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Research Article

Impact of *Stevia rebaudiana* Culturing in Liquid Medium: Elevation of Yield and Biomass, Mitigation of Steviol Glycosides

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Abstract: An efficient micropropagation system is developed by culturing nodal segments containing the axillary shoot buds on liquid Murashige and Skoog (MS) medium. Direct shoot and root formation are highly effective in both solid and liquid MS media without any plant growth regulators (PGRs). Interestingly, a significant difference in yield is obtained between solid and liquid cultures. It is revealed that a relatively higher amount of plant biomass is obtained after culturing for 4 weeks in a liquid MS medium. However, the shoots produced on solid MS medium produce a remarkable decline in all physiological parameters. On contrary, the bioactive steviol glycosides (rebaudioside-A and stevioside) content is higher in shoots grown in solid MS on a comparative basis, which could be compensated by higher yield. After hardening off, all the regenerants are effectively grown in the field with a negligible loss (<1 %), and steviol glycosides spectra is again obtained by conducting high-performance liquid chromatography (HPLC) analysis after 10 weeks of the plantation. This method has great potential to be applied on large scale in bioreactors.

Keywords: *Stevia rebaudiana*, Micropropagation, Liquid Culturing, Steviol Glycosides

1. INTRODUCTION

Stevia rebaudiana, belonging to the family Asteraceae, has common names viz. sweet leaf or candy leaf [1]. It is a perennial, bushy shrub native to Northeast Paraguay [2]. *Stevia* is of great commercial importance all around the globe because of the presence of steviol glycosides (SGs) in its leaves that play a vital role in reducing high blood pressure, diabetes (Type II), obesity, and issues related to dentistry [3, 4]. This naturally occurring zero-calorie sweetening compound is involved in the regulation of glucose and insulin in the blood which is crucial in maintaining a balanced diet for the well-being of humans [5]. The major steviol glycosides (SGs) responsible for approximately 300 times more sweetness than sucrose include rebaudioside A (Reb-A), stevioside (ST), and rebaudioside C (Reb-C) [6]. SGs are the potential bioactive compounds that have been approved in various countries because of their tremendous activity against microbes, cancer, inflammation, and diabetes [4, 7].

S. rebaudiana is very beneficial to plants both economically and commercially. Conventional methods for *Stevia* propagation involving vegetative propagation through stem cuttings are very slow. Another big hurdle is that *Stevia* seeds are very tiny and poor in germination [8]. The efficacious production of *Stevia* is limited by these factors, therefore, the adoption of modern techniques of biotechnology is the only solution. Tissue culture is one such biotechnological approach having the ability to mass propagate *Stevia* in a minimum period. In this process, true-to-type progeny is produced from a single mother plant [9]. Recently, few abiotic stress elicitors in appropriate

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concentrations have been found very useful in this regard [10-18] that alter the biosynthetic pathway for SGs production [19]. Future challenges involve the formation of plenty of biomass by employing a liquid suspension culture technique for explant cultivation. In this respect, the measurement of physiological parameters of plant growth in liquid culture media is immensely important. On the other hand, seed quality may be difficult to obtain as very often, and there is very low germination frequency, sometimes no germination at all.

Based on our literature survey, since the *Stevia* is a photosensitive plant for the SGs accumulation, only limited studies regarding clonal propagation of S. rebaudiana with their field performance are reported. Therefore, the current study in *S. rebaudiana* aims to establish a feasible and effective tissue culture protocol for increasing the mass proliferation of nodal explants in a liquid suspension medium. Moreover, the focus of our study is a comparison of different physiological parameters and SGs of commercial significance in *in vitro* and *ex vitro* (field) plants after being harvested.

2. MATERIAL AND METHODS

S. rebaudiana seeds were obtained from an agricultural company, Polisan Tarim, Istanbul, Turkey. The washing of seeds was carried out using distilled water and 0.1 % (w/v) mercuric chloride ($HgCl_2$) to get rid of disinfectants. Then the seeds were kept for germination, after which, the leaf nodes were removed to be used as explants to perform *in vitro* experiments. Murashige and Skoog (MS) medium [20] and 3 % (w/v) sucrose was used to prepare the culture medium. The pH of the medium was adjusted to 5.7–5.8 and then 0.8 % (w/v) plant agar was added for solidification. After autoclaving all media, shoot nodes present in culture plates were incubated in a 16 h light: 8 h dark photoperiod at a relative humidity of 55–60 % and 24 ± 1 °C temperature. The stock plants were obtained for further studies.

In the meanwhile, *in vitro* cultivation was also employed using axillary shoot nodes in liquid MS medium without any PGRs. The triplicate experiment was performed and all flasks were kept in a shaking incubator at 24 ± 1 °C in 16 h/8 h photoperiod for 4 weeks. The data regarding the mean length of roots and shoots, mean number of leaves and nodes per explant, and dry and fresh biomass were recorded. Afterward, shoots were shifted to glass jars (Magenta B-cap, Sigma-Aldrich, USA) having medium and maintained in the growth room.

Plantlets with healthy roots were taken out from jars and then placed into plastic trays (12 × 9 cells) having soil: compost: vermiculite: perlite mixture (10:10:1:1 (v/v/v/v)). After acclimatization in portable greenhouse (50 × 70 × 140 cm) conditions provided with cool-white fluorescent light (50 μmol $m^{-1}s^{-1}$ irradiance) for 4 weeks, well-growing plantlets were moved to the field (200 m^2), in Gebze, Turkey to carry out field trials for 10 weeks. Field trials were conducted with 10 plants per m^2 without the application of any fertilizer.

SGs analysis was conducted using leaves from in vitro regenerated plantlets and shoots. Plant material taken out from plates and jars was cleaned with water carefully and dried in the oven for 48 h at 60 °C. Later on, dried plant material was grounded into a fine powder and each sample (20 mg) was taken in 2 mL centrifuge tube. Incubation was done at 55 °C for 15 min in an ultrasonic bath, after which, samples were centrifuged for 10 min at 12,000 rpm and 25 °C in a centrifuge machine. 0.22 μm PTFE Millipore syringe filters were used for filtering the supernatant that was later on transferred to HPLC tubes for analysis.

The standards (rebaudioside A and stevioside) were prepared by dissolving 1 mg of standard glycosides in 30 % of distilled water and 70 % of acetonitrile. Chromatography was performed with an autosampler, a binary pump solvent delivery system, and a dual-wavelength absorbance detector operating at 350 and 210 nm. The column, with 150 × 4.6 mm length and 5 μm particle size was kept warm in a column oven system at 40 °C. Finally, the isocratic flow was performed using acetonitrile and 1 % (w/v) phosphoric acid buffer mixture at a ratio of 68:32 for 20 min.

All micropropagation experiments were performed in triplicate with the randomized design. Statistical analysis of data was done using SPSS software. ANOVA determined the statistical difference and Duncan's multiple range

test calculated the significance of the difference between means ± SE value at a confidence interval of 95 %.

3. RESULTS AND DISCUSSION

Interestingly, all physiological parameters were significantly higher in the liquid medium than the nodal explant regeneration in the solid medium. Under these circumstances, several parameters including the mean length of shoots (compare 8.5 ± 1.1 and 5.0 ± 0.9 cm in green bars of Figure 1), mean length of roots (compare 1. ± 0.5 and 1.1 ± 0.2 cm in blue bars of Fig.1) and mean number of roots (4.2 ± 0.8), nodes (7.44 ± 1.5) and leaves (17.5 ± 2.6) were found to be higher than those produced from a solid medium with a mean of 1.6 ± 0.1 roots, 14.0 ± 0.8 leaves, and a mean of 6.0 ± 0.4 nodes as shown in Figure 2. In this study, it was clearly shown that shoot organogenesis in a liquid medium can prove to be very cost-efficient to produce a large number of plants and to obtain excellent yield in a bioreactor system once established. Similarly, some earlier reports on temporary immersion [21] and bioreactor systems [22] claim the efficiency of liquid culture on a large number of plantlet formations. However, tissue browning during cultivation might be a drawback due to exogenously added growth regulators and that should be overcome by a separate multiplication medium [21]. Long exposure (more than 6 weeks) to the plant tissues in liquid culture might be incorporated with morphological and physiological abnormalities due to the presence of growth regulators [22]. Although some reports resolve using less constant immersion frequencies, well-rooted shoots increased fidelity in field conditions. However, many of these efforts are not available for large-scale production because of the additional passages of callus tissue needed for clonal propagation from microshoots. In the present study, we designed an effective regeneration system with a short regeneration process without callus formation.

Fresh biomass (0.67 g) and dry biomass (0.10 g)

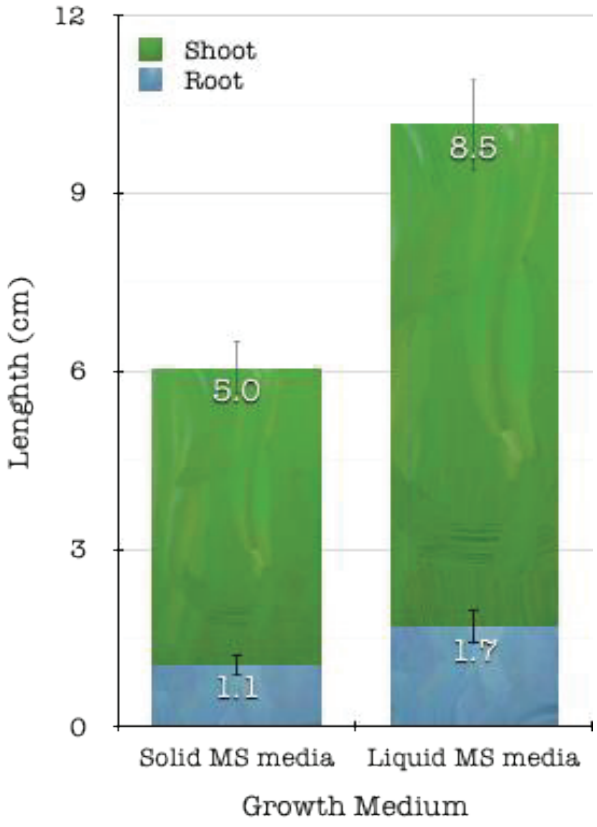

Fig. 1. Comparison of solid and liquid media in terms of shoot and root length for 4 weeks of cultivation. Bar lines represent the standard error of the mean values.

were also higher in MS liquid culturing system after 4 weeks of culturing. In this study, it was clearly shown that shoot organogenesis in a liquid medium can prove to be very cost-efficient to produce a large number of plants and to obtain excellent yield in a bioreactor system once established. Moreover, SGs content was also checked in in vitro plants. It was shown that Reb-A content (3.3 %) was significantly higher in solid MS than in liquid MS (1.4 %) medium. Similarly, ST content was almost doubled in solid MS, i.e., ST content was 0.4 % in leaves of liquid MS, much lower than 0.81 % obtained from the solid MS basal medium depicted in Figure 3. Earlier reports aiming at SGs production through the various cell and shoot cultures with or without elicitor [21, 23, 24] did not provide a reasonable amount for sustainable sweetener compound production unless in vitro raised samples were transferred progressively to ex vitro conditions [25]. Since S. rebaudiana is a photoperiod-sensitive plant, SGs accumulation is directly affected by a long day period (>14 h).

Healthy and vigorous shoots were shifted to the glass jars having MS fortified with or without rooting phytohormones, i.e., 0.25 mg/L IAA [25], and allowed to grow for 4 weeks. The percentage of rooting frequency obtained was approximately 89 %. All regenerants were healthy, therefore, shifted to about 500 jars of 450 ml volume filled with 0.4 dm^3 of soil mixture. The jars covered with the thin transparent film were placed in a growth chamber having 16 h/8 h photoperiod for 4 weeks. After hardening off in the greenhouse, almost all plants (> 98 %) were transferred to the field. Our findings revealed that all the regenerants produced in vitro were showing enough vigor to sustain the environmental conditions of the field and the average shoot length was measured to be ~ 62 cm after 10 weeks of the plantation. Figure 4 illustrates each step of the process in detail.

Table 1 shows that the Reb-A content increased from 2.6 % (w/v) in shoots to 3.7 % (w/w) in rooted-shoots, depending on the growth and development. The highest SGs content was observed after harvesting the leaf materials from the field 7.2 % (w/w). A significant difference was observed regarding ST content of 4 weeks old shoots (0.8 %, w/w) and 8 weeks old regenerants (2.0 %, w/w). Furthermore, in contrary to the rise of Reb-A content, ST content did not change and remained the same in field samples. The ratio of Reb-A and ST was obtained highest (3.6) in field-grown plants followed by 4-weeks old shoots (3.3). The ratio was lowest (1.9) in 4-weeks old

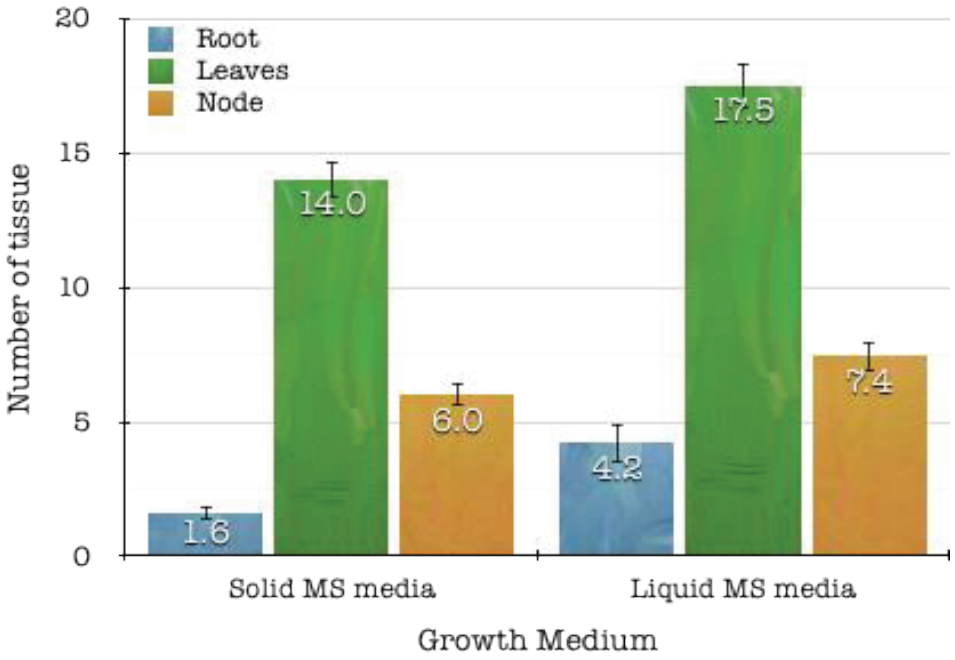

Fig. 2. Comparison of the mean number of roots, leaves, and nodes produced in solid and liquid medium for 4 weeks.

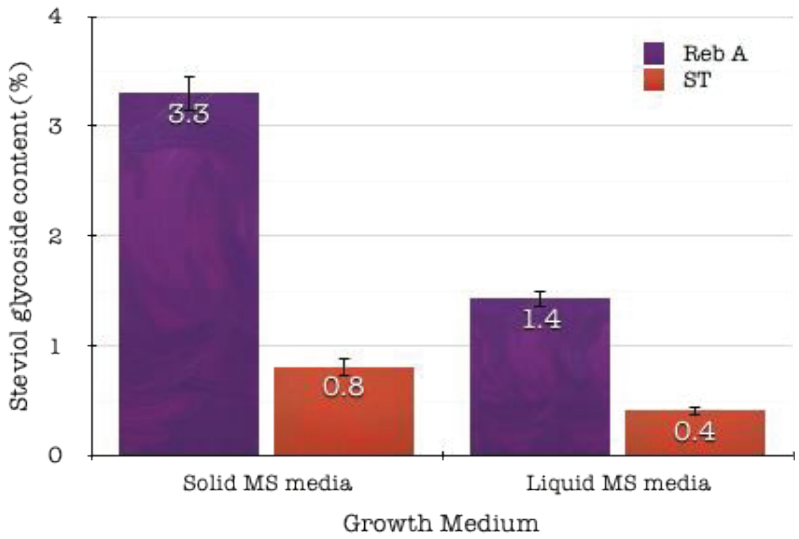

Fig. 3. Comparison of rebaudioside A (Reb-A) and stevioside (ST) content of the leaves produced in solid and liquid MS medium. Note: Bar lines represent the standard error of the mean values.

Fig. 4. Steps of tissue culture experiment conducted in S. rebaudiana from in vitro to ex vitro stages: Formation of shoots using nodes (explants) on solid MS medium for 4 weeks (A), Formation of shoots using nodes (explants) on liquid MS medium for 4 weeks (B), shoot and roots formation from liquid culture (C), root formation on MS medium having 0.25 mg/L IBA or absence of PGR (D), adventitious root formation from the shoots (E), hardening off the regenerants in jars filled with soil under plant growth room conditions (F), the general appearance of the potted regenerants one week before field plantation (G), flowering stage of the regenerants in field conditions after 12 weeks of plantation to the field (H).

Table 1. Comparison of SGs (rebaudioside A and stevioside) in the leaves of 4 weeks-old shoots, regenerants (rooted-shoots), and field samples (±: standard deviation).

Steviol glycosides	Plant Samples (%) DW*		
	Shoots (4-wk old)	Rooted-shoots (4-wk old)	Field samples (10-wk old)
Rebaudioside A	2.6±0.2[c]	3.7±0.3[b]	7.2±0.3[a]
Stevioside	0.8±0.0[b]	2.0±0.1[a]	2.0±0.1[a]
RebA/ST	3.3	1.9	3.6

* Same letter in the rows represents no significant difference at a 95 % confidence interval. Data were obtained from dried leaf materials.

regenerants due to higher ST content in them. The most probable reason might be the involvement of critical enzymatic reactions in the biochemical pathway of stevioside synthesis during root formation [26].

4. CONCLUSION

In conclusion, based on this protocol, about 32,500 healthy plantlets of ~16 cm in length can be produced within 7 months to conduct field trials. Most importantly, a significantly higher yield of *Stevia* plantlets has been obtained in MS liquid culture at a small laboratory scale. Commercial production of more SGs from these plantlets still needs further refinement of this technique by developing a suitable bioreactor system. Once an automation system is established for *Stevia* plants containing value-added SGs, it is noteworthy to mention a cost-efficient production targeting both agricultural practices and refinery systems for the natural sweeteners.

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. DECLARATION

The results of this study are original, the same material is neither published nor under consideration elsewhere, approval of all authors has been obtained, and in case the article is accepted for publication, its copyright will be assigned to the Pakistan Academy of Sciences

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