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Research article

INFLUENCE OF LIGHT, GROWTH REGULATORS, NITRATE AND SUGARS ON THE PRODUCTION OF STEVIOSIDE AND REBAUDIOSIDE-A IN THE LEAF CALLUS CULTURES OF *STEVIA REBAUDIANA* BERTONI

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ABSTRACT: Stevioside and rebaudioside-A are diterpenes steviol glycosides which are estimated to be 250-400 times sweeter than cane sugar. These natural sweeteners with zero calories are produced in green leaves of perennial shrub *Stevia rebaudiana* Bertoni, family- Asteraceae. In the present study, production of Stevioside and rebaudioside-A in callus cultures under the influence of light, growth regulators, carbohydrates and nitrate levels was investigated using LC-MS method. *Stevia* leaf explants showed 10.2 - 96.52% callus induction on Murashige and Skoog medium with different, 36 single and 28 combinations of growth regulators. Callus accumulated about 0.47 to 2.90µg/gdc (gram dry callus) Stevioside and 0.35 to1.91 µg/gdc rebaudioside-A on eight different media i.e. one concentration of each growth regulator 2,4-dichlorophenoxy acetic acid; α-naphthalene acetic acid; Indole-3-acetic acid; Indole-3butric acid; Kinetin and 6-benzyl amino purine and two combinations (6-benzyl amino purine + 2,4-dichlorophenoxy acetic acid and 6-benzyl amino purine + Indole-3-acetic acid) under dark conditions. A clear and significant increase in the accumulation of both Stevioside (132.9 µg/gdc) and rebaudioside-A (148.7 µg/gdc) was observed on exposure to 12 hours light conditions and sucrose (3%) containing medium with MS nitrates. Supplementation of glucose or fructose as carbon source, has further promoted production of both Stevioside (158.1 and 232.65µg/gdc) and rebaudioside-A (98.0 and 186.05 µg/gdc). Whereas increase in the total nitrate levels showed drastic reduction in the steviol glycosides accumulation in leaf derived callus cultures.

Key words: Stevioside, rebaudioside-A, callus culture, LC-MS, growth regulators.

Abbreviations: Stevioside St; rebaudioside-A Rb

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INTRODUCTION

Stevioside (St) and rebaudioside-A (Rb) are the major sweetening components used in today's sweetening industries as zero caloric sugar substitutes. These steviol glycosides are extracted from the leaves of *Stevia rebaudiana* Bertoni, an important member of Asteraceae family. Stevioside has been reported to reduce blood glucose levels in type II diabetics as well as blood pressure in mildly hypertensive patients (Gregersen et al., 2004; Hsieh et al., 2003). Metabolic studies have demonstrated that steviol glycosides (SGs) are poorly absorbed. Among the natural zero calorie sweeteners, steviol glycosides (SGs) are about 250-400 times sweeter than sucrose at their concentration in leaves is of about 4% (w/v) (Kinghorn et al., 1986). These SGs are diterpene glycosides synthesized in leaf cells through MEP pathway (Brandle et al., 2007).

In vitro studies on Stevia callus induction and propagation have been carried out using explants such as leaf (Sivaram and Mukundan 2003; Anbazhagan et al., 2010; Banerjee et al., 2008; Esmat et al., 2010; Rathi et al., 2009), stem tips (Tamura et al., 1984), node (Anbazhagan et al., 2010; Gupta et al., 2010; Kumar et al., 2014; Mohamed 2011; Sivaram and Mukundan 2003; Thiyagarajan et al., 2012; Ummi et al., 2014), and inter node (Gupta et al., 2010; Kumar et al., 2014). Compared to studies on micropropagation detailed studies on the effects of growth regulators (GR) (single and in combination) are limited. To the best of our knowledge few reports are avalable on the production of the SGs in callus cultures (Mathur and Shekhawat 2013; Sivaram and Mukundan 2003) and role of light on the accumulation of stevioside and rebaudioside-A. The present work has been carried to study the role of GRs, light, sugars and nitrate on production of St and Rb in callus cultures.

MATERIAL AND METHODS

Stevia rebaudiana Bertoni, germplasm obtained from IHBT, Palampur, Himachal Pradesh, India was maintained at the Botany Garden, Jiwaji University, Gwalior (India).

In vitro studies:

The Stevia leaves from field grown plants were used for the *in vitro* studies. Media preparation, leaf sterilization and inoculation were carried out as per Tejovathi et al.(1996).

The leaf explants of about 2x2 mm were cut and inoculated aseptically on to MS (Murashige and Skoog 1964) medium supplemented with 0.5 to 5.0 mg/l of growth regulators 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-butric acid (IBA), Kinetin (Kn) or 6-benzylamino purine (BAP) alone or in combinations. Thirty replicates per experiment were used and each experiment was repeated and the data collected was pooled. All the cultures were maintained under 25± 2 °C and continuous dark conditions for one month.

Effect of Light, growth regulators, type of carbohydrate and the level of nitrates in the medium, on the St and Rb accumulation in the callus was analysed using LC MS studies. All the LC-MS studies were carried out at IIIM, Jammu.

Effect of Growth regulators

Six single (one concentration from each GR which showed better callus) and two combinations (1.0 mg/l BAP + 1.0 mg/l 2,4-D and 1.0 mg/l BAP + 1.0 mg/l IAA) of GRs were selected to study the effect of GR on St and Rb production in *in vitro* cultures.

Effect of Light

Effect of light on accumulation of SGs in callus cultures were analyzed by exposing the callus under fluorescent light (800 -1000 Lux) in 12 hrs photoperiod and compared to cultures grown under dark conditions on same MS medium (1.0 mg/l 2,4-D and BAP + 3% sucrose).

Effect of Carbohydrates

Five carbohydrates- sucrose, glucose, fructose, dextrose and maltose were tested for better SGs production in callus cultures when supplemented as carbon source at 3% level in the MS with 1.0mg/l 2,4-D + 1.0mg/l BAP

Effect of Nitrate concentration

Effect of total nitrate levels on St and Rb accumulation was analyzed by using two concentrations of NH_4NO_3 and KNO_3 (1) as used in the MS medium and (2) double the MS nitrate concentration. Both nitrates were supplemented in the medium with 1.0mg/l 2,4-D + 1.0mg/l BAP +3% sucrose for the study.

LC-MS studies

Callus (from one concentration of each GR showing good response) was harvested and pooled from culture replicates from each selected experiment and dried in the oven at 60°C for 48 hrs. The dried callus was crushed into fine powder.

1g of dried callus was processed according to the procedure given by Jaitak et al. (2008) for LC MS analysis. 1g dried powder was mixed with 10 ml methanol (Merck, Germany) and incubated for 24 hr with continuous shaking. The extract was filtered through whattman filter paper No. 1. This step was repeated trice. Finally the filtrate were pooled and then dried by vacuum evaporation, until dry powder was obtained. The weight of the dry powder was checked and dissolved in 5ml methanol (HPLC grade, Merk, Germany) for quantification of St and Rb concentration.

LC-MS Analysis

The LC-MS work was carried out using a triple quadrupole LC MS spectrometer, Agilent 6410 (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source, at CSIR IIIM Labs at Jammu, India. Standard stevioside and rebaudioside-A (1mg/ 1ml methanol) were utilized for the preparation of five point calibration curve for both the compounds.

The LC was carried out on an Agilent 1260 infinity, equipped with a quaternary pump, online degasser, column heater, autosampler. A Chromolith High Resolution RP-$_{18e}$ column (Merck, Germany; id 100 x 4.6 mm) was used and the column temperature was maintained at 30^0C.

The mobile phase consisted of water (A) and acetonitrile (B). In the gradient programme following proportions of acetonitrile was applied; *t* (min), % acetonitrile: (15, 40), (20, 70), (22, 10), (25, 10), (3 min, time needed to reach initial conditions). Flow rate was 500 µl/min and injection volume was 5µl. Prepared callus sample solution injected directly, without pre-treatment, under the optimum condition mentioned earlier.

Full scan data was acquired by scanning from *m*/z 100 to 1000 in profile mode, using a cycle time of 4.94s, with a step size of 0.1 µs. Both the quadrupoles (Q1 and Q3) were operated at unit resolution. The ESI source in positive mode was chosen for the detection of stevioside and negative for rebaudioside-A with following operating conditions: Source and Mode; ESI, Polarity; positive (+/-), Collision gas; nitrogen, Capillary voltage; ±4000 V, Ion source temperature; 300 ^{o}C, Gas flow; 7 L/min, Dwell time; 200 ms, Resolution (Q1 and Q3); unit.

The spectra generated at 250V fragmentor voltage for stevioside and rebaudioside-A in positive and negative ion detection mode, gave the protonated molecule $[M+H]^+$ 827.4 and $[M-H]^-$, 965.4, respectively. Prepared callus sample solution injected directly, without pre-treatment, under the optimum conditions mentioned earlier.

RESULTS

The LC –MS analysis of standard stevioside and rebaudioside-A indicated the retention time of standard compounds as 14.2 and 14.3 minutes respectively (Figure 1). The calibration equation of stevioside and rebaudioside-A were obtained by plotting LC-MS peak area (y) versus the concentration (x, ng/mL) as y=1.630416x+8.547654 (R^2=0.9998) and y=5.285885x+16.206396 (R^2=0.998), respectively.

In vitro studies

Creamish to greenish, globular, fragile callus was found to be induced from the cut ends of the leaf explants on all the 64 media tested (Figure 2) with 10.2 to 92.5 % callus induction frequency (Figure. 3 and 4).

Effect of growth regulators

On the basis of callus induction frequency, callus initiated on MS medium with six single concentrations and two combination (1.0mg/l BAP+1.0mg/l 2,4-D and 1.0mg/l BAP+1.0mg/l IAA) of growth regulators was subcultured onto the same medium and maintained under dark conditions for one month, and was processed for stevioside and rebaudioside-A content by LC MS method.

The data (Table 1), showed 0.46 to 2.89 µg/gdc (gram dry callus) St and 0.33 to 1.89 µg/gdc Rb production in one month old cultures. Callus grown on BAP and 2,4-D (1.0mg/l) containing medium under dark conditions accumulated maximum level of St and Rb, while Kn supplemented medium recorded least amount (Table 1).

Effect of light

A comparitive study on the effect of light on St and Rb accumulation was conducted by culturing callus on MS + 1.0 mg/l BAP and 1.0 mg/l 2,4-D + 3% sucrose medium and maintaining callus cultures under complete dark conditions and as well as under 12 hours light period. Callus exposed to 12 hrs light showed a significant increase in the accumulation of both steviol glycosides (Figure 5), compared to the dark conditions. On exposure to light, the st levels elevated from 7.65 to 132.9µg/gdc and Rb level from 3.6 to 148.7 µg/gdc (Figure 5).

Effect of Carbohydrates

Five carbohydarates; sucrose, glucose, fractose, dextrose and maltose were tested for their effect on glycosides accumulation under light conditions. LC-MS analysis of callus extracts (Figure 6), indicated 132.9 ug/gdc St and 148.7 ug/gdc Rb production in callus raised on sucrose supplimented medium. Glucose supplimented medium accumulated highest levels of both the glycosides 233.1 ug/gdc (St) and 186.6 µg/gdc (Rb). While maltose resulted least accumulation (Figure 6). In general, callus cultures grown on glucose, fructose and maltose supplimented media showed more accumulation of stevioside and while on sucrose and dextrose, rebaudioside –A accumulation was more.

Effect of nitrate concentration

The data recorded on the effect of nitrates, given in the Figure 7 indicate that increase in the nitrate concentration in MS medium exhibited drastic inhibition in the St and Rb accumulation in callus cultures. Normal levels (MS medium; 1650 mg/l NH_4NO_3 and 1900mg/l KNO_3) appeared to support the glycosides accumulation whereas doubling the concentration of nitrates in MS medium, has drastically deduced the accumulation of St and Rb levels in callus cultures to 7.95 and 3.45 ug/gdc (Figure 7).

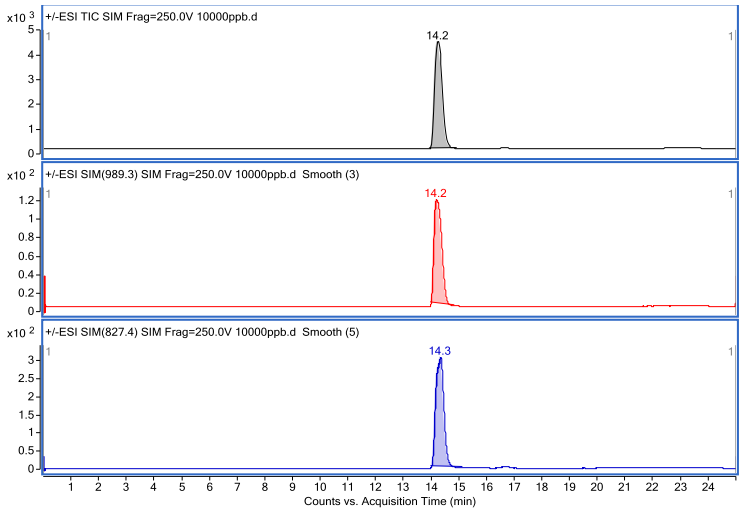

Figure 1: TIC (Total Ion Chromatogram) of standard mixture of stevioside and Rebaudioside (A) and EIC (Extracted Chromatogram of Stevioside (B) and Rebaudioside (C))

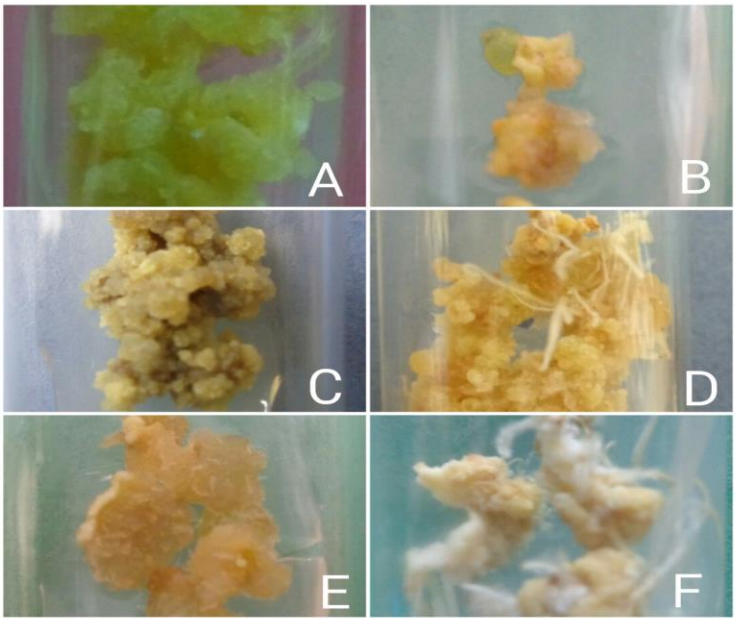

Figure 2: Callus regenerate from leaf explants of *Stevia rebaudiana* on MS medium supplemented with different growth regulators (A) BAP; (B) Kn; (C) 2,4-D; (D) IAA; (E) IBA and (F) NAA.

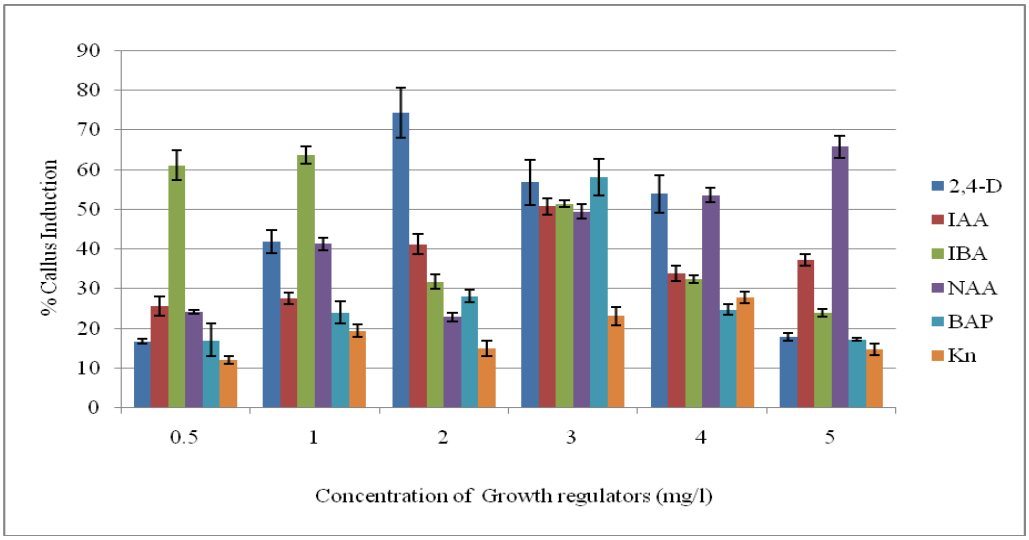

Figure 3: Percentage callus induction on MS medium supplemented with different concentration of growth regulators.

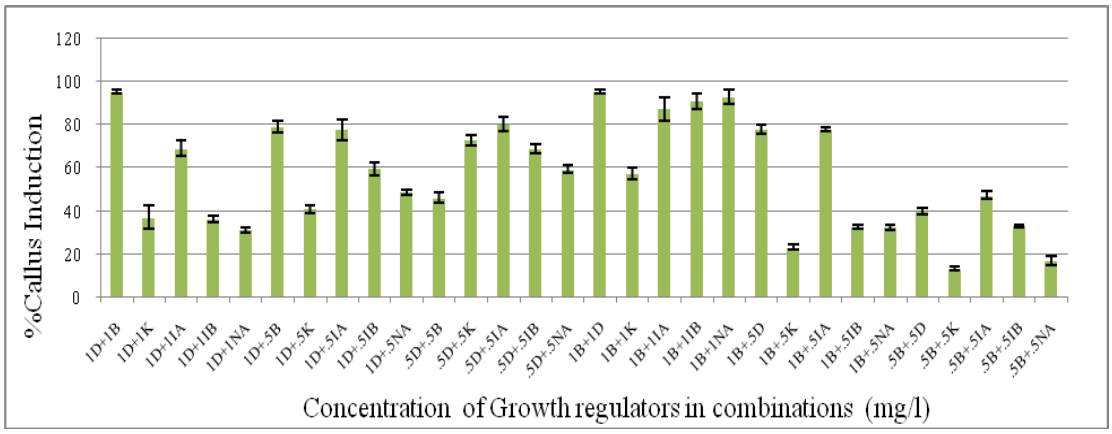

Figure 4: Percentage callus induction in MS medium supplemented with combination of two growth regulators.

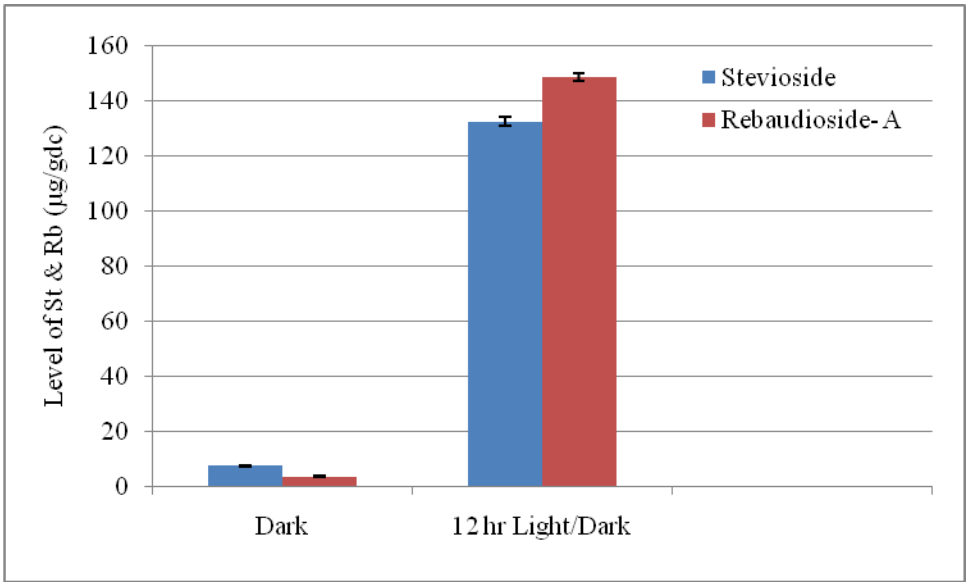

Figure 5: Effect of light on the accumulation of Stevioside and Rebaudioside-A in callus culture grown on MS+ 1.0mg/l 2,4-D + 1.0mg/l BAP + 3% sucrose medium.

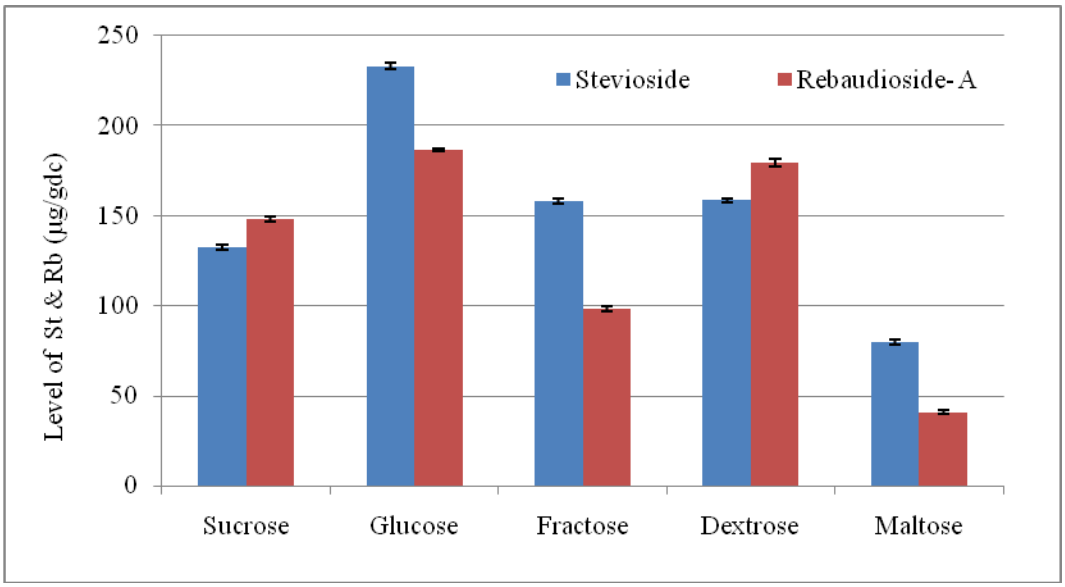

Figure-6: Stevioside and Rebaudioside-A production in callus cultures grown on MS medium supplemented with 3 % (w/v) of different sugars.

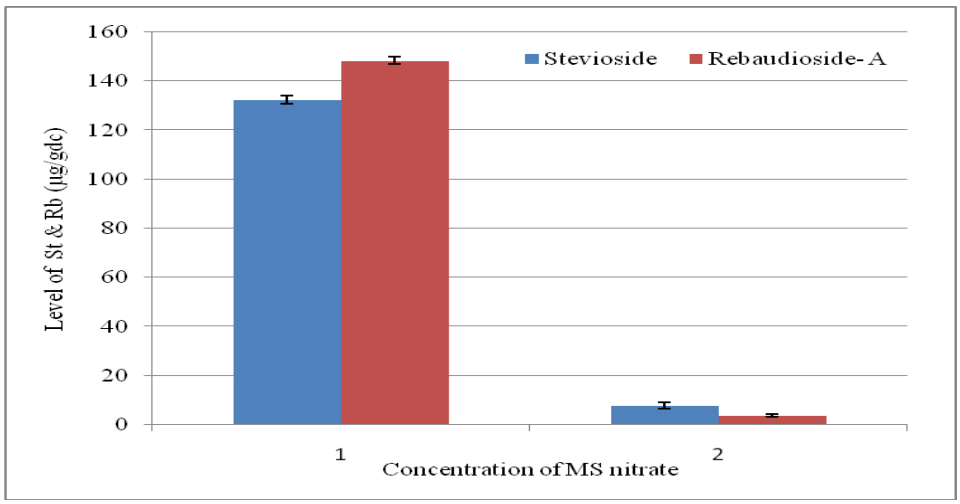

Figure 7: Effect of nitrate level on the accumulation Stevioside and Rebaudioside-A in callus cultures grown on 1- MS nitrate concentration and 2- double MS nitrate concentration.

Table-1: Concentration of stevioside and rebaudioside-A (ug/gdc) in callus cultures grown on MS medium supplemented with different growth regulators.

Name of callus	Stevioside (µg/gdc)	Rebaudioside-A (µg/gdc)
BAP	2.89±0.12	1.25±0.10
Kn	0.46±0.01	0.33±0.02
2,4-D	1.49±0.07	1.89±0.06
IAA	1.24±0.06	0.61±0.01
IBA	0.60±0.03	0.38±0.02
NAA	2.27±0.04	1.56±0.07
1.0 BAP+1.0 2,4-D	1.53±0.05	0.72±0.04
1.0 BAP+1.0 IAA	2.71±0.07	1.25±0.05

DISCUSSION

In natural conditions, SGs concentration in leaf tissue is dependent upon the Germplasm used and cultivation conditions like season, soil, temperature, humidity, irrigation, photo period and light intensity, even on the number and size of leaf (Madan et al., 2010). This leads to lack of consistency in the quantity and quality of the products. *in vitro* cultures are better option for secondary metabolites production.

Most of the tissue culture works reported on *Stevia rebaudiana* have been focused on plant regeneration alone. Influence of growth regulators on organogenesis (Abdul et al., 2014; Patil 2013; Shende et al., 2013; Wu et al., 2014) and micropropagation (Chotikadachanarong et al., 2013; Gauchan et al., 2014; Kumar et al., 2014; Tamura et al., 1984) are prominent in literature. However, studies on the production of SGs in callus cultures and influence of various physicochemical parameters are limited (Fu et al. 2015; Gupta et al., 2014 and 2015; Ibrahim et al., 2008; Lata et al., 2013; Singh et al., 2014; Upadhyay et al., 2013).

In present study on influence of GRs on callus induction, auxins were better compared to cytokinins and 2.0mg/l of 2,4-D was best among the 36 single concentrations tested. The combination of 2, 4- D with BAP (1.0mg/l each) showed significantly superior performance in callus induction over all the single and combination treatments tested (Figure 3 and 4).

Photoperiod is one of the important parameter in plant growth. Our LC-MS studies shows that the callus growth under 12hrs photoperiod enhanced St and Rb levels very significantly as compared to callus maintained in dark. Approximately 14 -17 fold elevation in the levels of both St and Rb was recorded in one month old callus cultures (Figure 5). Gupta et al. (2010) reported St accumulation in callus grown in photoperiod. Sivaram and Mukundan (2003) also reported highest levels of stevio glycosides accumulation in two month old callus cultures grown on BAP and IBA supplemented medium. However, no studies on the effect of light on St and Rb accumulation in callus cultures. Further, replacement of disaccharide (sucrose) with monosaccharide supported the accumulation of glycosides. This probably may be due to free availability of monosaccharide molecule for glycosylation of steviol ring. Doubling of nitrates in basal medium drastically inhibited production of St and Rb (Figure 7), indicate that the nitrate composition and concentrations in MS basal medium suit best for the *in vitro* production of glycosides in callus cultures.

CONCLUSION

In conclusion, the analysis of stevioside and Rebaudioside A in callus cultures by LC-MS method have shown that MS medium with 2,4-D and BAP (1.0mg/l) and glucose or dextrose (3%), exposure of callus cultures to light for 12hrs photoperiod has promoted accumulation of both stevioside and rebaudioside A in one month old leaf derived callus cultures.

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