Chloroplast ultra structure, photosynthesis and enzyme activities in regenerated plants of *Stevia rebaudiana* (Bert.) Bertoni as influenced by copper sulphate in the medium

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Received 2 April 2014; revised 5 June 2014

*Stevia rebaudiana* (Bert.) Bertoni is an important medicinal plant used as non-caloric commercial sweetener. Plants regenerated with higher levels of copper sulphate in the medium exhibited enhanced activity of peroxidase and polyphenoloxidase (PPO) enzymes. Transmission electron microscopy (TEM) revealed increase in size and number of electron dense inclusions in the chloroplasts of plants regenerated at optimised level of copper sulphate (0.5µM) in the medium. There was decrease in chlorogenic acid (CGA) content. Chl-a-fluorescence transient pattern (OJIP) showed that the photosynthesis process was more efficient at 0.5µM CuSO$_4$ in the medium.

**Keywords:** Chlorogenic acid, Chl-a-fluorescence transient pattern, Copper sulphate, Photosynthesis, *Stevia rebaudiana*

*Stevia rebaudiana* (Bert.) Bertoni is an economically important source of non-caloric natural sweetener and its extract is widely used in many countries as sugar substitute, making it a major source of high potency sweetener for the growing food market$^{1,2}$. *Stevia rebaudiana*, a Paraguayan perennial herb belongs to family Asteraceae; its leaves accumulate a mixture of eight different glycosides derived from the tetracyclic diterpene steviol$^3$. These products taste intensely sweet, e.g. Rebaudioside-A has been shown to be 320 times sweeter than sucrose on the weight basis$^4$. The accumulation of steviol glycosides in the cells of *S. rebaudiana* largely depends on the development of the chloroplast membrane system and the content of the photosynthetic pigments$^5$. The concentration of glycosides in the leaves increases with delayed flowering, long days and age of leaf tissue$^6$. The composition of tissue culture medium with different concentrations of micronutrients to improves growth and morphogenetic response, chlorophyll content and biomass of the regenerated plants$^7$$^9$. Copper is known to be a vital component of electron transfer reaction mediated by proteins such as superoxide dismutase, cytochrome-c-oxidase and plastocyanin but its concentration in the cells needs to be maintained at low levels$^{10}$. However, the ability of this essential metal ion to transfer electrons can be hindered becoming toxic to cells when present in excess as it is directly involved in the formation of toxic reactive oxygen species$^{11}$. Plants can regulate their intracellular concentration of metal ions in response to external stimuli, by modulating uptake, transport, storage and secretion rates of metal ions$^{12}$. Excess of heavy metal stress in plants, can lead to formation of reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals and hydrogen peroxide that damage the plant cells$^{13}$. Antioxidant enzymes such as peroxidases and polyphenoloxidases can convert H$_2$O$_2$ to H$_2$O in the plant cells and neutralizes the toxic effects of H$_2$O$_2$$^{14}$. Polyphenol oxidase (PPO) is a copper containing enzyme localised on the thylakoids of chloroplasts and involved in catalyzing the aerobic oxidation of different phenolic compounds to quinines which are autooxidised to dark brown pigments. Role of PPO as an “oxygen buffer” has been postulated by Hulya and Aylem$^{15}$. The involvement and the role of antioxidants in protection against oxidative stress has also been demonstrated using transgenic plants$^{16}$.

Plants are known to contain significant quantities of various polyphenolic acids, such as coumaric acid and caffeic acid, as well as their glycosides and esters. Among these 5 -caffeoylquinic or chlorogenic acid is widely distributed and is present in high content in

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plants. Chlorogenic acid is of interest as a natural antioxidant which had marked inhibitory activity in relation to enzymes such as arginase\textsuperscript{17} and xanthine oxidase\textsuperscript{16}.

Contrarily the presence of copper directly affects the photosynthetic process and its efficiency in the chloroplast of regenerated leaves of \textit{S. rebaudiana}. The fast Chlorophyll-a-fluorescence transient pattern is a powerful tool for assessing the photochemical electron transport as well as the overall vitality and physiological performance of oxygenic photosynthetic organisms and their tissues\textsuperscript{19}. It is a sensitive method for the detection and quantification of changes induced in the photosynthetic apparatus. Chl-a-fluorescence intensity of dark-adapted photosynthetic organisms follow a characteristic variation with time after the onset of illumination. This effect is well known as “Kautsky effect”\textsuperscript{20}. In fluorescence induction curve, at the minimal fluorescence (Fo) all the reaction centres are open, and at maximal fluorescence (Fm) all the reaction centres are closed. All oxygenic photosynthetic material investigated so far showed polyphasic fluorescence rise pattern consisting of a sequence of phases denoted as O, J, I and P\textsuperscript{21}. The O-J-I-P transient pattern is defined by a certain time during induction kinetics: O, first measurement at onset of illumination, J at about 2 ms, I at about 30 ms and P at about 500 ms\textsuperscript{22}. Polyphasic chlorophyll a fluorescence transient has been measured and quantification was done by JIP test to study the effect of copper sulphate on photosynthetic process in \textit{S. rebaudiana}. The aim of the study is to evaluate the effect of optimization of micronutrient copper on enzymatic activities, chloroplast ultrastructure and photosynthesis in the regenerated plants of \textit{S. rebaudiana}.

Materials and Methods

\textit{Plant material}—Stevia rebaudiana (SRB123 variety) plants were procured from Sun Fruit Pvt. Ltd. Pune, India. Young primordial leaves surrounding shoot tip and leaves of size < 1 cm from field grown plants were used as explants.

\textit{Explant sterilization}—The leaves were washed in tap water and gently rinsed with 20% (v/v)extran (Merck, India). The surface sterilization of explants was carried out in 0.1% sodium hypochlorite solution for 10 min. The explants were rinsed with five changes of sterile distilled water. Murashige and Skoog (MS) medium was prepared with 3% (w/v) sucrose along with BAP (2.2 µM) and IAA (2.8 µM). The medium was solidified with 0.8% Agar (Qualigen, bacteriological grade). The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C and 1.2-1.3 kg/cm\textsuperscript{2} pressure for 20 min.

\textit{Culture establishment}—Leaf explants were cut from petiolar end and placed on sterile medium (with dorsal side in contact with the medium) supplemented with 6-benzylaminopurine (BAP; 2.2 µM) and indole-3-acetic acid (IAA; 2.8 µM) as per Jain \textit{et al}\textsuperscript{8}. All the cultures were incubated in growth chamber at 26°C, 16 h photoperiod and 25 µ mol/ (m\textsuperscript{2} s\textsuperscript{1}) light intensity provided by white fluorescent tubes. After 4 weeks, the shoot buds induced directly on leaf explants were excised from base along with some portion of mother explant and placed on proliferation medium supplemented with 6-benzylaminopurine (BAP; 3.5 µM) and kinetin (Kn;1.8 µM). Cultures were established in different vessels and were observed regularly for 4 weeks. The leaves from regenerated plants were used further as explants for studying the effect of different concentration of copper sulphate in the culture medium. The explants were placed on culture medium supplemented with BAP (2.2 µM) and IAA (2.8 µM) and sucrose 3% (w/v). This was considered as control induction medium containing a usual CuSO\textsubscript{4} level of 0.1 µM. Different levels of CuSO\textsubscript{4} (0.1, 0.5, 1, 2, 3, 5 µM) were added in MS medium with BAP (3.5 µM) and Kn (1.8 µM) at the same levels as the control induction medium. The elongated shoots were sub cultured in proliferation medium supplemented with BAP (3.5 µM) and Kn (1.8 µM) along with different levels of CuSO\textsubscript{4} (0.1, 0.5, 1, 2, 3, 5 µM). The cultures were kept under controlled conditions in culture chamber and observed regularly for 4 weeks.

\textit{Peroxidase and polyphenoloxidase assays}—Fresh leaves (1 g) were taken from regenerated shoots of 3-5 week old cultures and grounded in 0.05 M potassium phosphate buffer (pH 7.0) with pre-chilled mortar and pestle. The homogenate was centrifuged for 20 min at 5000 rpm. After centrifugation, the pellet was discarded and supernatant was mixed with 70% cold acetone and centrifuged again at 5000 rpm for 10 min. The collected supernatant was used for further enzyme assays. The reaction mixture was prepared by mixing 0.1 mL enzyme extract, 0.01 mL 20 mM Guaiacol and 0.1 mL 50 mM H\textsubscript{2}O\textsubscript{2}. Peroxidase activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol at 470 nm after every 15 sec. One unit
of peroxidase activity corresponds to the level of enzyme activity which was expressed as moles H₂O₂ destroyed/min/g fresh wt²³. For PPO assay, the reaction mixture was made by mixing 1 mL enzyme extract, 3 mL catechol solution (0.01 M catechol freshly prepared in 0.1 M phosphate buffer, pH 6.0). PPO activity was determined spectrophotometrically by monitoring the formation of quinones at 495 nm after every 15 seconds up to 5 min. One unit of PPO activity corresponds to the level of enzyme activity which was expressed as moles of quinones formed/min/g fresh wt.

Estimation of chlorogenic acid—Standard curve was constructed using dilution of a 10 mg % commercial preparation of chlorogenic acid (MP Biomedical, USA). Quantitative assay was done spectrophotometrically at 315 nm. Fresh leaves (100-150 mg) were crushed by pestle and mortar in 100 volume of distilled water to prepare homogenous green suspension. It was allowed to stand for 1 min and was centrifuged at 9000 rpm for 5-6 min. The supernatant was immediately used for estimation of CGA content in leaves, spectrophotometrically at 315 nm.

Measurement of fast Chl-a fluorescence induction kinetics—The in vitro regenerated and potted plants were kept in darkness for 15 min and examined for Chl-a-fluorescence transient pattern (OJIP) using the procedure described by Strasser et al²⁴. Chlorophyll fluorescence was measured using a plant efficiency analyzer (Handy PEA, Hansa Tech Instruments Ltd., Norfolk, UK). The chlorophyll-a-fluorescence transients were induced by the red light of 3000 µE m⁻² s⁻¹ and recorded from 10 µs up to 1 s. All measurements were done on fully dark-adapted attached leaves. From the first OJIP transients, several bioenergetics parameters were derived according to the equation of the JIP test using Bioanalyzer program (R.M. Rodriguez, Bioenergetics Laboratory, University of Geneva; www.Unige.ch/sciences/biologie/bioen). Chlorophyll ‘a’ fluorescence at 20 µs, 2 ms, and 30 ms, and the time required to achieve maximum fluorescence are termed as the O, J, I, and P steps, respectively. The J-I-P test refers to the analysis of structural integrity and function of chloroplasts based on fluorescence emission data²⁵. Further details on OJIP measurements and the use of different formulae are described elsewhere²⁵. The basic level of fluorescence would vary with the amount of chlorophyll in the same sample as well as the duration of light and darkness. These differences in chlorophyll fluorescence could be overcome by normalizations to Fo, Fi, or Fm of the fast transients. This normalization makes a comparison of the fluorescence transients within the same figure easy²⁶. Various photochemical parameters like absorption, transmittance and electron transport per cross section of leaf and per reaction centre and photosynthetic yields are observed in regenerated shoots at normal MS level and at higher levels of copper sulphate in in vitro cultures and in potted field transferred plants.

Transmission electron microscopic studies—For Electron Microscopy, fixation of leaves of regenerated plants was done in 2.5% glutaraldehyde and 23% paraformaldehyde, made in 0.1 M Sodium phosphate buffer (pH 7.4). The fixed tissues were transferred in phosphate buffer to SAIF (Sophistical Analytical Instrumentation Facility (DST) AIIMS. Sample processing, microtomy and electron microscopy was done in AIIMS, New Delhi.

Statistical analysis and experimental design—Each treatment consisted of 3 replicates. One way analysis of Variance (ANOVA) was applied in order to evaluate the effect of different concentrations of micronutrient copper on Peroxidase and PPO enzyme activity and on CGA content. The statistical analysis was conducted by Fisher’s least significance difference (P + 0.05)²⁸.

Results

Effect of CuSO₄₂₃ on enzyme activities and CGA content—Activities of peroxidase and PPO enzymes increased in plants regenerated at higher levels of copper sulphate in the medium. With increase in copper levels in culture medium there was considerable decrease in antioxidant (CGA) content in the leaves of regenerated plants (Fig.1).

Effect of CuSO₄ on photosynthetic activity—Photosynthetic performance of leaf samples taken from potted plant (20 days old field transferred plant) as autotrophic control, in vitro regenerated plant at 0.1µM CuSO₄ as heterotrophic control, in vitro regenerated plant at 0.5 µM CuSO₄ and in vitro regenerated plant at 1 µM CuSO₄ was measured using the Handy plant efficiency analyzer. Effect of copper sulphate was observed on different photochemical and biochemical parameters during photosynthesis in radar plot which revealed that there was no variation at different parameters in leaves of field transferred
At MS level of copper sulphate a slight variation in different parameters was observed while plants regenerated at higher levels of copper showed maximum variations in different parameters (Fig. 2).

**Effect of CuSO$_4$ on chloroplast ultrastructure**—TEM studies were carried out to observe the effect of copper sulphate on ultrastructure of the chloroplast in *Stevia rebaudiana*. Plants regenerated at the MS level of CuSO$_4$ had symmetric and asymmetric oblong shape chloroplasts situated along thick cell wall. Magnified view of single chloroplast showed the presence of single electron dense inclusion body present in stroma (Fig. 3a). Plants regenerated at higher levels of copper in the medium had higher number of electron dense inclusion bodies in their chloroplasts and the size of the inclusion bodies were also larger along with large vacuoles present in mesophyll cells (Fig. 3 b-d).

**Discussion**

The appropriate level of micro nutrients is essential for the optimal functioning and growth of plants. Copper has been considered as one of the essential micronutrients$^{29}$ It has also been reported that copper plays an important role in several metabolic activities including protein and carbohydrate metabolism$^{31}$ Numerous copper containing enzymes (cytochrome oxidase, ascorbate oxidase, phenolase, laccase, diamine oxidase, super oxide dismutase, and quinol oxidase) have been identified in plants$^{31}$ Copper is directly involved in the photosynthetic electron transport chain as a constituent of plastocyanin, a copper containing protein$^{32}$ Plastocyanin operates as an electron carrier between the two photo systems.

OJIP transient pattern was used to monitor function of chloroplasts in mesophyll cells of *Stevia rebaudiana* under higher levels of copper sulphate in *in vitro* regenerated plants. Similarly OJIP transient pattern were used for revealing the effects of high salt stress on PSI in wheat leaves$^{26}$ Functional integrity of pea mesophyll protoplasts during isolation process was monitored by OJIP transient studies. Their results demonstrated that the OJIP transients could be successfully used to study the quality of mesophyll protoplasts at different isolation steps. The protoplast maintained their integrity and photosynthesis status well and their performance was similar to intact leaves$^{33}$ In the current study the effect of micronutrient copper was analysed on photosynthesis process. The plant efficiency analyser was used and OJIP transient pattern was generated for assessing the photosynthesis process in live Stevia plants. Shikanai *et al.*$^{34}$ also reported copper as an essential micronutrient required for photosynthesis process. The author reported that the leaves of copper deficient plants and *Arabidopsis thaliana* mutants, exhibited reduced chlorophyll content and photosynthetic electron transport into the chloroplast.

The ultra structure study of chloroplast showed increase in inclusion bodies in chloroplasts at higher level of copper sulphate. Earlier electron dense inclusion bodies inside thylakoids were reported in chloroplasts of some other plant species The experiments conducted on *Perilla ocymoides* showed that such a modified type of thylakoids were dominant when short day plants were grown under continuous illumination. It is of interest to note that both *Stevia* and *Perilla* are the short-day plant that display increased vegetative growth without transition
to flowering stage when they are grown under long
day conditions. Moreover, the content of the steviol
glycosides in Stevia leaves grown under long days
was found to be higher than that in the leaves of
the same plants grown under short day. However
the chemical nature of these inclusions remains so
far poorly understood and most likely may be
different for different plant species. According to
Ladygin et al., such inclusion bodies observed in
thylakoids of chloroplasts of Stevia cells are not
steviol glycosides, but might be their precursor.
However, in the thylakoids of Nymphoides indica
chloroplasts, the electron dense inclusion bodies
represented phenols. It was suggested that in
the chloroplasts of leaves of juvenile plants,
these inclusion bodies might serve as an inhibitors
of flowering and they could converge into
inactive forms, for example glycosides as may be
required. It is most likely that prolonged period
of intensive production of chloroplast isoprenoids
(phytols, carotenoids and plastoquinone-9) in
chloroplasts, the process essential for photosynthesis
as a whole precedes an active formation of the
steviol glycosides in these organelles during
ontogenesis. These metabolites are shown to be
capable of being synthesized in chloroplasts following
an alternative pathway. However, the observed
positive correlation between the development
and activity of the photosynthetic apparatus and
production of the steviol glycosides suggests that
synthesis of isoprenoids in Stevia chloroplasts is
somewhat related to secondary metabolite production
in chloroplast of Stevia rebaudiana. Therefore, the
production of both isoprenoids and steviol glycosides
in chloroplasts could be stimulated upon increasing
the concentrations of precursors of these compounds.
The initial step of synthesis of steviol glycosides occurs
in chloroplasts using alternative glyceraldehydes-3-
phosphate-puruvate (1-deoxy-D-xylulose-5-phosphate) pathway rather than mevalonate pathway operating in cytoplasm. In recent years, strong evidence indicated that the alternative pathway functions for production of some isoprenoids in higher plants.

Conclusion

The copper adapted cells showed higher photosynthetic activity and some morphological changes in chloroplasts and thylakoids ultrastructure at optimised levels (0.5 µM) of copper sulphate. The data support the role of copper as a positive element when used in lower optimal concentration in the culture medium thus affecting the structure/function of PSII and could be used as a potential elicitor for steviol biosynthesis in chloroplast of Stevia rebaudiana.

Acknowledgement

Pourvi Jain gratefully acknowledges Council of Scientific and Industrial Research (CSIR), New Delhi for the award of SRF and RA (Research Associateship) and Professor Reto J. Strasser, Director, Microbiology and Bioenergetics Laboratory, University of Geneva, Switzerland for plant efficiency analysis of Stevia rebaudiana.

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