC soil moisture and similar concentrations were observed at 60 and 50% soil moisture. Percent dry weight of rebaudioside A significantly differed (P<0.03) between 100 and 60% of FC soil moisture treatments. There was no difference among the 100, 80, 60 and 50 % of FC soil moisture treatments. Total SG content (calculated as leaf SG concentration multiplied by leaf mass) was significantly different between the treatments (P<0.03). Treatments with 50% and 60% of FC had significantly less SG content compared to that of 100% of FC.

Table 46: : Percent dry weight of stevioside and rebaudioside A in leaves of stevia (*Stevia rebaudiana*) and their sum (total SG) and total SG per plant grown at different soil moisture content. Values in each column with different letters are significantly different at P<0.005.

Soil moisture (% of FC)	stevioside (% dw)	rebaudioside A(% dw)	Total SG (% dw)	SG content (mg/plant)
120	5.6 a	2.5a	8.1a	163.9a
100	3.6 b	1.4 b	5.0 bc	141.8a
80	3.8 b	2.1 ab	5.8 b	86.3ab
60	2.1 c	1.2 b	3.2 c	43.5b
50	2.7 bc	1.8 ab	4.5 bc	48.0 b
lsd	1.45	0.884	2.1	90.2
P value (df=22)	<.001	0.033	0.001	0.035

3.4 *Discussion*

The yield of stevia is related to soil moisture. The maximum biomass was obtained when the crop was grown at the field capacity, with significant reductions noted at both increased (120% of FC) and decreased (<80% of FC) levels.

Increasing soil moisture up to field capacity increased leaf biomass, stem yield and root biomass, but yield decreased at 120 % of FC. A similar pattern was also observed in plant height. However, harvest index, the ratio of leaf dry weight and total above ground biomass, decreased with increased moisture content, reaching 46.6% at 120% of FC from 58.5% at 50% of FC (Figure 9). Lavini et al. (2008) have reported similar observations of reduced total biomass, harvest index and water use efficiency at increased amounts of water. Apparently, with increased water availability, the plant is investing proportionally more resources into stem, rather than leaf, growth.

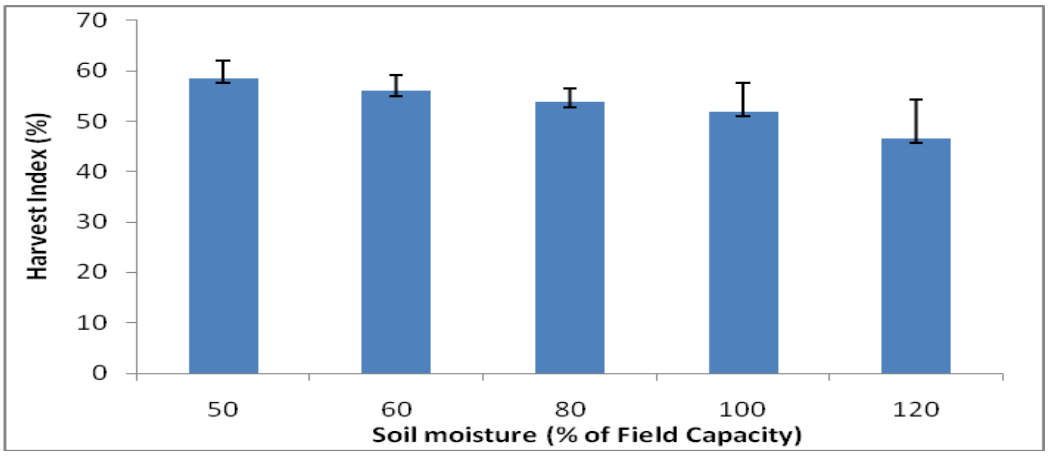


Figure 8: Harvest index of stevia at different soil moisture (% of field capacity).

Plants may osmoregulate using secondary metabolites to effect a water potential adjustment under water stress. Lavini et al. (2008) and Guzman (2010) have reported that SG concentration is not responsive to plant water stress, and Guzman

(2010) also reported plant water potential and leaf sap osmotic potential to decrease under stress. In comparison, in the current study, SG content was observed to increase in the 120% of FC treatment, and no difference in sap osmolality was noted, while plant water potential was significantly decreased only at soil water contents of 60 and 50% of FC. These differences may be due to differences in growth methods. In the current study, plants were maintained in the same moisture level throughout the growth period of 8 weeks, whereas Guzman (2010) imposed stress for 4-8 days.

Stevia is endemic to marshy land at the edges of grasslands. Stevia does not exhibit hydrophytic features, so presumably it has evolved to an environment experiencing fluctuating water availability. Curiously, plant water potential for stevia was between -0.6 and -0.7 MPa even in well watered conditions. Leaf sap osmolality was constitutive (i.e. it did not change with water stress, around close to 350 mmol/kg). This level of solutes will contribute significantly to cell water potential (viz. from the vant Hoff equation of solute potential = -miRT = -0.35*1.8* 0.00831 * 298 = -1.5 MPa). Given a measured total water potential of around -0.7 MPa, an average cell pressure potential of 0.8 MPa is inferred.

Total leaf SG concentration was assessed around 5% dw. Assuming a leaf moisture content of 90%, this is equivalent to a solution of 0.5% SG (assuming all cellular compartments are mixed), or 0.06 mM (MW of stevioside = 804 g). This concentration is low in terms of a contribution to the solute potential of cell cytoplasm. Apparently the measured osmolality of leaf cell sap is primarily due to solutes other than SGs.

Although SG content has been observed to be constitutive under other conditions, apparently it is responsive to soil waterlogging. The SG concentration was observed

to be high in plants grown at 120% FC. In water-logged conditions (120% of FC) plant growth was retarded and biomass yield was reduced. Such a result is expected due to a lack of oxygen supply to the root, which may also restrict translocation of materials from the leaves to the roots, and result in an accumulation of photo assimilate in the shoot (a girdling effect). Guzman (2010) has observed the conditions of high assimilate availability (e.g. increased photosynthetic conditions) do not result in increased leaf SG content, so presumably other triggers are involved in the elevation of leaf SG with soil water logging. The SG pathway is partly shared with that of gibberellic acid, so possibly the answer lies in an environmental trigger of the GA pathway. The net effect is that while growth was reduced under water logging, the increase in leaf SG more than compensated for this, resulting in an increase in total plant SG. This observation has obvious agronomic utility. Further investigation is required to look at the effect of water-logged conditions on stevia growth and accumulation of SG, and a cost/benefit analysis (cost of irrigation water relative to value of extra SG) should be undertaken.

Chapter Four: Nutrient deficiencies in stevia

4.1 Introduction

There are sixteen different elements required for the growth of plants, with thirteen commonly sourced by the plant from the soil, namely nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S) as macro elements; iron (Fe), boron (B), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo) and chlorine (Cl) as micro elements. The other elements required by plants are carbon (C), hydrogen (H) and oxygen (O_2) which are obtained from air and water. Each element plays a particular biochemical/physiological role, so deficiency of that element will result in a set of predictable metabolic and phenological disturbances. However, while conforming to a general set of symptoms, the exact phenological expression of a given disorder is specific to each species. For any crop typically there will exist a pictorial record of deficiency symptoms to assist field practical identification of disorders associated with nutrient limitation, e.g. for fruit crops, (Weir & Cresswell 1993), and for buffel grass (Makiela 2008). The expression of such symptoms is typically achieved by nutrient reduction or omission in hydroponics, with hydroponics offering greater control of the rhizosphere environment than in soil.

Stevia (*Stevia rebaudiana)* has a very small seed (typical weight of 1000 seeds is 0.3g). Given limited essential element storage in the seed, deficiency symptoms should be relatively easily achieved, even for micro elements, in seed grown plants. However, only two studies describing nutrient deficiencies in stevia have been found, and neither is in the English language (Lima Filho & Malavolta 1997a; Utumi et al. 1999).

Lima Filho & Malavolta (1997a) described the foliar symptoms of macro nutrient deficiency, and B and Zn toxicity in stevia grown in hydroponics. Biomass and chlorophyll content decreased with increasing level of concentration of B when applied as foliar spray. The uptake and accumulation of macro and micro nutrients by stevia in field conditions was also reported, with a calculation of the amount of macro and micro nutrients required before flowering and seed production presented.

Utumi et al. (1999) reported on the macronutrient deficiency in stevia in relation to plant growth, chemical composition and steviol glycoside (SG) content. They found that total above ground biomass decreased in all the macro nutrient deficiencies however the percentage of reduction was higher in treatments without N, P, and Mg, The concentration of stevioside (SG) decreased with the deficiency of all macro nutrients except for P. There are no reports on the effect of micro nutrients on SG concentration.

Sheu, Tamai & Motoda (1987) reported that a high concentration (10 mg/kg) of B reduced total biomass, decreased flowering percentage and SG content of stevia grown in hydroponics. When supplied at 5 mg/kg, good growth without any symptoms was noted, while at lower concentrations, symptoms included leaf spotting and decreased root weight.

No pictorial record of deficiency symptoms of stevia has been published. The aim of the current study was to document the effects of nutrient deficiencies on plant morphology and biomass accumulation, foliage symptoms (including a pictorial record), and SG content.

4.2 Materials and Methods

Location

The experiment was conducted at CQUniversity, North Rockhampton (23^{o} 22', 0.345"S, 150^{o} 31' 0.53"E), Australia, inside a screen-house with 67% full sunlight (Bhattarai, Huber & Midmore 2004)**.**

Plant material

Seeds of *Stevia rebaudiana* variety Shoutain-2 were sown on 17/08/09 in 1:1 perlite: vermiculite media in speedling trays inside the screen-house. Following germination, seedlings were watered with half strength Manutec hydroponic solution (Manutec Pty. Ltd.) for three weeks (6-9 weeks after sowing). After 9 weeks, seedlings were supplied with reverse osmosis (RO) water for two weeks, and were then transferred to 7 cm diameter poly pots lined with mesh and filled with perlite, and grown with RO water only. Seedlings were at the 8-10 leaf stage, with plant height ranging from 8-10 cm, at the time of transplanting. The duration of the experiment was 14 weeks (from August to December 2009).

Treatments and experimental design

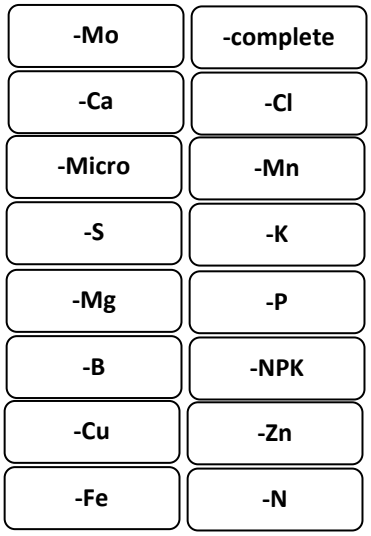
An omission nutrient trial was established, based on that of Roberts and Whitehouse (1976). The chemical composition of the nutrient solution is presented in Appendix 1 and 2. The experiment was conducted using non-circulatory hydroponics, following the method of Midmore (1994). Styrofoam boxes (53 cm x 23 cm x 25 cm) were lined each with a black plastic bag to prevent leakage. Four 7 cm diameter holes were made on the lid of each box to hold the 7 cm diameter poly-pots. The plant to plant and row to row distances were maintained at 13 cm and with 25 cm

respectively. To check the level of the solution a small hole was made in the lid with a measuring dip stick attached to it. The pH and electrical conductivity (EC) measurement was conducted through the same hole. The pH for the treatment was around 4.5 and a day later it changed to 5.5 to 6 and EC varied slightly between treatments. Mean ambient temperature during the experimental period was 25 - 36^{o} C. The temperature of nutrient solutions ranged from 25 - 30^{o} C.

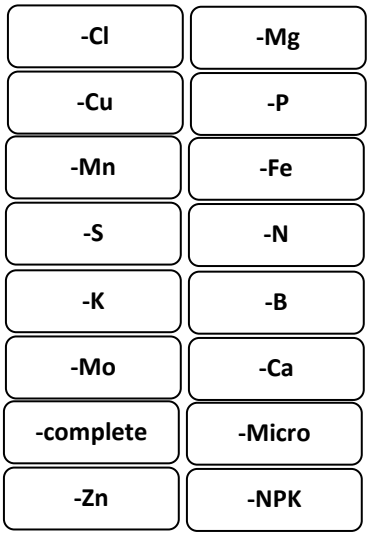
The experiment consisted of 16 treatments (Table 47) with two blocks in which treatment position was randomised. Each treatment in each block, i.e., each box, was comprised of four plants. Data were collected from each plant to estimate sampling error. Treatments were imposed after 11 weeks from the date of sowing.

Table 47: Different treatments and their symbols

Treatments	Symbols
Complete	Complete
no P	-P
no K	-K
no Ca	-Ca
no N	-N
no Mg	-Mg
no S	-S
no Fe	-Fe
no Mn	- Mn
no Cu	-Cu
no Zn	-Zn
no B	-B
no Mo	-Mo
no Cl	-Cl
no N, P, K (macro)	-NPK
no micro (Ca, Mg, S, Fe, Mn, Cu, Zn, B, Mo, Cl)	-micro



Block 1



Block 2

Plant description

A pictorial record of plant shoot and roots was made using a digital camera (Nikon Coolpix 5200). Plant height (measured from the base of the stem to the apical tip), leaf, stem and root fresh and dry weight was measured of each plant, four weeks after treatment imposition. At harvest, roots were thoroughly rinsed with tap water to remove perlite. The roots were blotted dry with paper towel and the plant samples were dried in a fan forced oven at 60 ^{o}C for 72 hours for dry weight measurement.

Leaf chlorophyll concentration was estimated using a SPAD meter (Konica, Minolta Japan), with readings taken 3 and 4 weeks after imposition of treatments. Youngest fully expanded leaves were used for the measurement.

Leaf gas exchange (photosynthesis, transpiration and stomatal conductance) for all the treatments were measured using an IRGA (Infrared Gas Analyser, model LCA-4 from ADC-UK), following the procedure of Bhattarai et al. (2008). Measurements were made just before the harvest (after 4 weeks of treatment application). The

IRGA readings were taken between 11:00 am and 2:00 pm, on the same leaves used for chlorophyll determination.

Steviol glycoside (SG) analysis

For the measurement of steviol glycoside concentration in the leaf, two youngest fully expanded leaves from each plant of every treatment were taken three weeks after treatment application. Samples from each box were combined. Plant samples were oven dried at 60^{o} C for 48 hours and stored in air-tight containers. The SG concentration of the leaves were analysed through HPLC as described in Chapter 2.

Statistical analysis

Data were analysed using the statistical package for ANOVA (analysis of variance) through Genstat version 11.1. Difference between means is reported significant at a 95% probability level.

4.3 Results

Foliar symptoms of nutrient deficiency

Plants in complete nutrient solution grew normally without any deficiency symptoms (Figure 9p) which indicates that solution pH value (4.5) and EC value (1.8) for the treatments were appropriate. When deprived of N, plants were slow in growth and developed leaves small in size, and slender stems without branches (Figure 9b). Leaf chlorosis was first noted in mature leaves, but by the later stage (28 days after treatment imposition) young and recently developed leaves became chlorotic.

Deprived of P, plants were stunted in growth and developed small leaves, as seen with N deficiency. However, marginal chlorosis and purple spots were observed on older leaves (Figure 9c).

Deprived of K, plants were also stunted in growth, and possessed small leaves and slender stems (Figure 9d). Older leaves developed brown margins with some brown spots on the leaf blade. Leaves curled downward as well as inward as the symptom development progressed.

Deprived of Ca, symptoms appeared after one week of treatment, and severe symptoms were observed within four weeks. At the beginning small dark necrotic spots were evident along the margin and middle of the leaf blade of young leaves and the shoot apex (Figure 9e). After two weeks, young leaves necrotic with inward curling and twisting of the older leaves was observed., followed by necrosis of the growing points.

Deprived of Mg, symptoms appeared after two weeks of treatment imposition. Leaves curled downward, and chlorosis of the leaf blade occurred but the veins remained green (Figure 9f). Chlorosis of the leaf started at the leaf tip and developed basipetally. As symptoms progressed leaves curled inward and some brown necrotic spots were seen at leaf margins and at the tip of the leaf. The surface of the leaf was rugose.

Deprived of S, growth was stunted, leaves were small, slender and the main stem was without branches (Figure 9g). Chlorosis of older leaves was common, with this symptom also developing later in young leaves.

Deprived of N, P and K growth was slow, and developing stems were slender and with small chlorotic leaves. Yellowing of the leaves started in older leaves and progressed towards the young emerging leaves (Figure 9a).

When deprived of B only, cracking of the main stem was observed, appearing after two weeks of treatment application. The leaf surface was rugose (Figure 9h).

The micronutrient deficiency treatment involved supply of N, P and K, and deprivation of B, Ca, Mg, S, Cu, Cl, Mo, Fe, and Mn. Symptoms were quite similar to those of Fe deficient plants (see below). Plants showed stunted growth, chlorosis of the leaves and small leaf size. Necrosis occurred at the tip of the young growing points (Figure 9i).

When deprived of Mn only, no distinct symptoms were observed over the four weeks of observation. Older leaves were dark green in colour but the main and lateral veins were slightly light green in colour (Figure 9j).

When deprived of Cu only, symptoms were apparent after two weeks of treatment imposition. Younger leaves curled inward and bending and rolling of older leaves was observed. After four weeks of treatment application, chlorotic areas were noted to have developed on the older leaves, starting from the base of the leaf and progressing towards the tip of the leaf (Figure 9k).

When deprived of Fe only, symptoms were observed after one week of treatment imposition, with the young growing leaves changing from dark green to light green and later to pale yellow. As the symptoms progressed in the leaf blade, leaf veins along with the stem changed to whitish colour (Figure 9l). After three weeks of treatment, necrosis of the growing points was evident.

When deprived of Mo, no visible symptoms were observed. There was a massive growth of leaves and branches. Plants had thick stems with short internodes (Figure 9m). They were similar to the complete nutrient plant except for short internodes.

When deprived of Zn, plants developed short internodes, with a clustered leaf arrangement at the growing tip. The main branch was thin above the ground surface but it was thicker towards the apex of the plant. Older leaves curled downward (Figure 9n).

When deprived of Cl, a mild inward curling of the leaves was seen. No other distinct symptoms were observed (Figure 9o).

Nutrient deficiencies on root growth of stevia

Symptoms of nutrient deficiency were also observed in the roots of stevia (Figure 10). In treatments lacking macro nutrients (N, P, K, NPK, Ca, Mg and S), root growth was very poor compared with complete treatment. In treatments without Ca, the roots showed black necrotic spots on the tip of the roots. This was observed after four weeks of treatment application. Plant root growth was markedly stunted in treatments without B, Zn, Cl, Cu, Mn, Mo, micro and Fe.

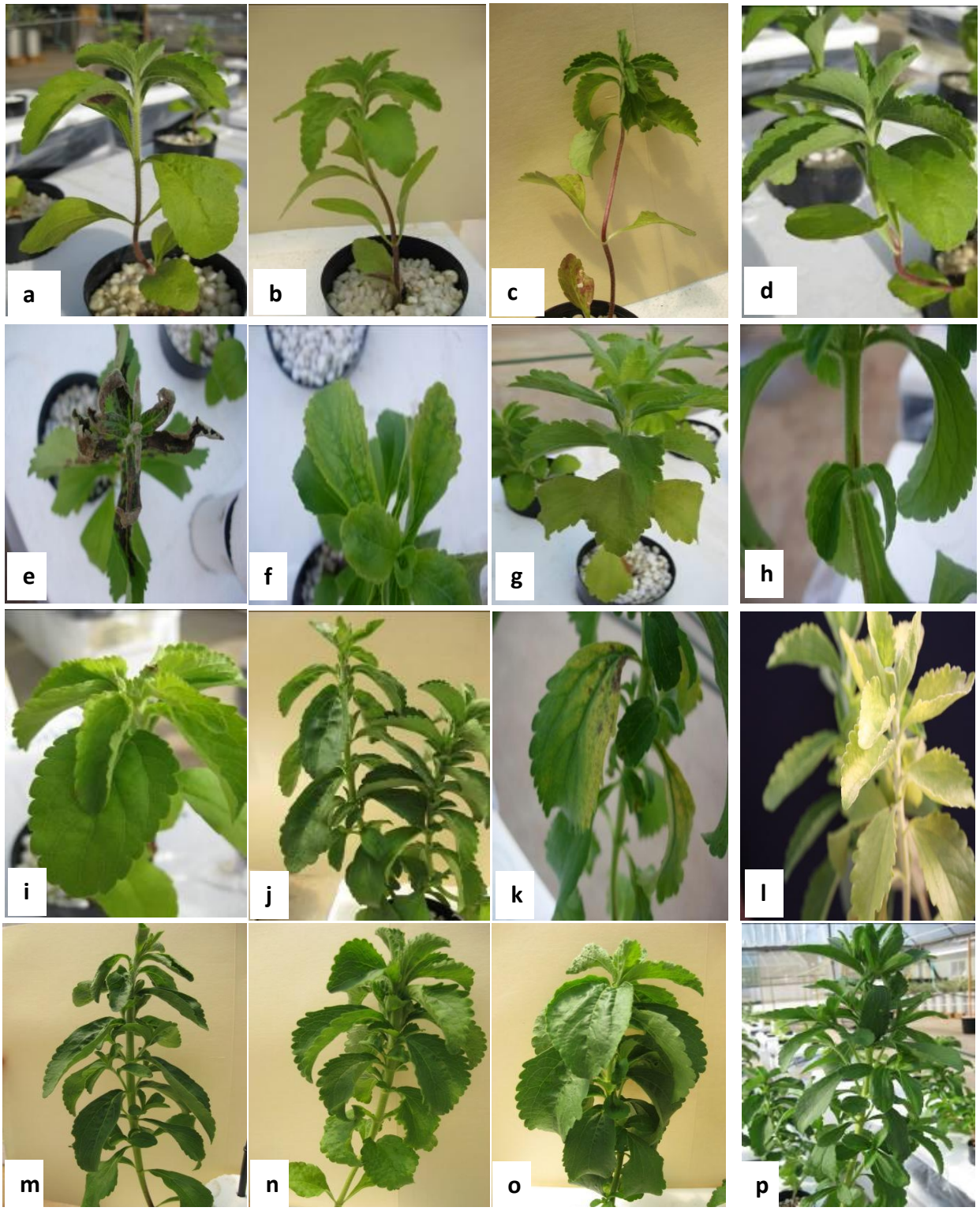


Figure 9: Nutrient deficiency symptoms in stevia; a) NPK, b) N, c) P, d) K, e) Ca f) Mg, g) S, h) B, i) Micro nutrient, j) Manganese, k) Cu, l) Iron, m) Mo, n) Zn, o) Cl and p) Complete. Nutrient omission was imposed on 13 week plant for four weeks.

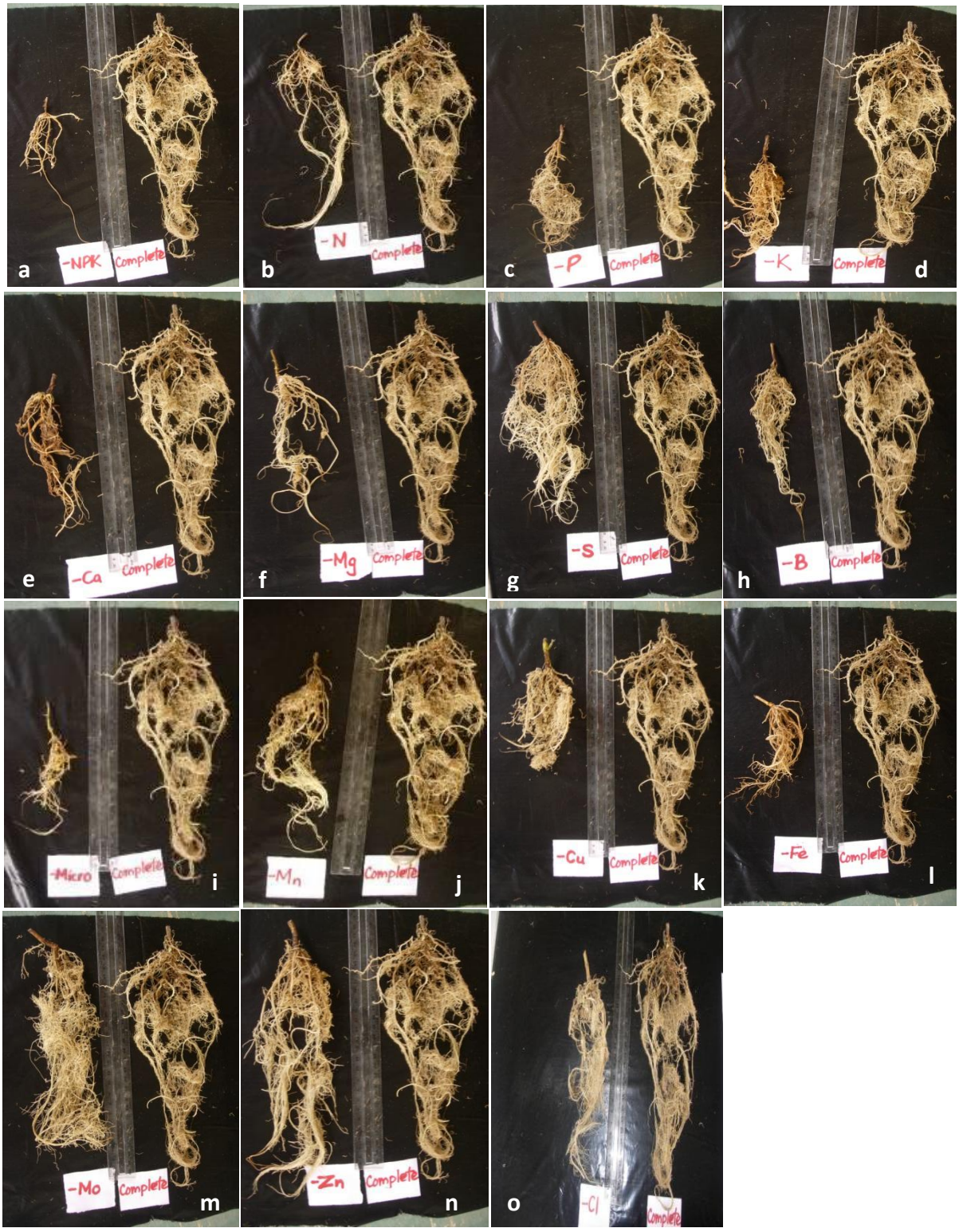


Figure 10: Effects of nutrient deficiency in stevia roots; a) NPK, b) N, c) P, d) K, e) Ca f) Mg, g) S, h) B, i) Micro nutrient, j) Manganese, k) Cu, l) Iron, m) Mo, n) Zn, o) and Cl).

Growth parameters in relation to nutrient deficiency

Total above ground (shoot) biomass of stevia grown in solution without the micronutrients Mo, Cu, Cl, Mn, and Zn and the macronutrient K, did not differ significantly from plants grown in the complete nutrient treatment (5.7 g/plant) (Figure 11). Significant differences in the biomass was found in all other treatments ($P<0.001$). Similarly there was no significant difference in total biomass in treatments without NPK, N, micro, P, S, Ca, Mg, Fe B and Zn. Plant biomass ranged from 3.1 g/plant (B deficient treatment) to 0.12 g/plant (NPK deficient treatment).

There was no significant difference in leaf yield between the treatment without Mo and complete nutrient (Figure 12) with 4.1 and 3.9 g/plant, respectively. Treatments without Cu, K, Cl, Mn, Zn and B had similar leaf weight to the complete nutrient treatment. However, plants without Mg, Ca, Fe, S, no micro, N, P, and NPK had less leaf yield. Stevia grown without NPK had the lowest leaf yield (0.3 g/plant).

Stem dry weight was highest (1.7 g/plant) in the complete treatment, with comparable mass achieved by treatments without Cl, Cu, Zn, Mn, Mo (Figure 13). However, stevia in the complete treatment significantly differed from treatments without Mg, Ca, Fe, S, micro nutrient, N, P, and NPK.

Root weight for the complete treatment was 0.6 g/plant, and the treatments without Cl, Mo, Fe, Cu, Zn, Mn, S, P, N, B, K, and Mg was not significantly different from the complete treatment (Figure 14). However, stevia grown without NPK, Ca, and micro nutrients had lower root growth than the complete treatment. Treatment without micro nutrients had 40% less root than the complete treatment.

Maximum plant height was obtained in the complete nutrient treatment (25 cm), with comparable height achieved in Zn, Mo, and Cl deficiency treatment. Plants were significantly shorter in treatments without Fe, B, K, Ca, Mg, S, P, micro, N and NPK (Figure 15). The treatment without NPK had the lowest plant height (9.5 cm).

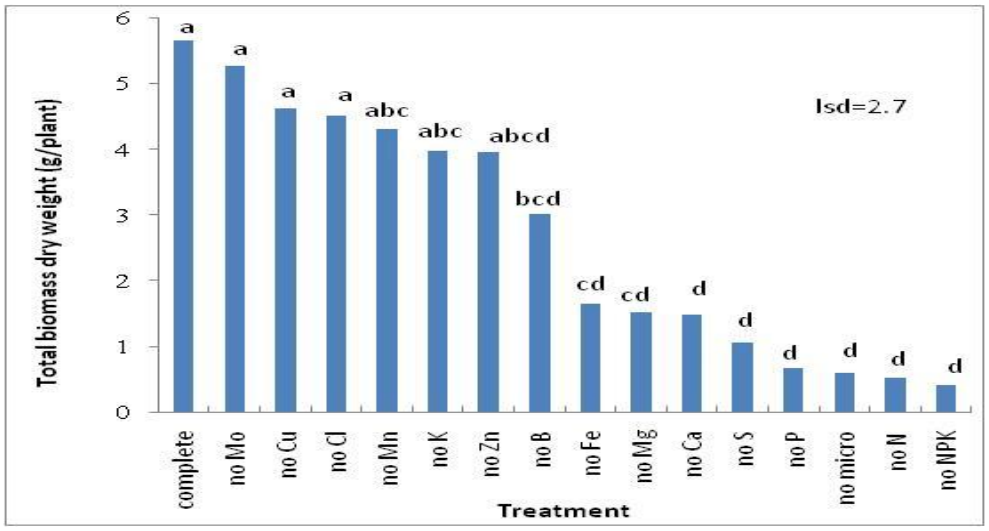


Figure 12: Average total above ground dry weight of stevia (*Stevia rebaudiana*) grown with various nutrient deficiencies. Plants harvested after 4 weeks of treatment imposition. Treatments with the same letter did not differ significantly from each other.

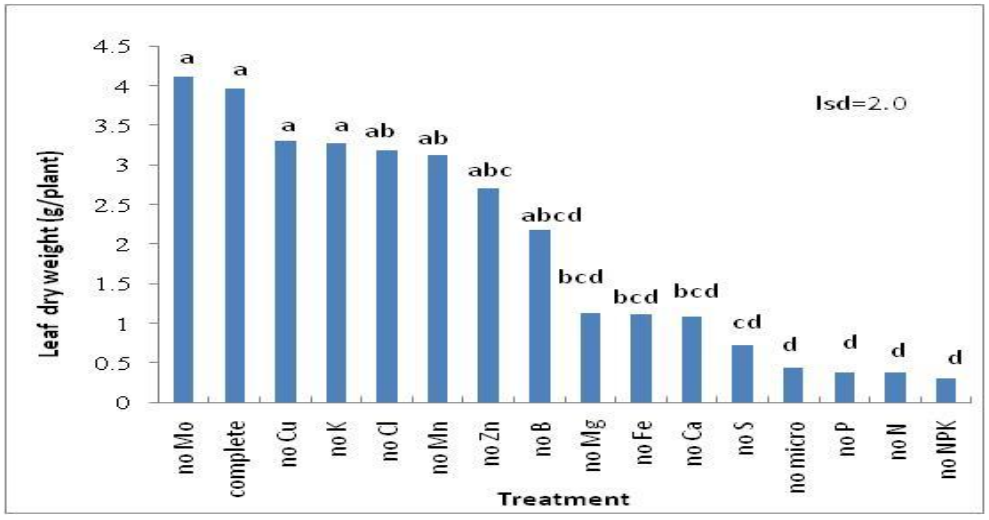


Figure 11: Average leaf dry weight of stevia (*Stevia rebaudiana*) grown with various nutrient deficiencies. Treatments with the same letter did not differ significantly from each other at P<0.05.

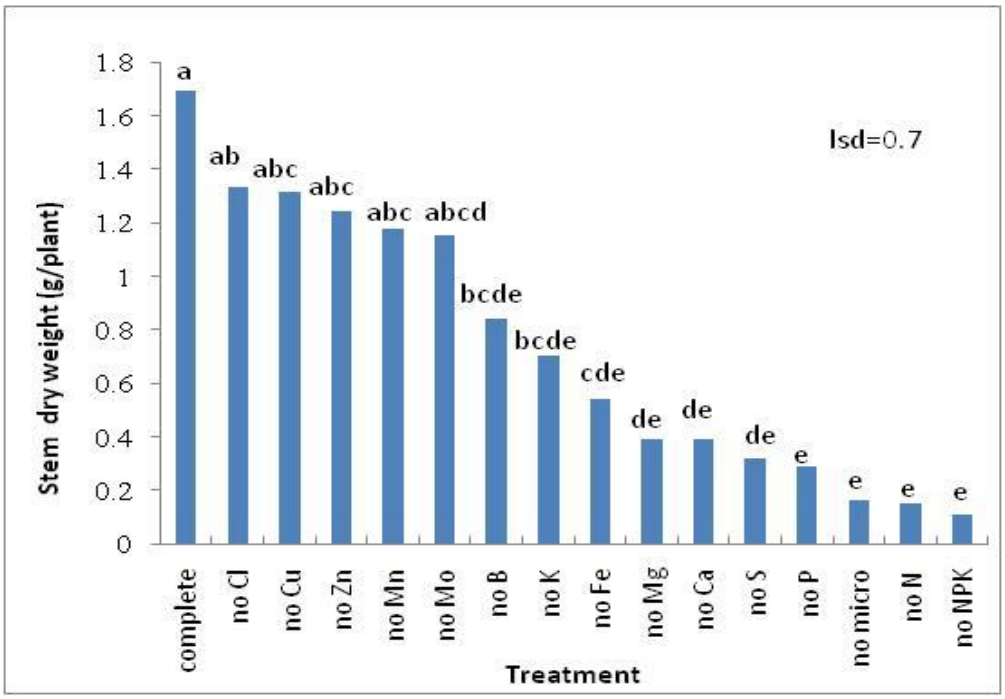


Figure 13: Average stem dry weight of stevia (*Stevia rebaudiana*) grown on various nutrient deficiencies. Treatments with the same letter did not differ significantly from each other at P<0.05.

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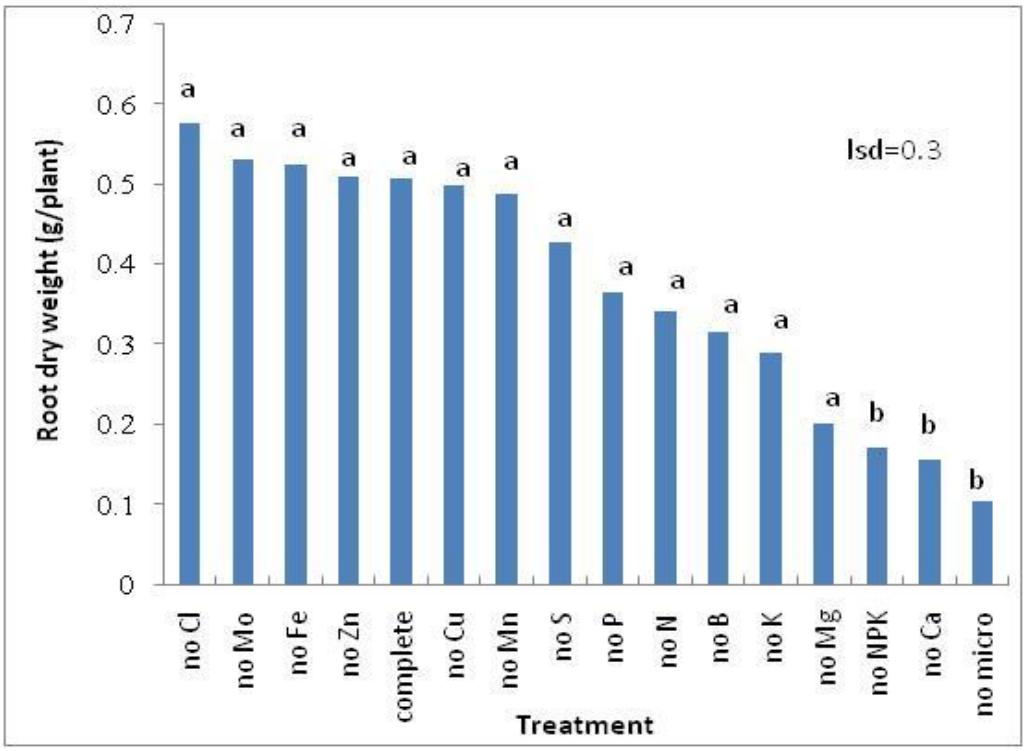

Figure 14: Average root dry weight of stevia (*Stevia rebaudiana*) grown on various nutrient deficiencies. Treatments with the same letter did not differ significantly from each other at P<0.05.

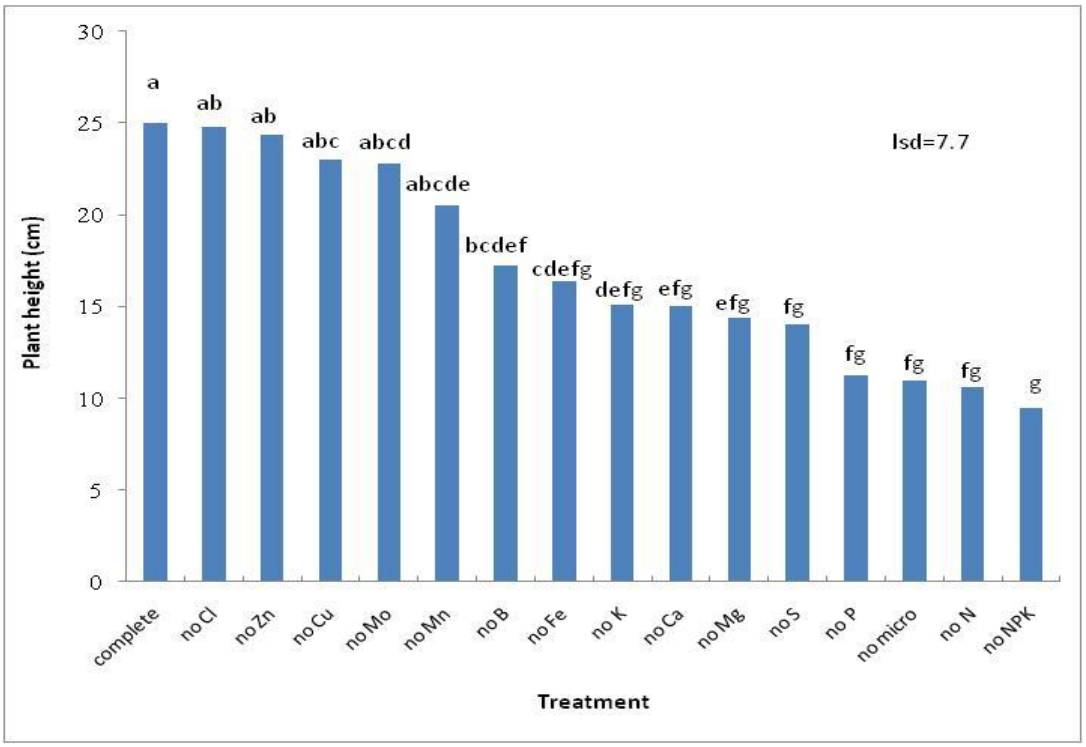

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Figure 15: Average plant height of stevia (*Stevia rebaudiana*) grown on various nutrient deficiencies. Treatment with the same letter did not differ significantly from each other at P<0.05.

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SPAD reading (surrogate for chlorophyll concentration)

After three weeks of treatment application, the SPAD reading for the complete treatment was 51.4 units, and was higher in treatments without Mo, Cl and Mn by 1.7, 1.8 and 1.9 percent, respectively (Table 48). The treatment without NPK had the lowest SPAD reading (21.9), 57% less than that of the complete treatment.

After four weeks, the SPAD reading was highest in plants grown in complete nutrient solution (53.5), followed by the treatments without Mn, Zn, Mo, B, Cu, Cl, and K (Table 2). The treatment without NPK had the lowest SPAD reading at 61 % of the complete treatment. The SPAD reading increased from week 3 to week 4 by 3-4 % in complete treatment and in most of the other treatments.

Table 48: Average SPAD readings for stevia (Stevia rebaudiana) leaves on plants grown in various nutrient solutions. Same letters in each column showed no difference between the treatments. Percent differences were calculated compared to the complete treatment.

Treatments	Chlorophyll content (SPAD unit)			
	Third week	% difference from the complete	Fourth week	% difference from the complete
Complete	51.38 a		53.45 a	
No Mn	52.36 a	+1.9	52.90 a	-1.0
No Zn	47.51 a	-7.5	50.45 a	-5.6
No Mo	52.27 a	+1.7	50.15 a	-6.2
No B	50.72 a	-1.3	50.00 a	-6.5
No Cu	49.96 a	-2.8	49.90 a	-6.6
No Cl	52.31 a	+1.8	46.50 a	-13.0
No K	49.41 a	-3.8	46.45 a	-13.1
No Ca	30.81 a	-40.0	33.50b	-37.3
No S	33.32 a	-35.1	31.7 bc	-40.7
No Mg	33.17 b	-35.4	28.00 bcd	-47.6
No Fe	28.24 bc	-45.0	25.75 bcd	-51.8
No P	24.92 bc	-51.5	22.70cd	-57.5
No N	23.87 bc	-53.5	22.05 d	-58.7
No Micro	24.41 bc	-52.5	21.35 d	-60.1
No NPK	21.91 c	-57.4	20.90 d	-60.9
Lsd	9.68		9.08	
P value (df=15)	<0.001		<0.001	

Leaf gas exchange

The photosynthetic rate of plants grown in the complete nutrient solution differed significantly (P<0.001) from the treatments without S, Fe, N, P, Mg, micro, Ca and NPK. However, treatments without B, Cu, Mo, Zn, Mn, and K were similar to the control. The treatment without Cl had the highest photosynthetic rate (15.89 µmol $m^{-2}s^{-1}$) and the treatment without NPK had the lowest (0.28 µmol $m^{-2}s^{-1}$) photosynthetic rate (Table 49).

The transpiration rate differed significantly (P<.006) with treatments and followed the same pattern as for photosynthetic rate. Transpiration rate in treatment without Cl

was highest (12.46 mmol $m^{-2}s^{-1}$) and lowest (2.75 mmol $m^{-2}s^{-1}$) was observed in the treatment without micro nutrients (Table 49).

The stomatal conductance differed significantly between the treatments (P<0.02) and followed the same pattern as for the photosynthetic and transpiration rates. The stomatal conductance was the lowest (0.06 mol $m^{-2}s^{-1}$) in the treatment without Fe (Table 49).

Table 49: Photosynthetic rate, transpiration rate and stomatal conductance of stevia at the time of harvest (at four weeks of treatment application). Means with the same letters in each column did not significantly differ from each other at P<0.005.

Treatments	Photosynthetic rate (µmol $m^{-2}s^{-1}$)	Transpiration rate (mmol $m^{-2}s^{-1}$)	Stomatal conductance (mol $m^{-2}s^{-1}$)
No Cl	15.89 c	12.46 g	0.37 g
No B	15.51 c	11.18 fg	0.3 efg
No Cu	13.12 c	10.99 efg	0.3 fg
No Mo	13.03 c	9.73 cdefg	0.22 bcdefg
Complete	12.73 bc	10.01 defg	0.24 cdefg
No Zn	11.57 bc	9.16 cdefg	0.19 abcdef
No Mn	11.28 bc	10.57fg	0.27 defg
No K	10.43 bc	9.68 cdefg	0.2 abcdefg
No S	5.78 ab	6.6 abcde	0.13 abcdef
No Fe	2.98 a	3.87ab	0.06 ab
No N	2.60 a	7.34 bcdef	0.12 abcde
No P	1.49 a	5.39 abc	0.07 abc
No Mg	1.45 a	5.89 abcd	0.1 abcd
No Micro	0.87 a	2.75 a	0.04 a
No Ca	0.66 a	4.54 ab	0.07 abc
No NPK	0.28 a	5.40 abc	0.08 abc
Lsd	7.07	4.51	0.17
Pvalue (df=15)	<0.001	0.006	0.02

SG concentration and content in stevia leaves

There was significant difference in total SG concentration between the treatments (P<0.02). The SG concentration did not significantly differ between plants grown with complete nutrients, or deficient in P, N, Cl, Mn, S, Fe, B, Mo or NPK (Table 50). The highest SG content was noted in plants grown without Zn (12.1% w/dw). The lowest SG concentration was noted in plants grown without microelements,

especially Cu. Plants deficient in Mg also had lower concentration than that of the complete nutrient plant.

There was significant difference in stevioside concentration between the treatments ($P<0.01$). Plants grown without Zn had highest stevioside concentration (Table 50). There was no significant difference in rebaudioside A concentration between the treatments. The second highest stevioside level was obtained in P deficient plants, followed by plants grown without N, Cl, Mn, S, and Fe. The lowest stevioside content was obtained in plants without Cu.

The SG content on per plant basis was highest in plants grown without Mo (414.3 mg/plant) followed by complete, Zn, Mn, Cl, K, and B. The SG content was related to the total leaf yield. For example, in treatments involving S, P, N and Ca deficiency the SG concentration was high but due to a reduction on leaf biomass the total plant SG yield was low. For Cu deficient plant the total SG concentration was the lowest (3%) but higher leaf yield (3.5 g/plant), so the total plant SG content was higher in this treatment than that in a number of other treatments, particularly the all microelement deficiency treatment (Table 50).

Table 50: Effect of different nutrient deficiencies on stevioside and rebaudioside A concentration and content. Values within a column followed by the same letter are not significantly different.

Treatments	Stevioside (% dw)	Rebaudioside A (% dw)	Total SG (stev + rebA) (% dw)	Total SG content (mg/plant)
no Zn	7.8 a	4.2a	12.1a	350.0ab
no P	7.2 ab	3.5a	10.7a	18.3d
no N	6.4 abc	2.4a	9.0 ab	38.6d
no Cl	6.1 abcd	3.0a	9.2 ab	314.5 abc
no Mn	6.0 abcd	3.5a	9.6 ab	323.0abc
no S	6.0 abcd	3.1a	9.2 ab	78.2 cd
no Fe	5.6abcd	3.9a	9.6 ab	109.6 bcd
complete	5.2 bcde	3.6a	8.9 ab	379.7a
no B	5.1 bcde	3.4a	8.6 ab	198.6 abcd
no Mo	5.1 bcde	3.7a	8.9 ab	414.3a
no NPK	4.7 bcde	1.3a	6.1 bc	14.3d
no Ca	4.3cdef	3.7a	8.1 bc	96.1cd
no K	4.0cdef	2.4a	6.5 bc	209.3abcd
no Mg	3.8def	2.1a	5.9bc	125.0 bcd
no micro	2.8ef	1.1a	4.0 c	20.8d
no Cu	1.9 f	1.1a	3.0 c	116.8bcd
Lsd	2.51	2.05	4.03	248
P (df=12)	0.01	0.06	0.020	0.031

4.4 Discussion

Nutrient deficiency symptoms

Nutrient deficiencies can be diagnosed from visual symptoms. A pictorial record of the deficiency symptoms associated with each essential element exists for most crops. It is intended that the images collected in this study can serve a similar purpose. The next requirement is for a set of tissue concentration levels for each element, indicating deficiency, sufficiency or toxicity. Typically, such a recommendation would be made of a given tissue (e.g. most recently matured leaf) at a given phenological stage (e.g. flowering).

The symptoms associated with macro nutrient (N, P, K, Ca, Mg and S) deficiency of poor root development, stunted growth and chlorosis of the leaves were consistent with that expected in plants in general (e.g. Weir and Cresswell, 1993), and also matched the symptoms reported by previous authors for macro nutrient deficiency in stevia (Lima Filho & Malavolta 1997a; Utumi et al. 1999). For example, in Ca deficient plants, necrosis of the shoot tip and young leaves was observed, both in this study and by Utumi et al. (1999).

As expected, biomass yield was decreased relative to the control treatment for all the macro nutrient deficiency treatments. Utumi et al. (1999) reported biomass to be decreased by 47% and 11% when N and S were omitted, respectively. Shoot biomass was proportionally more decreased than root biomass by the macronutrient deficiencies, a result consistent with the interpretation that the plant assigned resources to the growth of the root, as the main nutrient absorption organ for the plant.

The symptoms associated with micro nutrient deficiency were also consistent with that expected in plants in general (e.g. Weir and Cresswell, 1993). For example, B deficiency symptoms with cracked stems were first noted after two weeks of treatment, and browning of internal tissue was noted after four weeks of treatment application. Deficiency of B commonly leads to cracking of roots and stems, a response which has been reported in other species such as cauliflower, carrot and celery (Weir & Cresswell 1993).

Fe deficiency symptoms appeared after two weeks as general chlorosis, and were severe after four weeks, with necrosis of young leaves. Deficiency of this element

leads to decreased in chlorophyll content (Salisbury & Ross 1992), as was very evident by the low SPAD reading in this study.

Cu deficiency was similar to that of Mg. Chlorosis was first noted on older leaves. Inward curling was observed in younger leaves whereas bending and rolling was observed in the older leaves. Chlorosis of the leaf started from the base and progressed towards the tip of the leaf. At the later stage, mature leaves developed brown necrotic spots, similar to that reported for tomato (Weir & Cresswell 1993). Plant biomass, stem yield, leaf yield, root weight was slightly decreased but not significantly so compared to the control. Plant height was similar to the complete treatment. Jain, Kachhwaha & Kothari (2009) reported that the increased Cu level (0.1 to 0.5µM) enhances biomass and chlorophyll content in stevia grown on culture media.

Deficiency symptoms of Mo, Cl, Zn and Mn were not prominent. Plant height, total biomass, root weight and SPAD readings were not significantly different to the complete treatment. A possible reason might be that the stevia plants were grown on half strength nutrient solution before transplanting to the treatment solutions. This pre-treatment may have allowed the development of a sufficient store of these micro nutrients, avoiding symptom expression during the four week nutrient omission trial.

Effect of nutrient deficiency on SG concentration and content

Nutrient deficiencies can affect the accumulation of secondary metabolites. For example, when grown under K (Ferreira 2007) and P (Usha & Swamy 2002) deficient conditions, *Artimisia annua* produces high concentrations of the secondary metabolite, Artimisinin. Freitas, Monnerat & Vieira (2008) also reported an

increased concentration of vitexin in the leaves of *Passiflora alata* when grown under N deficiency conditions. Utumi et al. (1999), also reported increased SG concentration in P (11.03%) and N (9.2%) deficient plants, and a lower SG content per plant due to reduced biomass.

Zinc deficient plants demonstrated a significantly higher stevioside leaf concentration (at 7.8% dw basis) than that of the control (5.2%). Rebaudioside levels, while apparently increased in this defiency treatment, were not significantly increased relative to the control treatment, and total plant yield was apparently decreased, although not significantly.

Neither the leaf concentration of SG nor the overall shoot SG content was significantly different to that of the complete nutrient treatment (leaf SG concentration of 6.5% dw basis) in plants subjected to K deficiency. Leaf SG concentration was higher in plants subjected to P and N deficiency, but SG content per plant was decreased relative to the control because of the reduced biomass.. Indeed the reduction of any macro nutrient (N, K, Ca, Mg or S) led to a decrease in plant SG content. Utumi et al. (1999) also mentioned that deficiencies of N, K, Ca, Mg and S reduce the SG content.

The decreased SG content of Fe deficient plants was primarily due to the reduced biomass, as the SG concentration was not reduced in Fe deficient plants. However, in Cu deficient plants the yield of the biomass was not reduced, but the SG concentration was significantly decreased. The possible role of Cu for reduced SG content is unknown. In micro nutrient deficient treatment, all the nutrients except N, P and K were omitted. Overall, omission of N, P or less K, or all micro nutrients resulted in the most serious reductions of SG content per plant.

Chapter Five: Role of pH on growth and SG content of stevia grown in hydroponic system

5.1 Introduction

pH is one of the important factors that influences plant growth. Different crop species require different pH for optimum growth. pH is a measure of the degree of acidity and alkalinity. The degree of acidity and alkalinity is influenced by soil type and climate of the surrounding areas. In areas with heavy rainfall, soils in a solid growing medium are more acidic because most of the base forming cations are leached out and the aluminium and hydrogen ions remain. Similarly, in areas with low rainfall (dry areas) most of base forming cations remain in the soil which leads to alkalising in the soil (Brady & Weil 1999). Crops such as cassava and Napier grass and to some extent barley can tolerate high acidity level. Similarly, there are crops which prefer moderately acid soils, for example beans, lettuce and cauliflower (Salisbury & Ross 1992).

Nutrient availability for plants is highly dependent on the pH of the growing medium. Nutrients such as phosphorous, magnesium and calcium are less available when the pH is below 5. Similarly, with high pH nutrients such as iron, manganese, copper, zinc and boron ions are available to the plants only in small quantities (Jones 2005). Most of the nutrients are not soluble at high pH of 8, for example iron. Roots cannot absorb nutrient from the media so as a result deficiency symptoms are seen on the plant. Many studies have been conducted to identify cultivars which are tolerant to acidic or alkali growing conditions.

For plants grown in soilless culture, pH is maintained by adding either acid or alkali to the nutrient solution. In common practice pH is raised by adding NaOH and

lowered by adding H_2SO_4 or HNO_3 to the solution (Jones 2005). Plant growth depends on availability of nutrients from the media.

In its natural habitat, stevia has been found to be grown in infertile, acids sands or muck soils. Most of the previous studies on stevia have been conducted on different types of soil and pH, ranging from 5-7. However, Shock (1982) indicates that stevia can be grown in acid soils with pH 4-5 and grows well in pH ranging from 6.5-7.5. Rank & Midmore (2006) also reported that plants grown on neutral to alkali soils had reduced plant yield. There is still a lack of understanding on the effects of pH on biomass yield and SG content. There is no experimental work published on this. This study sets out to identify the optimum pH levels required for the growth of stevia and its effect on steviol glycoside (SG) concentration.

5.2 Materials and Methods

Location

The experiment was conducted at CQUniversity, North Rockhampton (23 ^{o} 22', 0.345"S, 150^{o} 31' 0.53"E), Australia. The experiment was conducted inside a screen house with 67% light transmission (Bhattarai, Huber & Midmore 2004). The experiment was carried out using a non-circulatory hydroponics (controlled conditions) system following the method of Midmore (1994). Mean ambient temperature during the experiment period was 22-23^{o}C.

Nutrient solution preparation

Commercially available hydroponics fertilizer (Manutec Pty. Ltd) was used as a nutrient medium. Half strength of the solution (with pH of 6.7 and EC 1.45 µS) was used for this study. This solution was modified to result in different pH levels. At the

start of the experiment, 170 L of nutrient solution was prepared in a 200 L capacity drum. The solution was prepared in reverse osmosis (RO) water. The solution was prepared by mixing 60 g of part A (N 7.6%, P 3.1%, K 18.2%, S 4.5%, Mg 3.5%, Fe 0.34%, Mn 0.008%, Zn 0.04%, Cu 0.03%, B 0.003% and Mo 0.001%) and 40 g of part B (Ca 19% and N 15.5%) in 100L of water. To bring the pH to the desired level different amounts of acid or alkali was added to the solution as indicated in Table 51.

Table 51: pH and the amount of buffer added to the total volume of half-strength hydroponic solution (Manutec)

pH of the original stock solution	Acid or alkali	Amount of adjuster added (ml)	Total amount of solution (L)	Final pH value of the solution
6.7	0.25M H_2SO_4	710	170	4
6.7	0.25M H_2SO_4	580	170	5
6.7	0.25M H_2SO_4	355	170	6
6.7	1M NaOH	35	170	7
6.7	1M NaOH	125	170	8

Experimental design and set up

Five treatments were set up, with pH 4, 5, 6, 7 and 8, with 6 replications in a completely randomised design. The duration of the experiment was 8 weeks (19/07/2010-15/09/2010). Styrofoam boxes with 40 L capacity were used for this experiment. They were each lined with one black plastic bag to prevent leakage. Twenty eight litres of solution was supplied to each box. Four holes were made on the lid to hold 7 cm diameter poly-pots. Plant to plant and row to row distance was maintained at 13 cm and 25 cm respectively. A 2 mm air dripper was connected to each box to mix the solution within each box. To check the level of the solution a small hole was made on the lid with a measuring dip stick attached to it. The pH and EC measurements were conducted through the same hole. pH of the solution was

adjusted by adding buffers. To lower the pH of the solution 0.25 M of H_2S0_4 and to raise sodium hydroxide of 1 M was used. The level of the solution and pH was monitored daily and later modified to bring the pH to the desired value. Each solution was completely replaced after 6 weeks because the EC of the nutrient solution was reduced to half than that at the start of experiment.

Plant material

Seedling preparation

Seeds of variety Shoutain were sown in 25/04/10 in vermiculite media in germination trays. Growth of the seedling was very slow during that period. From germination trays they were transferred to cup trays after one month and fertilized with half-strength Manutec solution for 4 weeks. Seedlings were then transferred to 7 cm diameter pots in perlite media and grown in half-strength Manutec solution for four weeks. They were then transplanted into the different treatment solutions on 19/07/2010. Plant height during transplanting was 3-4 cm.

Data collection

pH of the solution was measured each morning between 9:00-10:00 am. Plant height and chlorophyll content were measured at fortnightly intervals. Chlorophyll concentration was estimated with a Minolta ^{TM} SPAD meter. Readings for chlorophyll concentration were taken from three fully expanded leaves and the average value calculated. Other physiological parameters such as photosynthetic rate, transpiration rate, and stomatal conductance were measured with an IRGA (Infrared Gas Analyser) model LCA-4 from ADC-UK following the method used by

Bhattarai, Midmore & Pendergast (2008). These measurements were made at fortnightly intervals, each using one leaf per plant per box.

Harvesting

Plants were harvested after two months growth, on 15/09/2010. After harvest, leaves and stems were separated from each plant and the fresh weight was taken. These were placed in the oven at 60^{o}C for 48 hours and dry weight was measured. For root dry weight analysis, all the roots (four plants) were combined in one sample. Each box was regarded as one replication. Roots were thoroughly rinsed with water to remove perlite and dried in the oven at 60^{o}C for 48 hours for dry weight measurement.

Steviol glycoside analysis

Leaf samples for HPLC analysis from each box were taken from 8^{th} -10^{th} leaf from the apex. Fresh leaf samples were oven dried at 60^{o} C temperature for 48 hours. Dried leaves were ground to a fine powder using mini beat-beater. SG content in the leaf was analysed using the same method as outlined in Chapter 2.

Statistical analysis

Data were analysed using the statistical package Genstat version11.1, employing the procedure for a completely randomised design. Mean differences were calculated through least significant difference test at $P<0.05$ level.

5.3 Results

Effect of pH on growth parameters

Above ground plant biomass was greater in plants grown at pH 4 and 6 than at pH 7 and pH 8. The plant biomass at pH 8 was one half that at pH 4 and pH 6 (Table 52). Leaf dry weight did not differ between plants grown at pH 4, 5, 6, and 7 all were greater than that at pH 8. The leaf weight of the plants grown at pH 8 was 43% less than that at pH 4.

There was no significant difference in stem dry weight between the treatments. However, plants grown at pH 8 had the highest stem dry weight followed by pH 4, 6 and 5. Lowest stem yield was at pH 7 (Table 52).

Root dry weight was not separated on a per plant basis. Average root weight per plant was calculated by dividing total root weight by four (the number of plants). Root weights were significantly lower in plants grown at pH 8 from those at pH 4, 5 and 7 (Table 52).

The shoot to root ratio differed significantly between the treatments (Table 52). The highest shoot to root ratio was obtained at pH 6 followed by pH 5 and pH 4. The shoot to root ratio was significantly lower at pH 7 and pH 8 than at lower pH.

There was no significant difference ($P \leq 0.05$) in plant height at harvest for plants grown in different pH treatments (Table 52). Branch number was highest at pH 6 that with the highest shoot to root ratio, followed by pH 5, 4 and 7. Lowest branch number was observed at pH 8 (Table 52).

Table 52: Dry weight of stem, leaf, root and total biomass, shoot to root ratio, plant height and number of branches per plant of stevia (Stevia rebaudiana) grown at different pH. Means within the columns with different letters are significantly different (P<0.05).

Solution pH	Dry weight (g/plant)				Shoot to root ratio	Average plant ht (cm)	Branch no. (per plant)
	Stem	Leaf	Root	Total above ground biomass			
4	12.2 a	14.1 b	8.8 c	26.3 c	0.74 bc	48.8 a	8.3 ab
5	10 a	13.8 b	7.6 b	23.8 b c	0.78 c	47.2a	8.8 b
6	11.9a	14.3 b	7.3 ab	26.3 c	0.89 c	50.4a	10.7 b
7	7.8a	12.8 b	8.5 bc	20.6 b	0.60 ab	47.7a	7.7 ab
8	13.1a	8.8 a	6.4 a	13.8 a	0.53 a	41.6a	4.9 a
Lsd	11.1	2.0	1.2	4.7	0.2	6.3	3.4
P value (df)	0.87(25)	<0.001 (25)	0.003 (20)	<0.001(25)	<0.001(20)	0.077(25)	0.032 (25)

Effect of solution pH on gas exchange and chlorophyll content

There was no significant difference in photosynthetic rate, transpiration rate and stomatal conductance between the treatments (Table 53). Transpiration rate was low at pH 8 with low stomatal conductance. The SPAD reading shows that the chlorophyll concentration was low at pH 8 compared to the maximum at pH 5.

Table 53: Photosynthetic rate, transpiration rate, stomatal conductance and estimated of chlorophyll concentration (SPAD) of stevia (Stevia rebaudiana) grown at different pH. Means within columns with the same letters are not significantly different (P<0.05)

Solution pH	Photosynthetic rate ($\mu mol\ m^{-2}s^{-1}$)	Transpiration rate ($mmol\ m^{-2}s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}s^{-1}$)	SPAD reading
4	4.60 a	3.20 a	0.15 a	49.4 a
5	3.58 a	2.71 a	0.11 a	49.9 a
6	4.00 a	2.81 a	0.12 a	44.7 b
7	4.60 a	2.02 a	0.08 a	47.6 b
8	0.38 a	2.19 a	0.08 a	44.4 b
lsd (5%)	5.71	1.24	0.09	4.2
P value (df)	0.52 (20)	0.30 (20)	0.45 (20)	0.03 (25)

Effect of pH on SG concentration and content

No significant difference between the treatments was found in leaf concentration of either stevioside or rebaudioside A (Table 54). The total SG concentration did not differ between the treatments.

Table 54: Percent dry weight of stevioside and rebaudioside A in leaves of stevia (Stevia rebaudiana) grown at different pH. Values are means of six replicates with one missing value.

Solution pH	SG (% dry weight)	rebaudioside A (% dry weight)	Total SG (% dry weight)	Total SG (mg/plant)
4	2.8	0.7	3.5	510
5	2.7	0.7	3.4	477
6	2.5	0.5	3.0	436
7	2.9	0.8	3.8	505
8	3.0	0.9	3.9	354
Lsd (5%)	0.83	0.58	1.28	199
P value(df=19)	0.67	0.65	0.58	0.47

5.4 Discussion

The growth of plants is related to the uptake of the nutrients through the roots. Root growth and function is influenced by the surrounding environment such as nutrient concentration and solution temperature. The availability of nutrients is known to depend on the pH level. In our study we found stem yield was highest at pH 8. Plants grown at pH 8 had thick stems (although the difference from other treatments was not significant) with fewer leaves. Leaf yield was greatly reduced at pH 8, and was 37.5 percent less than the maximum at pH 6. Rank & Midmore (2006) also reported that plants grown on neutral to alkaline soil had thicker stems with poor growth during research trial in northern Queensland, Australia. Leaves showed symptoms of iron-deficiency, this may be due the lower availability of iron in the

solution of high pH. Iron is responsible for increasing chlorophyll content (Salisbury & Ross 1992) and the iron-like deficiency symptoms were corroborated by the low SPAD reading, also indicating a low leaf chlorophyll concentration and by the visual symptoms observed for iron deficient plants in Chapter 4. Islam, Edwards & Asher (1980) also studied the effect of pH on six different plant species in flowing solution culture and found that species like ginger and cassava tolerate low solution pH. Most of the species achieved maximum growth between the range at pH 5.5 to 6.5. However, high pH resulted with iron deficiency in maize and wheat in their studies. In our studies we found that root dry weight was highest at pH 4 followed by pH 5, 6 and 7. The root dry weight was the lowest at pH 8 indicating that growth was retarded due to the lack of nutrient uptake by the roots. In our study we found that the leaf yield between pH 4 and pH 6 was higher compared to plants grown at pH 7 and pH 8. Out results revealed that stevia should be grown between the range of pH 4 and pH 6 to get maximum yield.

The SG concentration for this variety (Shoutain) was low in this experiment. The possible reason might be the growing conditions. During the experiment stevia was attacked by white flies, aphids and spider mites. We sprayed contact insecticide Kendon, active ingredient pyrethrins (5 gL^{-1}) and piperonyl butoxide (22.5 gL^{-1}) to eliminate the insects. This insecticide did not work. So after 4 days the systemic insecticide Roger 100 was (3 ml L^{-1}). This possibly might have had an effect on the SG concentration. The other reason might be plants were grown under the screen house with 67% light intensity during the winter period. Zaidan, Deietrich & Felippe (1980) have reported that irradiance effects the SG concentration. In their research

they found that plants grown under the greenhouse had lower SG concentration than those grown outside in natural sunlight at the same time.

Australian agricultural soils are highly to moderately acidic. According to CSIRO (2004), about 33 million hectare of agricultural land are highly acidic and about 55 million hectare of land are moderately acidic soils, and are under the severe risk of degradation. As stevia can tolerate highly acidic soil, there is a great scope of growing stevia profitably in such soil.

Since the pH affects the nutrient uptake from the solution further study is required to find out the uptake of nutrients at different pH for stevia.

Chapter Six: Conclusions

Stevia is a non-calorie sweetener derived from the leaves of the stevia plant and is heat and pH stable. Being a calorie free sweetener, stevia is regarded as being helpful for people with hypertension, diabetes and obesity. Stevia is a new crop to Australia therefore agronomic requirements of the crop are yet to be identified for commercial cultivation.

Stevia is regarded as a short day plant with the requirement of 12 hours day length for flowering. Stevia seeds were imported from China and were grown in controlled conditions in Australia. Average number of days to reach flowering and the biomass yield were influenced by transplanting age, seedling or ratoon cropping and the genotype, in both first and ratoon crops. The effect of the age of seedling at the transplanting varied with the varieties. Irrespective of the varieties, the SG concentration was higher when younger (five weeks old) seedlings were transplanted compared to older 6 to 8 week old seedlings. Older seedlings were on average smaller than younger seedlings at transplanting. The concentration of SG depends on genotype and growing conditions. SG content is the product of leaf biomass yield and the SG concentration in the leaves of stevia. The total SG concentration and content was significantly lower in two (separate for each parameter) of the three varieties. Under long day conditions, plant accumulate more biomass whereas in short day conditions plants tend to flower and most of the subsequent captured energy is accumulated to the reproductive organs. Selections were made in each variety for early, medium and late flowering, and nine plants of each within a variety x flowering group were crossed to produce seed. There was some evidence that the crosses between late selections were also later than the other selections, giving some

optimism to being able to develop later flowering. Selection of early, medium and late flowering plants should be carried out for a number of generations in order to select the plants with desired traits (early flowering for seed production and late flowering for SG production).

The cuttings from the early, medium and late flowering groups were grown under constant light with different photoperiods .Commencement of flowering under 12 hours day length was early and also the number of flowering plants (100%) was significantly higher as compared to those grown under longer photoperiods (24, 16 and 14 hours), confirming stevia as a short day species.

The biomass yield and SG concentration of stevia was related to the availability of nutrients. Deficiency of a single element reduced the quantity of leaf yield (leaf being the important part of stevia) and also decreased the SG concentration. A pictorial record is the first step to help in identifying the symptoms of nutrient deficiency and further to correct the deficient element required for the plant growth. Stevia grown without micro-nutrients had both decreased leaf yield and reduced SG concentration. Total SG content decreased in most of the macronutrient deficient plants because of the decreased leaf yield.

Biomass yield of stevia was also related to the availability of soil water. Plants resulted in low yields under both low and very high moisture content. High leaf yield was obtained on plants grown under field capacity and 80% of FC. The SG concentration was significantly higher for plants grown on water-logged conditions but because of reduced leaf yield, total SG content was similar to that of the plants grown under 100 and 80% of the FC. Decreased SG content in water stressed (i.e.

drought) stevia plants was more due to the reduced yield of the leaf biomass than the SG concentration.

Plant growth and leaf yield of stevia was maximum at pH ranging from 4-6. High pH levels from neutral to alkali reduced plant growth and leaf yield. The SG concentration did not vary between the treatments. As most of the Australian agricultural soils (more than 88 million hectares) are either highly or moderately acidic in nature, stevia could be the ideal crop to grow in such soils and can contribute to the national economy significantly.

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Appendix

Appendix 1: The final concentration of each stock solution when used in nutrient solution. The stock used for any given treatment are in detailed in Roberts and Whitehouse (1976)

Chemicals	Dilution rate (1:100)mM	N	P	K	Ca	Mg	S	Zn	Fe	Cu	Mo	B	Cl	Mn	Na
Ca(NO3)2.4H2O	3.3454	6.691			3.345										
KNO3	3.3629	3.363		3.363											
NaNO3	3.3532	3.353													3.3532
K2SO4	1.6642			3.328			1.6642								
Na2SO4.10H2O	0.6828						0.6828								1.3656
CaSO4.2H2O	0.0987				0.099		0.0987								
MgSO4.7H2O	1.5012					1.5012	1.5012								
Na2H2PO4.2H2O	1.1732		1.173												2.3464
Mg(NO3)2.6H2O	1.4820	2.964				1.4844									
Na2(E.D.T.A)	0.0597	0.119													0.1194
FeCl3	0.0514								0.0514				0.1541		
MnCl2.4H2O	0.0101												0.0202	0.0101	
CuCl2.2H2O	0.0012									0.0012			0.0023		
ZnCl2	0.0022							0.0022					0.0044		
H3BO3	0.0323											0.0323			
Na2MoO4	0.0002										0.0002				0.0005
MnSO4.H2O	0.0101						0.0101							0.0101	
CuSO4.5H2O	0.0012						0.0012			0.0012					
ZnSO4.7H2O	0.0022						0.0022	0.0022							

Appendix 2: Elemental compositions of the 16 different nutrient solutions (mM) used in deficiency trials with stevia

Treatments	mM solution													
	N	P	K	Ca	Mg	S	Fe	Mn	Cu	Mo	B	Zn	Cl	Na
Complete	10.054	1.17	3.36	3.35	1.50	1.50	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	0.0005
(-N)		1.17	3.33	0.10	1.50	1.76	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	2.4658
(-P)	10.17		3.36	3.35	1.50	2.18	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	0.1194
(-K)	10.04	1.17		3.35	1.50	1.50	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	7.0652
(-Ca)	6.836	1.17	3.36		1.50	1.50	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	0.1194
(-Mg)	10.05	1.17	3.36	3.35		0.68	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	1.3656
(-S)	13.14	1.17	3.36	3.35	1.48		0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1767	2.4658
(-Fe)	10.05	1.17	3.36	3.35	1.50	1.50		0.010	0.0012	0.0002	0.0323	0.0022	0.0270	2.3464
(-Mn)	10.05	1.17	3.36	3.35	1.50	1.50	0.051		0.0012	0.0002	0.0323	0.0022	0.1565	2.4658
(-Cu)	10.05	1.17	3.36	3.35	1.50	1.50	0.051	0.010		0.0002	0.0323	0.0022	0.1787	2.4658
(-Mo)	10.05	1.17	3.36	3.35	1.50	1.50	0.051	0.010	0.0012		0.0323	0.0022	0.1767	2.3464
(-B)	10.05	1.17	3.36	3.35	1.50	1.50	0.051	0.010	0.0012	0.0002		0.0022	0.1767	2.4658
(-Zn)	10.05	1.17	3.36	3.35	1.50	1.50	0.051	0.010	0.0012	0.0002	0.0323		0.1767	2.4658
(-Cl)	10.17	1.17	3.36	3.35	1.50	1.50	0.051	0.010	0.0012	0.0002	0.0323	0.0022		0.0049
(-NPK)				3.35	1.50	1.60	0.051	0.010	0.0012	0.0002	0.0323	0.0022	0.1811	0.1199
(-all micro)	6.72	1.17	3.36											2.3464