Optimization of micronutrients for the improvement of \textit{in vitro} plant regeneration of \textit{Stevia rebaudiana} (Bert.) Bertoni

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Optimization of micronutrients in the MS basal culture medium resulted into enhanced \textit{in vitro} plant regeneration, chlorophyll content and biomass in \textit{Stevia rebaudiana} (Bert.) Bertoni. The shoot bud induction response improved on medium with increased levels of MnSO$_4$ (400 µM), KI (10 µM) and CoCl$_2$ (2 µM). Plant regenerated on higher levels of micronutrients showed significant increase in leaf chlorophyll content. The cumulative effect of all micronutrients at the optimum level in the composite medium also positively influenced the biomass and chlorophyll content of the plantlets.

Keywords: Chlorophyll, clonal plants, micronutrients, micropropagation, \textit{Stevia rebaudiana}

Introduction
\textit{Stevia rebaudiana} (Bert.) Bertoni (Family: Asteraceae), a herbaceous perennial plant, is used as a natural noncaloric sweetener$^1$. It has hypoglycaemic, hypotensive and cardiotonic properties$^1$. Medicinal and other commercial value of \textit{Stevia} led to increased demand for elite germplasm, which could be met through \textit{en mass} production of clonal plants through micropropagation. Micropropagation of \textit{S. rebaudiana} using shoot tip, nodal segments or leaf cultures has earlier been reported$^{2-5}$. Micronutrient optimization in the basal culture medium has been reported to improve plant regeneration in many dicot and monocot plants, such as, \textit{Paspalum, Eleucine, Jatropha, Withania} and \textit{Terminalia}$^6$-$^9$. Use of higher amount of copper in \textit{Stevia} cultures has earlier been reported to improve regeneration, biomass and the leaf chlorophyll content.$^2$ In the present study, authors have explored the optimization of other micronutrients in the culture medium in order to enhance the regeneration of \textit{Stevia} plants.

Material and Methods

Plant Material
\textit{S. rebaudiana} plants (var. SRB123) were procured from Sun fruit Pvt. Ltd., Pune, India. Young nodal segments taken from the field grown plants were used as explants, while nodal explants were taken from regenerated shoots.

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Explant Culture
The nodal explants were washed in tap water and gently rinsed with 20% (v/v) Extran (Merck, India). They were surface sterilized in 5% sodium hypochlorite solution for 10 min and then rinsed 5 times with sterile distilled water. Murashige and Skoog medium$^{10}$ was prepared with 3% (w/v) sucrose and solidified with 0.8-0.9% agar (Qualigens, bacteriological grade). The pH of the medium was adjusted to 5.8 before autoclaving at 121.8°C and 1.2-1.3 kg/cm$^2$ pressure for 20 min. Nodal explants were cut to 1 cm length and placed on the primary culture medium (PCM) consisting of MS+BAP (2.2 µM)+IAA (2.8 µM). All the cultures were incubated in a growth chamber at 26°C and under 16 h photoperiod with 25 µmol m$^{-2}$ s$^{-1}$ light intensity, provided by white fluorescent tubes as previously reported by the authors$^2$.

Shoot Bud Proliferation
Shoot buds induced on nodal explants on the PCM were excised from the base along with some portion of the mother tissue and placed on the second stage proliferation medium [SPM: MS+BAP (3.5 µM)+Kn (1.8 µM)] as reported earlier$^2$.

Micronutrient Manipulations in Culture Medium
Concentration of the micronutrients, viz., cobalt, iron, boron, manganese, potassium iodide and copper (Co, Fe, B, Mn, KI & Cu) used in MS medium were varied individually in the PCM as well as in the SPM to optimize the level of individual micronutrient for best response towards induction of shoot buds.
from the nodal segments, biomass content of the cultures and chlorophyll content in regenerated plants. Different levels of micronutrients used in the PCM were (100-600 μM) Mn, (0.1-5 μM) Co, (100-600 μM) Fe, (100-600 μM) B, (0.1-5 μM) Cu and (5-30 μM) KI. Similarly, micronutrient concentrations were also varied in the second stage proliferation medium SPM.

**Composite Medium**

The optimized level of all the micronutrients was added together to constitute the composite medium (CM) and influence of the CM on shoot bud induction, biomass and chlorophyll content was investigated.

**Chlorophyll Estimation**

The chlorophyll (Chl) content was determined by extraction of pigments with 80% acetone. Fresh leaves (1 g) were ground in small volumes of acetone and the extract obtained was diluted to a final volume of 4 cm³. Absorbance at 662 nm (Chl a) and 645 nm (Chl b) was measured with spectrophotometer (Shimadzu, India).

**Statistical Analysis and Experimental Design**

Each treatment consisted of 5 replicates. One-way analysis of variance (ANOVA) was applied in order to evaluate the effect of different concentrations of micronutrients. Statistical analysis was conducted by Fisher’s least significant difference (P=0.05).

**Results**

**Effect of Micronutrients on Shoot Bud Induction**

Induction of multiple shoot buds occurred directly from the nodal segments cultured on the PCM. Higher level of micronutrients in the PCM influenced the shoot bud induction and increased levels of MnSO₄ (400 μM), CoCl₂ (2 μM) and KI (10 μM) resulted into increased number of shoot buds as compared to control (Figs 1 & 2 a-c). However, micronutrients Fe, Zn and B did not have any influence on the induction of shoot buds at their increased or decreased levels in the medium. On the composite medium, the shoot buds induced from the nodal segments were much elongated with broad dark green leaves as compared to leaves of plants regenerated on control PCM, while no considerable increase in shoot bud number per nodal explant was observed in composite medium as compared to control PCM (Fig. 2d).

**Effect of Micronutrients on Chlorophyll and Biomass**

The chlorophyll content of the leaves regenerated on the medium with optimum levels of micronutrients [MnSO₄ (300 μM), H₃BO₃ (300 μM), Fe₂ (SO₄)₃ (200 μM), ZnSO₄, (57.82 μM) and CoCl₂ (3 μM)] was
higher as compared to control PCM medium (Fig. 3). Similarly, total biomass content of the cultures was also influenced by higher levels of micronutrients in the medium. As a result, increased levels of Fe (SO₄)₃ (200 µM), CoCl₂ (3 µM) and KI (10 µM) yielded better growth in cultures (Fig. 4). It was observed that different concentrations of individual micronutrient were found to be optimum for different parameters (shoot bud induction, chlorophyll and biomass) studied. The regenerated plants on composite medium also had higher content of chlorophyll in the leaves as compared to control MS medium (Fig. 5).

**Effect of Micronutrients on Shoot Bud Proliferation**

Shoot buds induced on PCM were subcultured on SPM. An increase in shoot bud number was observed at CoCl₂ (2 µM), CuSO₄ (1 µM), MnSO₄ (500 µM), ZnSO₄ (89.73 µM) and H₃BO₃ (400 µM) as compared to shoot proliferation in control MS medium (Table 1). The KI level present in control MS medium was found to be optimum for shoot bud proliferation.

**Discussion**

Nutrient levels in the medium have profound effect on callus induction and plant regeneration. Optimization of individual micronutrients in the culture medium have also been reported to result in

![Fig 2 (a-d)—a. Shoot bud induction on control medium MS+BAP (2.2 µM)+IAA (2.8 µM); b. Shoot bud induction on MS+BAP (2.2 µM)+IAA (2.8 µM)+CoCl₂ (2 µM); c. Shoot bud induction on MS+BAP (2.2 µM)+IAA (2.8 µM)+MnSO₄ (400 µM); & d. Effect of composite medium on shoot bud induction.

![Fig. 3—Effect of micronutrients on chlorophyll content of regenerated shoots in *S. rebaudiana*](image)
the improvement of plant regeneration in several monocot and dicotyledonous plants, such as, *Paspalum*, *Eleusine*, *Jatropha*, *Withania*, *Terminalia* and chilli peppers. In addition, the optimized level of micronutrients also improved the growth of tissue cultures resulting in higher level of biomass of the cultures and the regenerated plants had more chlorophyll content in their leaves. These findings are in accordance with the earlier reports on similar studies as the micronutrients are essential component of several enzymes and electron transport proteins.

Although minerals are major component of plant tissue culture media, only a small number of studies have been made on the direct effect of mineral nutrients on growth and morphogenesis in plants. The reports published in the last decade have demonstrated that individual nutrient composition
should be optimized for every plant to obtain best regeneration. The result obtained in the present study fills such void for Stevia cultures. Development of composite media was thought out with the view that the best levels of all individual nutrients would be having synergistic effect on shoot formation, biomass and chlorophyll content but no synergistic effect was observed. The observed positive correlation between development and activity of the photosynthetic apparatus and production of steviol glycoside in Stevia cultures suggests that synthesis of isoprenoids in Stevia chloroplasts is somewhat related to the production of both isoprenoids and steviol glycoside. Micronutrient optimization yielded highly improved in vitro plant regeneration in S. rebaudiana. Optimal micronutrients in the medium positively influenced growth of the cultures and chlorophyll content in the leaves of regenerated plants. Chlorophyll content and leaf biomass are important as steviol glycosides are synthesized in chloroplast and accumulated in leaves. Therefore, the production of both isoprenoids and steviol glycosides in Stevia chloroplast could be stimulated upon optimizing the concentration of micronutrients in the culture medium.

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References