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# Influence of pH and plant growth regulators on secondary metabolite production and antioxidant activity of *Stevia rebaudiana* (Bert)

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**Abbreviations:**
PGR – plant growth regulators
BA – 6-benzylaminopurine
GA3 – gibberellic acid
IAA – indole-3-acetic acid
IBA – indole-3-butyric acid
DPPH – 1,1-diphenyl-2-picrylhydrazyl

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## Abstract

***Background and purpose:*** *Beside being rich with sweet glycosides, Stevia rebaudiana Bertoni is an interesting source of flavonoids and phenols. The current study aimed to assess the potential for increasing total phenols and flavonoids in S. rebaudiana tissue by varying concentrations of plant growth regulators (PGR) and pH levels of media.*

***Materials and methods:*** *The culture was established from seeds and propagated shoots were cultured on media of different pH levels (4.6, 5.8 and 7.4) and PGR. Total polyphenolics and free radical scavenging capability of leaf, callus and root extracts were determined. Shoot height, root length, shoot and root number were also recorded.*

***Results:*** *Shoot elongation and root development was stimulated by singly applied growth regulators though the values of both parameters were also satisfactory in PGR-free media. In the latter media, pH value of 4.6 was a main factor for increasing leaf metabolite levels. A most significant rise in phenols and flavonoids was evident in response to combination of BA either with GA3 or IAA compared to singly applied regulators indicating synergistic effects of PGR (especially of auxins and cytokinins). However, polyphenolics levels and their distribution between different tissues were also influenced by medium pH value. A positive correlation was found between antioxidant activity of S. rebaudiana extracts and phenols and flavonoids.*

***Conclusions:*** *The results show that PGR and medium pH value strongly affect accumulation of secondary metabolites and can lead to significant enhancement in productivity of bioactive polyphenolics in S. rebaudiana plant cultures.*

## INTRODUCTION

*Stevia rebaudiana* Bertoni is a small perennial shrub of the Asteraceae family, native to South and Central America. *S. rebaudiana* occurs naturally on infertile, acid (pH 4 to 5) soils, but thrives with soil pH as high as 7.5 [1, 2]. The plant is known worldwide principally for its high content of sweet diterpene glycosides, rebaudioside A and stevioside (the most prevalent glycoside) in leaf tissue and is being used as a low-calorie and non-toxic sweetener. Moreover, antimicrobial, antiviral, anticarcinogenic and antioxidant activity of *S. rebaudiana* leaf extracts was demonstrated by a number of studies. *S. rebaudiana* is also a good source of inulin-type carbohydrates, proteins, essential oils, minerals, vitamins as

well as flavonoids and phenolic compounds [3]. Similar to other plant secondary metabolites, polyphenols have roles in protection against infections, attraction of pollinators and seed-dispersing animals, as allelopathic agents and are generally involved in plant defense against ultraviolet radiation. However, these bioactive compounds have attracted much scientific attention due to their beneficial effects on human health, notably in the prevention of various diseases associated with oxidative stress. The antioxidant effects of polyphenols are connected with their free radical scavenging activity, metal chelating ability and inhibition of enzymes involved in the oxidative processes, the properties mostly attributed to the phenolic hydroxyl groups attached to ring structures [4]. The relationship between antioxidant activity of *S. rebaudiana* leaf extracts and their polyphenolic compounds has been demonstrated by several studies [5, 6, 7]. However, propagation of *S. rebaudiana* by seeds results in heterogeneous populations and consequently varying composition including polyphenolics levels. Moreover, sexual reproduction is generally restricted due to the poor seed viability and low germination rate [8, 9]. On the other hand, vegetative propagation is also limited as specific habitat conditions are mandatory to grow the plants in addition to low acclimatization rate in soil [10]. Due to above-mentioned limitations of conventional propagation of *S. rebaudiana* plants, in vitro plant culture seems to be an alternative which offers the possibility to obtain greater quantity of homogeneous populations with enhanced production and uniform levels of polyphenolics.

Metabolite production in tissue culture conditions can be influenced by modulation of culture conditions and medium composition among which PGR act as one of the critical determinants affecting cell growth and differentiation. The effects of different PGR, medium strength and pH value on secondary metabolite accumulation have previously been studied in various plants [11, 12, 13] though limited data exist on the topic regarding *S. rebaudiana* [7, 14]. In the present study, half- rather than full-strength Murashige and Skoog (MS) media was used for the experiments as our preliminary data showed far better germination, growth and development of plantlets in lower salt media. The positive effect of lower strength MS media on growth and secondary metabolite production was observed in several plant species [11,15, 16]. Based on these studies, we hypothesized that the different PGR combinations and different medium pH values may influence the levels of secondary metabolites and their accumulation in different plant tissues of *S. rebaudiana*.

Thus, this study was undertaken to investigate the effects of different combinations and concentrations of auxins, gibberellins and cytokinins and three medium pH levels (4.6, 5.8 and 7.4) on the production and distribution of phenols and flavonoids in micropropagated *S. rebaudiana*. With an objective to elucidate the antioxidant potency, we evaluated a relationship between measured metabolites and radical scavenging activity estimated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of leaf, callus and root methanolic extracts. We have also determined the germination percentage and morphogenetic response under experimental conditions.

## MATERIALS AND METHODS

### Plant material and germination media

*S. rebaudiana* seeds were obtained from the commercial seed source company Stevia-Paraguay, Paraguay (PY). The seeds were stratified for a month at 4 °C, surface sterilized by washing with 70% ethanol for 30 s followed by 2% (w/v) water solution of chlorine preparation Izosan-G (99% sodium dichloroisocyanurate dihydrate) with few drops of Tween 20 for 5 min and thoroughly washed in sterile distilled water. Following sterilization, the seeds (one seed per experimental tube) were germinated in half-strength MS media at three pH levels (4.6, 5.8 and 7.4) supplemented with 0.1g $L^{-1}$ myo-inositol, 0.1 mg $L^{-1}$ thiamine×HCl, 0.5 mg $L^{-1}$ pyridoxine×HCl, 0.5 mg $L^{-1}$ nicotinic acid, 30 g $L^{-1}$ sucrose, 8g $L^{-1}$ agar (basal media). Regarding growth regulators, germinating media were supplemented with 1.0 mg $L^{-1}$ of gibberellic acid (GA3) or combination of 0.5 mg $L^{-1}$ GA3 and 1.0 mg $L^{-1}$ 6-benzylaminopurine (GA3+BA) or contained no PGR (further referred to as control or PGR-free media). The percentage of germination was determined after 8-week growth period. Elongated shoots (one shoot per experimental tube) were transferred to control media pH 5.8 for several 4-week subculture periods. Regenerated shoots (clones) were used as experimental material for subsequent experiments.

### Plant propagation

#### Media with different pH values and growth regulators

Nodal segments (approximately 1cm) of regenerated shoots were aseptically excised and cultured (one segment per experimental tube) on basal media with three pH levels (4.6, 5.8 and 7.4) and following concentrations of PGR: 1.0 mg $L^{-1}$ of GA3, combination of 0.5 mg $L^{-1}$ GA3 and1.0 mg $L^{-1}$ BA (GA3+BA) or combination of 0.5 mg $L^{-1}$ indole-3-acetic acid (IAA) and 1.0 mg $L^{-1}$ BA (IAA+BA). PGR-free media with different pH levels were also prepared. Shoot height, root length, shoot and root number were determined after 4-week growth period.

#### Media with different concentrations of auxins

Nodal segments of regenerated shoots were aseptically excised and cultured (one segment per experimental tube) on basal media with pH value 5.8 and with IAA or indole-3-butyric acid (IBA) in concentrations 0.5, 1.0 or 1.5 mg $L^{-1}$. Shoot height, root length, shoot and root number were determined after 4-week growth period.

### Culture conditions

All in vitro cultures were maintained in a growth chamber at 24 ± 2 °C under a 16:8 h light:dark period of cool fluorescent light (70 µmol $m^{-2}s^{-1}$; TLD 36W/54-765; Philips, Poland).

### Sample preparation and analytical determinations

Following 4-week growth period, leaves, calli and roots of *S. rebaudiana* cultivated on different media were collected separately and dried at 40°C until constant weight (30h). Dry plant material was ground with a mortar and pestle to a fine powder and homogenized with aqueous methanol solution (60% v/v). The samples were then sonicated (35 kHz, 360 W; Elma, Germany) for 1h at 60°C and afterwards centrifuged (3K18 centrifuge Sigma-Aldrich) for 10 mins at 15 000 µg and 4°C. The supernatant was used for secondary metabolites and radical scavenging activity determination.

Total soluble phenols in the shoots, callus and root extracts of *S. rebaudiana* were determined with Folin–Ciocalteau reagent (Sigma-Aldrich) according to Zhishen et al. [17] using gallic acid (Sigma-Aldrich) as a standard. Supernatant (20 µl) was diluted with distilled water after which 100 µL of Folin-Ciocalteau reagent was added. Afterwards, 300 µL 1,88M $Na_2CO_3$ was added and the mixture was incubated for 45 minutes at 85°C. The absorbance of the mixture was measured at 765 nm using a UV-visible spectrophotometer (Specord 40, Analytic Jena, Germany). Total phenolics content was expressed as mg of gallic acid (GAE) per gram of dry weight (DW).

Total flavonoid content in the shoots and callus extract of *S. rebaudiana* were determined with $AlCl_3$ according to the method described by Zhishen et al. [17] using quercetin (Sigma-Aldrich) as a standard. Supernatant (100 µl) was diluted with distilled water and 60 µL 5% $NaNO_2$ was added. After 5 min of incubation, 60 µL 10% $AlCl_3$ was added and mixture was incubated at room temperature for 6 mins, and finally 400 µL 1M NaOH was added in the mixture. The absorbance of reaction mixture was measured at 415 nm. Total flavonoids content was expressed as mg of quercetin (QUE) per gram of DW.

DPPH free radical scavenging activity was determined according to Germano et al. [18]. The extracts of stevia leaves, calli and roots were evaluated in terms of their radical scavenging ability using DPPH radical. *S. rebaudiana* extracts (200 µl) was mixed with 800 µl freshly prepared 0.2 mM metanolic DPPH solution (Sigma-Aldrich) and left in the dark for 30 mins incubation at 25°C. The decrease in absorbance was measured at 517 nm. Gallic and ascorbic acid (Sigma-Aldrich) were used as standard antioxidants. DPPH radical scavenging activity was expressed as the inhibition percentage of free radical by the extract.

## Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) for comparison of means, and significant differences were calculated according to Duncan's multiple range test at the 5% level. Correlation coefficients between measured parameters were recorded. All statistical analyses were performed using the STATISTICA 12.0 (StatSoft, Inc., USA) software package. All the experiments were independently carried out two times, each time with 24 replicates for germination, 12 replicates for growth parameters or with four replicates for analytical determinations.

## RESULTS AND DISCUSSION

### Effects of growth regulators and medium pH on germination

Following eight weeks, the percentage of germinated seeds on control media was 16.7 at pH 5.8 and 7.4 or non-existent at pH 4.6. Germination values on media with 1.0 mg $L^{-1}$ GA3 increased with an increase in pH – the percentages were 14.6, 35.4 and 39.6 at pH 4.6, 5.8 and 7.4, respectively. The highest germination percentage (52.1%) was obtained on GA3+BA media at pH 5.8. Germination percentages on the same media at pH 4.6 and 7.4 were 20.1 and 12.5, respectively. Obtained results (less than 50% of germinated seeds on majority of tested media) are in accordance with most available data [2, 3,19]. Regarding growth regulators and pH value, there is little data on their effects on *S. rebaudiana* germination. Recent research reported relation between germination percentages and origin of *S. rebaudiana* seeds [20]. The authors germinated seeds on GA3 (0.4 mg $L^{-1}$) media at pH 5.8 and obtained 30 and 55% germination values from seeds originated from Paraguay and United States, respectively. Gibberellins promoted seed germination in a number of plant species [21, 22], though treatment with cytokinins was also reported to break seed dormancy in some plant species [23, 24]. Here, the maximum germination was obtained by combination of GA3 and BA. Stimulative effects of jointly applied GA3 and cytokinins on germination were also observed in celery seeds [25]. The authors implied that exogenous cytokinins (when applied together with GA's) increased the uptake of gibberellins by the seeds.

### Effects of growth regulators and medium pH on plant growth

Several studies reporting *S. rebaudiana* micropropagation recorded best shoot multiplication (shoot number per explant) on full-strength MS medium supplemented with cytokinins (BA or kinetin) though newly formed microshoots were usually thin and in some cases showed hyperhydric symptoms [9, 26, 27, 28, 29]. Consequently, such microshoots were not suitable for multiplication through

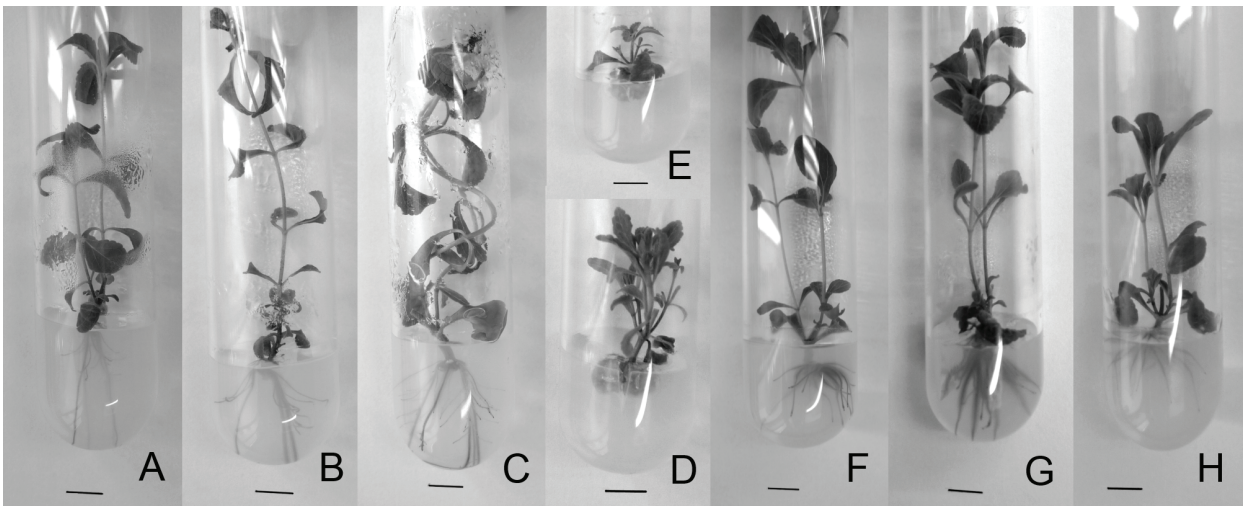


**Fig 1.** *Micropropagated S. rebaudiana plants.* **a** *Control (hormone-free) medium pH 4.6,* **b** *control medium pH 5.8,* **c** *medium at pH 5.8 level supplemented with 0.5 mg L$^{-1}$ GA3,* **d** *combination of 0.5 mg L$^{-1}$ GA3 and 1.0 mg L$^{-1}$ BA media at pH level 7.4,* **e** *combination of 0.5 mg L$^{-1}$ IAA and 1.0 mg L$^{-1}$ BA media at pH level 5.8,* **f** *medium at pH 5.8 level supplemented with 1.0 mg L$^{-1}$ IAA,* **g** *medium at pH 5.8 level supplemented with 1.0 mg L$^{-1}$ IBA,* **h** *medium at pH 5.8 level supplemented with 1.5 mg L$^{-1}$ IBA. Bar = 1 cm.*

several subcultures or were poorly acclimatized for outdoor growth [28, 30]. A few reports noted the beneficial effects of PGR-free MS media with full or half concentrations of macroelements on *S. rebaudiana* shoot growth and/or root induction [10, 29, 31, 32]. In our preliminary studies, full-strength MS media (irrespective of either media pH value or presence of growth regulators) induced development of smaller number of microshoots (approximately 1cm) which rooted sporadically (data not shown). However, when nodal segments were cultured on half-strength control media (irrespective of media pH value), axillary buds appeared in a few days and developed in morphologically normal shoots which easily rooted (Fig. 1a, b). With respect to growth parameters, medium pH only affected shoot height; the parameter was significantly increased at pH 5.8 and 7.4 levels compared to values obtained at pH 4.6 (Table 1). Addition of GA3 to control media caused further elongation of *S. rebaudiana* shoots which was again more pronounced at 5.8 (Fig. 1c) and 7.4. Moreover, GA3 media with higher pH levels (5.8 and 7.4) stimulated both root length and number in comparison with the parameters values obtained at GA3 media with pH 4.6 or at control media. The plant hormone also induced formation of small callus at the segment base. Similar effects of GA3 on *S. rebaudiana* shoot elongation and root growth were also noted by Verma et al. [30] though in our case the effect of the plant regulator was more pronounced with respect to shoot height. GA3 is known to promote, among other important biochemical and morphogenetic responses, stem and leaf elongation [33]. When BA was applied together with either GA3 or IAA, the formation of roots and elongation of shoots was inhibited and the callus growth strongly stimulated, the effects being especially noticeable in combination with IAA (Table 1, Fig. 1e). The latter treatment (BA+IAA media) failed to induce more than two shoots per nodal segment; in addition, the newly formed shoots were reduced to microshoots regardless of pH value. On the other hand, BA+GA3 combination at pH 5.8 and 7.4 levels induced around four shoots per explants and those showed normal morphology (Table 1, Fig. 1d).

Inhibition of shoot elongation and root formation in the case of BA+GA3 combination might be explained by reciprocal interactions between the two hormones on those processes; it has been shown that cytokinin inhibits the production of GA and promotes its deactivation and GA inhibits cytokinin responses [34, 35]. Regarding auxin-cytokinin interaction on root growth, it was found that cytokinins prevent the formation of an auxin gradient required for lateral root initiation [36]. The development of *S. rebaudiana* microshoots and the callus tissue upon simultaneous application of BA and IAA was also evident in the study of Sivaram and Mukundan [9] though a much higher number of shoots per explants (10 shoots/ nodal segment) was recorded than in our study. The reasons for the discrepancy might lie in the facts that authors used twofold higher concentration of IAA (1.0 mg L$^{-1}$) and BA (2.0 mg L$^{-1}$) and scored data after longer growth period. In recent study on *S. rebaudiana* growth, combination of 1.0 mg L$^{-1}$ BA and 0.1 mg L$^{-1}$ IAA produced 3.7 shoots per nodal segment [20] which is similar to our results in the case of BA+GA3 combination (pH 5.8 and 7.4) or singly applied IAA (1.5 mg L$^{-1}$) and IBA (0.5 mg L$^{-1}$) (Table 1, Table 2).

With respect to the effects of medium pH values on *S. rebaudiana* growth (namely shoot height), it was evident that neutral or slightly alkaline media were superior in

**Table 1.** *Shoot height (cm), root length (cm), shoot and root number in media with different growth regulators (mg L-1) or without them (control) at three pH levels after 4-week growth period.*

| Parameter | pH | Control | 1.0 GA3 | 0.5 GA3+1.0 BA | 0.5 IAA+1.0 BA |
|---|---|---|---|---|---|
| Shoot height | 4.6 | 6.2 (0.69) $^{d}$ | 7.4 (0.56) $^{c}$ | 1.8 (0.18) $^{ef}$ | 1.1 (0.08) $^{f}$ |
| | 5.8 | 8.5 (0.53) $^{b}$ | 11.6 (0.73) $^{a}$ | 2.5 (0.21) $^{e}$ | 1.2 (0.07) $^{f}$ |
| | 7.4 | 8.2 (0.61) $^{b}$ | 11.9 (0.69) $^{a}$ | 3.0 (0.28) $^{e}$ | 1.5 (0.08) $^{f}$ |
| Shoots / explant | 4.6 | 2.3 (0.31) $^{b}$ | 2.5 (0.34) $^{b}$ | 2.3 (0.31) $^{b}$ | 2.0 (0.00) $^{b}$ |
| | 5.8 | 2.0 (0.39) $^{b}$ | 2.2 (0.25) $^{b}$ | 3.5 (0.19) $^{a}$ | 2.0 (0.00) $^{b}$ |
| | 7.4 | 1.9 (0.25) $^{b}$ | 2.0 (0.00) $^{b}$ | 4.1 (0.35) $^{a}$ | 2.0 (0.00) $^{b}$ |
| Root length | 4.6 | 2.5 (0.29) $^{d}$ | 2.3 (0.41) $^{d}$ | - | - |
| | 5.8 | 2.9 (0.55) $^{c}$ | 3.2 (0.43) $^{b}$ | - | - |
| | 7.4 | 2.6 (0.41) $^{cd}$ | 3.8 (0.40) $^{a}$ | - | - |
| Roots / explant | 4.6 | 4.5 (0.85) $^{bc}$ | 4.3 (0.74) $^{c}$ | - | - |
| | 5.8 | 5.8 (0.79) $^{b}$ | 9.3 (1.01) $^{a}$ | - | - |
| | 7.4 | 4.7 (0.77) $^{b}$ | 8.5 (0.92) $^{a}$ | - | - |
| Callusing et base | 4.6 | - | + | ++ | +++ |
| | 5.8 | - | + | ++ | +++ |
| | 7.4 | - | + | ++ | +++ |

Values represent mean ± SD (parenthesis) of 12 replicates. Different letters within parameter indicate significant difference at $p < 0.05$. + denotes increase in the amount of callus.

comparison to acidic media which is contrary to some data about *S. rebaudiana* natural occurrence on soils of pH 4 to 5 [1]. Concerning data on *S. rebaudiana* in vitro culture, medium pH was generally set in the range of 5.7–5.8 which is recommended for the majority of plant species [37]. In our study, the difference between the effects of low (4.6) and higher pH (5.8 and 7.4) media was also noticeable with regard to shoot number and root growth on GA3+BA and GA3 media, respectively (Table 1). However, *S. rebaudiana* growth was not significantly affected by pH value when media were supplemented with 0.5 mg $L^{-1}$ IAA or IBA alone (data not shown) but it was affected by PGR concentration (Table 2, Fig. 1f, g, h). Cultivation of nodal segments at media supplemented with auxins resulted in satisfying growth with healthy shoots, maximal (100%) rooting induction and callus growth at the base. The same rooting percentage was achieved also on PGR-free and GA3 media though the morphology of roots was different than on auxin media; those media induced formation of thin and long roots compared to auxin (especially IBA) media which induced formation of shorter and thicker roots. Both IAA and IBA showed similar effect on shoot height, root length and number – the value of the parameters decreased with an increase in auxin concentrations, though the effect of IBA was stronger (Table 2). The same trend of changes was observable with number of

**Table 2.** *Shoot height (cm), root length (cm), shoot and root number in media with different concentrations of auxins (mg $L^{-1}$) or without them (control) after 4-week growth period.*

| | Shoot height | Shoots / explant | Root length | Roots / explant | Callusing at base |
|---|---|---|---|---|---|
| Control | 8.5 (0.53) $^{bc}$ | 2.0 (0.39) $^{c}$ | 2.9 (0.55) $^{c}$ | 5.8 (0.79) $^{c}$ | - |
| IAA 0.5 | 10.1 (1.29) $^{a}$ | 2.8 (0.30) $^{bc}$ | 2.0 (0.28) $^{b}$ | 13.3 (1.48) $^{a}$ | ++ |
| IAA 1.0 | 7.6 (0.79) $^{cd}$ | 2.3 (0.21) $^{c}$ | 1.2 (0.11) $^{c}$ | 9.5 (1.34) $^{b}$ | ++ |
| IAA 1.5 | 7.3 (0.78) $^{cd}$ | 4.0 (0.42) $^{a}$ | 1.2 (0.11) $^{c}$ | 9.2 (1.37) $^{b}$ | ++ |
| IBA 1.5 | 9.4 (1.01) $^{ab}$ | 3.5 (0.43) $^{ab}$ | 2.1 (0.16) $^{b}$ | 12.1 (0.65) $^{a}$ | ++ |
| IBA 1.5 | 6.8 (0.69) $^{d}$ | 2.6 (0.33) $^{bc}$ | 1.0 (0.10) $^{c}$ | 9.1 (1.08) $^{b}$ | ++ |
| IBA 1.5 | 4.1 (0.35) $^{e}$ | 2.0 (0.00) $^{c}$ | 0.9 (0.06) $^{c}$ | 6.0 (0.90) $^{c}$ | ++ |

Values represent mean ± SD (parenthesis) of 12 replicates. Different letters within parameter indicate significant difference at $p < 0.05$. + denotes increase in the amount of callus.

shoots under IBA influence. On the other hand, number of shoots increased (four shoots per explants) at the highest IAA concentration. Our results agree with those of Sivaram and Mukundan [9] who achieved 100% rooting (with 11–12 roots per explants after 30d of culture) of *S. rebaudiana* on half-strength MS medium supplemented with 0.5 or 1 mg $L^{-1}$ IBA. The authors also observed that IBA media acted well as shoot elongation media. Maximal or nearly maximal rooting (90%) and satisfying *S. rebaudiana* shoot growth was also obtained on half-strength MS medium supplemented with 1 mg $L^{-1}$ IAA [10] or with 0.01 mg $L^{-1}$ NAA [31]. The negative effect of increasing IAA or IBA above 0.5 mg $L^{-1}$ on *S. rebaudiana* root growth (but not on shoot height) was reported in the study of Ibrahim et al. [28] whereas, on contrary, some authors noted better rooting and root growth with an increase of IAA or IBA concentration [27, 30].

## Effect of growth regulators and medium pH on total phenolics, flavonoids and DPPH activity

Polyphenolic compounds in *S. rebaudiana* leaves, calli and roots were extracted by using aqueous methanol solution (methanol/water:60/40 v/v). Application of different growth regulators (used either singly or in combination) and medium pH value significantly affected accumulation of total phenolics and total flavonoids and their distribution between different tissues (Table 3, 4; Fig 2).

The total phenolics ranged from 16.2 to 59.8 mg GAE $g^{-1}$ DW in leaves, from 24.6 to 65.8 mg GAE $g^{-1}$ DW in callus and from 15.5 to 30.3 mg GAE $g^{-1}$ DW in roots whereas total flavonoid contents ranged between 16.6 and 62.4 mg QUE $g^{-1}$ DW in leaves, between 28.1 and 70.4 mg QUE $g^{-1}$ DW in callus and between 12.9 and 25.3 mg QUE $g^{-1}$ DW in roots. Regardless of the pH value or growth regulator, total phenols strongly correlated with total flavonoids, especially in the case of leaves and roots (Table 6).

Cultivation of *S. rebaudiana* on PGR-free media and pH set at 4.6 resulted in significant accumulation of phenols (39.7 mg GAE $g^{-1}$ DW) and flavonoids (35.0 mg QUE $g^{-1}$ DW) in leaves and roots compared to respective metabolite contents obtained on media with higher pH values (Table 3, 4). Alongside with *S. rebaudiana* cultivation in vitro, plants were also germinated and grown in soil (data not shown) and their secondary metabolite levels and antioxidant activities of leaf tissue were measured after 4-week growth period. Obtained secondary metabolite contents of plants grown in soil (about 25 mg $g^{-1}$ DW for both phenols and flavonoids) were comparable to those of plants cultivated on PGR-free media with pH 5.8. Similar phenols (24.01–25.3 mg $g^{-1}$ DW) and flavonoids (20.0–23.5 mg $g^{-1}$ DW) levels of *S. rebaudiana* leaves were also reported in research of Muanda et al. [5], Tadhani et al. [7] and Abou-Arab and Abu-Salem [14] although the extracts were prepared by different methods. In the first study leaves were extracted using methanol-water (50/50 v/v) solution in a similar way as in our study whereas in two latter studies leaves were extracted in HCl-methanol solution, then evaporated to dryness and re-extracted with hot water. However, in the study of Shukla et al. [6] aqueous extract of *S. rebaudiana* leaves showed higher total phenolic contents (56.7 mg $g^{-1}$ DW) than in abovementioned studies whereas in the study of Kim et al. [38] leaf aqueous extract exhibited even higher values of phenolics (130.8 mg $g^{-1}$ DW) though with a relatively low flavonoids levels (15.6 mg $g^{-1}$ DW). The influence of different solvents on the secondary metabolite contents of *S. rebaudiana* leaves was demonstrated by Jahan et al. [39]. Dependent on the extract solvent, phenolic contents ranged between 15.3–65.2 mg $g^{-1}$ DW whereas those of flavonoids ranged between 23.6–125.6 mg $g^{-1}$ DW. Nevertheless, apart from particular extraction method or solvent, origin of *S. rebaudiana* seeds cannot be disregarded as a factor contributing to variability of *S. rebaudiana* polyphenolic contents noted in several reports.

Application of GA3 in media with pH 5.8 significantly increased leaf and root secondary metabolite levels (especially those of flavonoids) compared to the levels noted in respective control media, whereas in media with pH 4.6 and 7.6 GA3 was not so effective (Table 3, 4).

Cultivation of *Salvia miltiorrhiza* Bunge hairy roots and *Artemisia annua* shoots in media supplemented with GA3 and pH set at 5.8 also resulted in higher accumulation of secondary metabolites compared to hormone-free-media [40, 41]. The increase of artemisinin and leaf biomass in *A.annua* upon exogenous GA3 was observed in the study Banyai et al. [42]. The authors stated that accumulation of the terpenoid is associated with increased synthesis of key enzymes in the artemisinin biosynthesis pathway due to the GA3 application. Regarding the effect of GA3 on callus tissue, phenolics content decreased with increase of media pH value; the phenolics level of callus tissue in media with the lowest pH value was significantly higher compared to the level of leaf tissue noted in respective control media (Table 3). Accumulation of flavonoids in callus tissue was highest on GA3 media with pH 5.8 (Table 4). The combination of GA3 with BA was much more effective in modulation of leaf secondary metabolite levels; the levels were markedly increased compared to the levels measured in hormone-free and GA3 media with respective pH values (increase by 27, 56 and 109% compared to values at pH 4.6, 5.8 and 7.4, respectively). Regarding phenols and flavonoids of callus tissue on GA3+BA media, the levels increased with an increase of media pH value (Table 3, 4). A most significant rise in leaf secondary metabolite levels was seen on IAA+BA media with pH set at 4.6 whereas the levels dropped with an increase of media pH value. Still, the secondary metabolite contents in the latter media with 5.8 and 7.4 pH levels were higher compared to those measured in PGR-free

**Table 3.** *Total phenols in different tissues of micropropagated S. rebaudiana plants cultivated on media at three pH levels (4.6, 5.8 and 7.4) and supplemented with different concentrations of GA3, GA3+BA or IAA+BA.*

| | pH | Control | 1.0 GA3 | 0.5 GA3+1.0 BA | 0.5 IAA+1.0 BA |
|---|---|---|---|---|---|
| Leaf | 4.6 | 39.7 (2.6) $^{de}$ | 26.7 (3.1) $^{hi}$ | 50.2 (2.6) $^{c}$ | 59.8 (2.1) $^{b}$ |
| | 5.8 | 26.6 (2.8) $^{hi}$ | 33.2 (4.7) $^{fg}$ | 41.5 (4.7) $^{de}$ | 39.8 (3.3) $^{de}$ |
| | 7.4 | 24.2 (3.0) $^{ij}$ | 16.2 (0.3) $^{k}$ | 50.4 (4.2) $^{c}$ | 31.9 (1.1) $^{gh}$ |
| Callus | 4.6 | - | 48.3 (5.2) $^{c}$ | 24.6 (1.7) $^{ij}$ | 38.0 (4.2) $^{de}$ |
| | 5.8 | - | 40.3 (2.9) $^{de}$ | 33.9 (1.2) $^{gh}$ | 65.8 (1.7) $^{a}$ |
| | 7.4 | - | 34.2 (2.5) $^{fg}$ | 51.5 (2.6) $^{c}$ | 63.7 (4.6) $^{a}$ |
| Root | 4.6 | 30.3 (4.0) $^{gh}$ | 20.8 (1.5) $^{jk}$ | - | - |
| | 5.8 | 19.3 (1.1) $^{jk}$ | 28.0 (2.5) $^{hi}$ | - | - |
| | 7.4 | 19.5 (2.5) $^{jk}$ | 15.5 (2.2) $^{k}$ | - | - |

Values represent mean ± SD (parenthesis) of 4 replicates. Different letters indicate significant difference at $p < 0.05$.

**Table 4.** *Total flavonoids in different tissues of micropropagated S. rebaudiana plants cultivated on media at three pH levels (4.6, 5.8 and 7.4) and supplemented with different concentrations of GA3, GA3+BA or IAA+BA.*

| | pH | Control | 1.0 GA3 | 0.5 GA3+1.0 BA | 0.5 IAA+1.0 BA |
|---|---|---|---|---|---|
| Leaf | 4.6 | 35.0 (0.4) $^{ef}$ | 30.3 (3.8) $^{fg}$ | 58.4 (1.5) $^{b}$ | 62.4 (1.3) $^{b}$ |
| | 5.8 | 25.7 (1.2) $^{hi}$ | 46.7 (1.7) $^{d}$ | 46.3 (1.9) $^{d}$ | 38.2 (0.2) $^{ef}$ |
| | 7.4 | 24.8 (1.4) $^{hij}$ | 16.6 (1.0) $^{k}$ | 56.5 (5.5) $^{bc}$ | 27.7 (1.5) $^{gh}$ |
| Callus | 4.6 | - | 30.5 (3.9) $^{fg}$ | 28.1 (2.8) $^{gh}$ | 37.9 (3.7) $^{e}$ |
| | 5.8 | - | 48.8 (0.8) $^{cd}$ | 38.1 (2.2) $^{e}$ | 68.2 (4.0) $^{a}$ |
| | 7.4 | - | 33.7 (3.8) $^{fg}$ | 56.5 (5.2) $^{b}$ | 70.4 (1.9) $^{a}$ |
| Root | 4.6 | 24.3 (2.3) $^{hi}$ | 18.1 (1.6) $^{jk}$ | - | - |
| | 5.8 | 17.5 (0.8) $^{jk}$ | 25.3 (0.7) $^{hi}$ | - | - |
| | 7.4 | 18.0 (2.2) $^{jk}$ | 12.9 (1.6) $^{k}$ | - | - |

Values represent mean ± SD (parenthesis) of 4 replicates. Different letters indicate significant difference at $p < 0.05$.

media with respective pH values. The most significant accumulation of phenols and flavonoids in general was observed in callus tissue on IAA+BA media with pH 5.8 and 7.4 levels (Table 3, 4). Compared to our results, almost two times lower phenolic (33.99–36.0 mg $g^{-1}$ DW) and flavonoid (30.03–32.0 mg $g^{-1}$ DW) contents of *S. rebaudiana* callus tissue induced by combination of auxins and cytokinins has been reported in the studies of Tadhani et al. [7] and Abou-Arab and Abu-Salem [14]. However, in both latter studies different type of auxin (1-napthaleneacetic acid, NAA) as well as different auxin to cytokinin ratio (2.0 mg $L^{-1}$ NAA and 0.3 mg $L^{-1}$ BA) was used to initiate callus growth than in our study (0.5 mg $L^{-1}$ IAA and 1.0 mg $L^{-1}$ BA). These results imply that in vitro production of bioactive secondary metabolites is significantly influenced by the exogenous addition of different types and concentrations of cytokinins and auxins. The influence of auxins alone on *S. rebaudiana* secondary metabolites contents are presented in Fig. 2.

It is evident that both type and concentration of auxin play a significant role in the production of polyphenols. Two lower concentrations of IAA (0.5 and 1.0 mg $L^{-1}$) either did not influence or markedly enhanced leaf phenolics (20% increase compared to control) and flavonoids (30–42% increase compared to control) contents. The highest used IAA concentration inhibited the production of polyphenols in leaves (for more than 60% compared to control) but markedly stimulated their accumulation in callus. The lowest IBA concentration showed the similar effect on leaf polyphenols as the highest IAA concentration. Increasing concentration of IBA led to higher production of leaf secondary metabolites with the highest values noted at 1.5 mg $L^{-1}$ IBA (twofold increase compared to control). The secondary metabolites in *S. rebaudiana* roots showed a lower degree of variability than those in leaf or callus; the most significant effect was seen at 1.0 mg $L^{-1}$ IBA (65–72% increase in root polyphenols compared to control). Comparison of the effects of

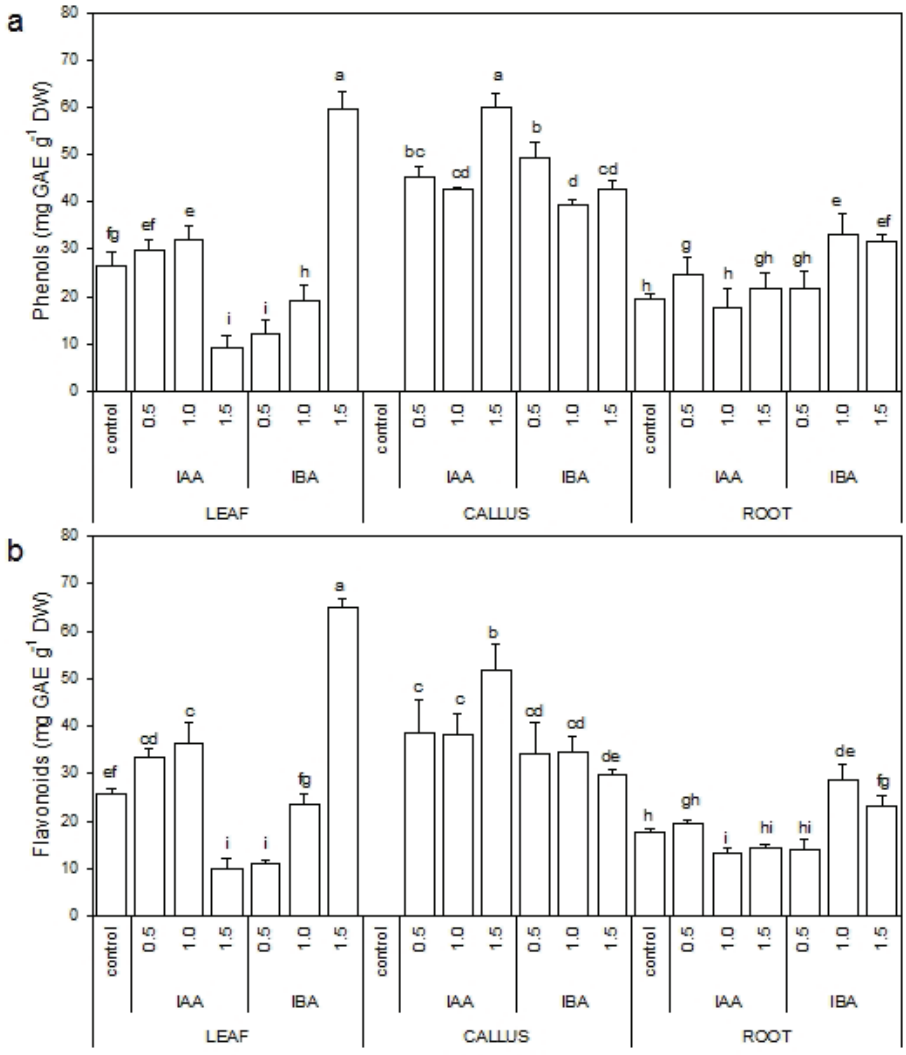


**Fig 2.** **a** *Total phenols and* **b** *total flavonoids contents in different tissues of micropropagated S. rebaudiana plants cultivated on media at 5.8 pH level and supplemented with different concentrations (0.5, 1.0, or 1.5 mg L$^{-1}$) of auxins. Values are mean ± SD based on four replicates. Bars with different letters are significantly different at $p < 0.05$.*

IAA+BA and IAA alone on *S. rebaudiana* polypenols in leaves and callus suggests a possible synergistic interaction of cytokinin with low auxin concentration on accumulation and distribution of recorded secondary metabolites. The interaction was especially apparent in the case of callus tissue as the supplementation of medium with BA and low IAA concentration caused 45% and 77% increase in phenols and flavonoids contents, respectively, when compared to medium containing the lowest IAA concentration alone.

DPPH is a free radical compound and it has been widely used to test the free radical scavenging ability of antioxidant compounds [3]. The antioxidant activity of *S. rebaudiana* extracts and ascorbic and gallic acid as standards was expressed in terms of percent inhibition of DPPH radicals (Table 5).

Among the extracts and standard antioxidants used in the experiment, gallic acid was found to be the strongest one. Nevertheless, in general both leaf (with few exceptions) and especially callus showed very high DPPH radical scavenging activity which was, comparatively, about 10% lower values in roots. Antioxidant activities of *S. rebaudiana* tissues were affected, in some cases significantly, by different plant hormones and medium pH levels. Unlike root, antioxidant activity of leaves (on average 91.4%) was not significantly affected by pH value of control media. Our results on DPPH activity of leaf extracts obtained from plants cultivated on hormone-free media are comparable to those of Muanda et al. [5] though in the study the activity was a few percents higher (96.9). GA3 media with pH levels 4.6 and 7.4 had negative effect on DPPH radical scav-

**Table 5.** *DPPH radical scavenging activity (%) of plants cultivated in different media after 4-week growth period.*

| | | Leaf | | | Callus | | | Root | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH | 4.6 | 5.8 | 7.4 | 4.6 | 5.8 | 7.4 | 4.6 | 5.8 | 7.4 |
| Control | | 92.9 $^{bcd}$ | 90.3 $^{de}$ | 91.1 $^{cd}$ | - | - | - | 90.6 $^{de}$ | 82.7 $^{g}$ | 86.3 $^{f}$ |
| 1.0 GA3 | | 90.7 $^{de}$ | 93.1 $^{abc}$ | 86.5 $^{f}$ | 92.6 $^{bcd}$ | 93.9 $^{abc}$ | 92.2 $^{bcd}$ | 83.7 $^{g}$ | 89.9 $^{de}$ | 77.7 $^{h}$ |
| 0.5 GA3+1.0 BA | | 95.5 $^{ab}$ | 93.4 $^{abc}$ | 95.2 $^{ab}$ | 90.7 $^{de}$ | 92.8 $^{bcd}$ | 94.7 $^{ab}$ | - | - | - |
| 0.5 IAA+1.0 BA | | 95.8 $^{ab}$ | 92.1 $^{bcd}$ | 89.8 $^{e}$ | 92.7 $^{bcd}$ | 96.1 $^{a}$ | 95.8 $^{ab}$ | - | - | - |
| IAA 0.5 | | - | 91.1 $^{bcd}$ | - | - | 92.7 $^{abc}$ | - | - | 87.7 $^{de}$ | - |
| IAA 1.0 | | - | 91.7 $^{bcd}$ | - | - | 92.5 $^{abc}$ | - | - | 81.9 $^{f}$ | - |
| IAA 1.5 | | - | 66.2 $^{h}$ | - | - | 95.2 $^{b}$ | - | - | 83.8 $^{ef}$ | - |
| IBA 0.5 | | - | 76.9 $^{g}$ | - | - | 93.3 $^{abc}$ | - | - | 80.7 $^{fg}$ | - |
| IBA 1.0 | | - | 89.8 $^{cd}$ | - | - | 92.8 $^{abc}$ | - | - | 91.2 $^{bcd}$ | - |
| IBA 1.5 | | - | 96.0 $^{a}$ | - | - | 92.1 $^{bcd}$ | - | - | 90.2 $^{cd}$ | - |
| Ascorbic acid | | 93.1 | | | | | | | | |
| Gallic acid | | 96.6 | | | | | | | | |

Values are means of 4 replicates. SD were less than 10% of the averages. Different letters indicate significant difference at $p < 0.05$.

**Table 6.** *Correlation coeficients (R) between polyphenols and DPPH in different media.*

| Media – different pH and growth regulators | | | | | | |
|---|---|---|---|---|---|---|
| | Leaf | | Callus | | Root | |
| | Flavonoids | DPPH | Flavonoids | DPPH | Flavonoids | DPPH |
| Phenols | 0.91** | 0.87** | 0.85* | 0.87** | 0.95** | 0.86** |
| Flavonoids | | 0.92** | | 0.95** | | 0.93** |
| **Media – auxins** | | | | | | |
| | Leaf | | Callus | | Root | |
| | Flavonoids | DPPH | Flavonoids | DPPH | Flavonoids | DPPH |
| Phenols | 0.99** | 0.80* | 0.89* | 0.90** | 0.90** | 0.86** |
| Flavonoids | | 0.82* | | 0.91** | | 0.91** |

Significance level at *$p < 0.05$, **$p < 0.01$

enging activity of leaves and roots while those activities were slightly stimulated by concurrent application of GA3 and BA. Regarding the effects of IAA and BA combination as well as auxins alone on antioxidant activity of leaf and callus, similar trend of changes was noted as in the case of secondary metabolite levels. The highest values of DPPH radical inhibition were recorded in callus (96.1%) and leaf (96.0%) extracts obtained from plants cultivated on media supplemented with IAA+BA and 1.5 mg $L^{-1}$ IBA, respectively. Conversely to IBA, the highest concentration of IAA caused the greatest reduction in antioxidant activity of leaf extracts. As in several investigations on *S. rebaudiana* antioxidant activity [5, 6, 7], significant correlations between the contents of total phenolics or flavonoids and DPPH radical scavenging activity were found in our study as well.

The correlation was however stronger between DPPH antioxidant activity and flavonoids regardless of the type of organ.

The present study demonstrates that application of certain growth regulators and pH value markedly influences in vitro production and distribution of secondary metabolites in *S. rebaudiana*. Taking into account growth and secondary metabolite levels, several conditions seem promising for the production of bioactive compounds in *S. rebaudiana* leaves and/or callus. Also, *S. rebaudiana* leaves and callus were found to posses strong antioxidant

activity and may be rich sources of antioxidants. Obtained results could be exploited in selection of plant cultures producing a higher amount of valuable polyphenolics compared to in vivo grown plants.

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