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Effect of nutrient omission and pH on the biomass and concentration and content of steviol glycosides in stevia (*Stevia rebaudiana* (Bertoni) Bertoni) under hydroponic conditions

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ABSTRACT

Steviol glycosides (SGs), have recently been approved in western countries as sources of intense natural sweeteners. SGs are found in *Stevia rebaudiana*, mainly in the leaves, and industry prefers rebaudioside A (Reb-A) over other steviol glycosides for its superior flavour profile. Hence leaf biomass and concentration of SGs (and their product, SG content) are of primary agronomic interest. Experiments were conducted under controlled conditions in nutrient solution to assess the effects of nutrient deficiencies and pH on biomass production, and concentration and plant content of SGs. Total SG content was low in plants deficient in the macronutrients N, P, S, Mg or Ca because of reduced photosynthesis and because of the decreased leaf yield, even though lack of N resulted in greater concentration of stevioside in the leaves. Lack of N or P reduced the proportion of Reb-A to total SGs. Plants deficient in K had less yield than in the nutrient-complete control, but not significantly so and SG concentration in the leaves was similar to that of the control. Deficiency of the micronutrients Cu and Fe led to low SG yield, because of reduced SG concentration in leaves, and because of reduced leaf yield, respectively. Lack of other micronutrients did not influence SG content. Neutral to alkali conditions reduced plant growth and leaf yield, most likely due to deficit of P, but pH had no effect on SG concentration. Our results are indicative, but preliminary, and require confirmation in open field trials over several years.

1. Introduction

Stevia (*Stevia rebaudiana* (Bertoni) Bertoni) is known for its sweet tasting compounds, steviol glycosides (SG), found in greatest concentration in the leaves (Kulasekaran et al., 2006). Production of high SG concentration in plentiful leaf biomass is an important goal in commercial stevia production. Interest in commercial production of stevia has intensified with approval, firstly in Australia and then in the USA and more recently in Europe, of steviol glycosides as intense sweeteners (FSANZ, 2008; Anon., 2011). In its natural habitat, stevia is found in infertile, acid sands or muck soils (Shock, 1982) although commercial production tends to take place on somewhat better soils. It is well known that plant growth in general is stunted when deprived of nutrients, whether due to insufficient quantity or to pH-conditioned non-availability in the growing medium, or to insufficient water for uptake. Each element plays one or more particular biochemical/physiological roles, so deficiency of that element will result in a set of predictable metabolic and physiological disturbances.

Reports on the influence of nutrient deficiency (and toxicity) on stevia growth and SG yield are scarce. Low availability of soil N leads to low leaf N concentration, reduction of photosynthesis (Sharma et al., 2016) and low leaf yield compared to adequate soil N, but it also leads to a higher concentration of SG in leaves than with adequate soil N (Barbet-Massin et al., 2015). In a similar manner, higher rates of NPK or farmyard manure led to higher leaf yield but lower SC concentration in trials in the western Himalayas (Kumar et al., 2012), and highest leaf SG concentration (16.7% w/w) was achieved by omission of N compared to that found in the N-supplied control (11.5% – Das et al., 2006). Utumi et al. (1999) found that total above ground biomass decreased with deficiencies of any macronutrient, however, the percentage of reduction was significantly higher in treatments without N, K, or Mg than with deficiencies of S, Ca or P. The concentration of SG decreased with the deficiency of all macronutrients except for P (by 27% for Ca, 24% for N, to 41% for S), causing a reduction in content of SG per plant. This contrasts with the earlier mentioned increased SG concentration in leaves of plants lacking N, and the reported lack of response of total leaf SG concentration to deficiency of any macro or micronutrient by Jarma et al. (2012), although concentration of Reb-A did decline with lack of P, S, K or Cu.

Plant nutrient availability is highly dependent on the pH of the

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growing medium. Nutrients such as phosphorous, magnesium and calcium are less available when the pH is below 5 (Jones, 2005). Similarly, at high pH iron, manganese, copper, zinc and boron are only sparingly available due to their low solubility (Jones, 2005). As a result, when the pH is outside of the optimal range, deficiency symptoms are seen on the above ground plant, although it is commonly difficult to ascribe the symptoms to deficiencies of individual elements. For stevia, Shock (1982) reports that even though native to low pH (4–5) soils, it grows well on less acid soils ranging from 6.5–7.5. Rank and Midmore (2006) reported that plants grown on neutral to alkali soils showed less plant yield compared to that on acidic soils.

The specific effects of pH and of nutrient deficiency on biomass yield and SG content in stevia are largely unknown. This study therefore set out to determine the consequences of macro and micronutrient deficiency on morphology, biomass accumulation, and SG concentration and content in stevia plants. This was complemented by a study to identify the optimum pH required for the same. Visual symptoms of nutrient deficiency on stevia shoot and root colour and morphology from these experiments are reported by Midmore et al. (2012).

2. Material and methods

2.1. Location

Two experiments (one nutrient omission and one with differing pH) were conducted at CQUniversity, Rockhampton (23° 22′, 0.345”S, 150° 31′ 0.53”E), Australia, using a non-circulatory hydroponics system (Midmore, 1994) inside a screen-house with 67% full sunlight.

2.2. Plant material

Seeds of *Stevia rebaudiana* variety ‘Shoutain-2′ were sown into 1:1 perlite:vermiculite media in speedling trays inside the screen-house on 17/08/09 for the nutrient omission and on 25/04/10 for the pH experiment. Following germination, seedlings were watered with half strength Manutec hydroponic solution (Manutec Pty. Ltd.) for three weeks in the nutrient experiment and for four weeks in the pH experiment. They were then transferred to 7 cm diameter poly-pots lined with mesh and filled with perlite, for a further 4 weeks and watered as before. At the 6–8 leaf stage, with plant height ranging from 6 to 8 cm, seedlings for the nutrient experiment were supplied with reverse osmosis (RO) water for two weeks, and then subjected to treatment. Seedlings for the pH treatments were not preconditioned with RO water.

2.3. Treatments and experimental design for the nutrient omission experiment

The chemical composition of the nutrient-deficient solutions was similar to that reported by Roberts and Whitehouse (1976). In essence, the following (in mmol) was the composition of the complete nutrient solution: N (10.054), P (1.17), K (3.36), Ca (3.35), Mg (1.5), S (1.5), Fe (0.51), Mn (0.01), Cu (0.0012), M0 (0.0002), B (0.323), Zn (0.0022), Cl (0.1767) and Na (0.0005). Omission treatments were so designed that, with exception of Na (which ranged from 0.119 to 7.065), S in the −Mg (0.68), and Cl in the −Fe (0.27), concentrations did not differ from the control. Styrofoam boxes (53 cm x 23 cm × 25 cm) were lined each with a black plastic bag to prevent leakage. Four 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 25 cm, respectively. Solution was filled to a depth of 15 cm, and height of the solution and pH and electrical conductivity (EC) were monitored daily between 9:00 and 10:00 am and adjusted accordingly. The pH for the treatments was *c*. 5.5–6.0 and EC varied between treatments. Mean maximum and minimum temperatures during the experimental period were 33.1 and 20.1 °C. The temperature of nutrient solutions ranged from 25 to 30 °C.

Table 1
Effect of different nutrient deficiencies on leaf chlorophyll concentration, photosynthetic rate, leaf dry biomass and shoot to root dry weight ratios of stevia at the time of harvest (at four weeks after treatments imposed). Values within a column followed by the same letter are not significantly different ($P < 0.05$).

Treatments	Chlorophyll concentration (SPAD units)	Photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$)	Leaf biomass (g/plant)	Shoot to root ratio
Complete	53.45 a	12.73 bc	3.96 d	12.6 ef
No Mn	52.90 a	11.28 bc	3.12 cd	8.6 cde
No Zn	50.45 a	11.57 bc	2.70 bcd	7.6 bcde
No Mo	50.15 a	13.03 c	4.11 d	10.2 de
No B	50.00 a	15.51 c	2.19 abcd	9.6 de
No Cu	49.90 a	13.12 c	3.31 d	9.5 de
No Cl	46.50 a	15.89 c	3.18 cd	8.9 cde
No K	46.45 a	10.43 bc	3.27 d	17.5 f
No Ca	33.50 b	0.66 a	1.09 abc	10.9 de
No S	31.70 bc	5.78 ab	0.73 ab	2.6 ab
No Mg	28.00 bcd	1.45 a	1.12 abc	9.8 de
No Fe	25.75 bcd	2.98 a	1.11 abc	3.6 abc
No P	22.70 cd	1.49 a	0.38 a	2.3 ab
No N	22.05 d	2.60 a	0.38 a	1.7 a
No Micro	21.35 d	0.87 a	0.43 a	6.5 abcd
No NPK	20.90 d	0.28 a	0.30 a	2.8 ab

The experiment consisted of 16 treatments (Table 1) each randomised in two blocks. Each treatment in each block, i.e., each box, comprised four plants, giving a total of 128 plants in the experiment. Data were collected from each plant to estimate sampling error.

2.4. Treatments and experimental design for the pH experiment

In common practice for plants grown in soilless culture pH is raised by adding NaOH or KOH and lowered by adding H_2SO_4 or HNO_3 to the solution (Jones, 2005). Half strength commercially available hydroponics fertilizer (Manutec Pty. Ltd) was used as a nutrient medium with pH of 6.7 and EC 1.45 dS/m. To bring the pH to the desired level 710, 580 or 355 ml of 0.25 M H_2SO_4 were added to achieve pH values of 4, 5, or 6, and likewise 35 or 125 ml of 1.0 M NaOH per 170 l were added to achieve pH values of 7 or 8, respectively. Styrofoam boxes and solution monitoring were as in the nutrient experiment, and each treatment was replicated six times, in a completely randomised design. Solution pH was maintained at 4, 5, 6, 7 and 8 daily by adding appropriate amounts of H_2SO_4 or NaOH. Mean maximum and minimum temperatures during the experimental period were 24.1 and 13.2 °C.

2.5. Data collection

Leaf chlorophyll concentration was estimated using a SPAD meter (Konica, Minolta Japan), with readings taken 3 and 4 weeks after the start of treatment in the nutrient experiment and at fortnightly intervals for the pH experiment. Youngest fully expanded leaves were used for the measurement.

Leaf gas exchange (photosynthesis, transpiration and stomatal conductance) for all the treatments in both experiments was measured using an IRGA (Infrared Gas Analyser, model LCA-4 from ADC-UK). Measurements were made at 4 weeks following the start of treatments in the nutrient experiment and at fortnightly intervals for the pH experiment. The IRGA readings were taken between 11:00 a.m. and 2:00 p.m., on the same leaves used for chlorophyll determination.

For the measurement of leaf steviol glycoside concentration (i.e., amount of SG per unit dry weight of leaf, expressed as %), two youngest fully expanded leaves from each plant of every treatment were removed three weeks after treatments began. The eight leaves from each box were combined and oven-dried at 60 °C for 48 h, ground to a fine powder with a mini bead-beater, and stored in air-tight containers. The