Chlorocholine chloride (CCC) - induced modulation of growth, metabolism and stevioside productivity of *Stevia rebaudiana* Bertoni under *in vitro* and field condition

SYNOPSIS OF THE THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (SCIENCE) OF THE UNIVERSITY OF BURDWAN

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Introduction:

The development of traditional medicinal systems incorporating plants as means of therapy can be traced back to the Middle Paleolithic age some 60,000 years ago as found from fossil studies (Solecki, 1975). The research and development thrust in the pharmaceutical sector is focused on development of new innovative and indigenous plant-based drugs through investigation of leads from the traditional system of medicine (Patwardhan et al., 2004). The World Health Organization has also recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines.

Stevia rebaudiana Bertoni is a perennial semi-herb of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil). It is often referred to as “the sweet herb of Paraguay”. For centuries, the Guarani tribes of Paraguay and Brazil used Stevia species primarily S. rebaudiana as sweetener. The constituents responsible for Stevia’s sweetness were called glycosides. Of these glycosides, stevioside is considered the sweetest and has been tested to be approximately 300 times sweeter than sugar. Stevioside, comprising 5-10% of the leaf is also the most prevalent glycoside. The other sweet constituents include steviolbioside, rebaudiosides A-E and dulcoside A.

Biotechnology has a role to play in accelerated development of reliable plant medicines. Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as in-vitro regeneration and genetic transformations. Plant tissue culture is an example of a biotechnology with a multitude of applications to medicinal plant improvement programs, and it is not included in the controversy involving genetically modified organisms.

In-vitro propagation of plants holds tremendous potential for the production of high quality plant-based medicines (Murch et al., 2000). Numerous factors are reported to influence the success of in-vitro propagation of different medicinal plants (Bhagyalakshmi et al., 1988). It has been shown that 6-Benzylaminopurine (BA), at high concentration (1–5ppm), stimulates the development of the axillary meristems and shoot tips of Atropa belladonna (Benjamin et al., 1987). With the stimulus of endogenous growth substances or by addition of exogenous growth
regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. Plant regeneration has been achieved from leaf callus of *Cephaelis ipecacuanha* on Murashige and Skoog medium supplemented with 4.5 mg/L kinetin and 0.1 mg/L a-Naphthaleneacetic acid (NAA) (Rout et al., 1992). Embryogenic calluses and germination of somatic embryos in nine varieties of *Medicago sativa* has been achieved (Fuentes et al., 1993).

Plants are the traditional source of many chemicals used as pharmaceuticals. Most valuable phytochemicals are products of plant secondary metabolism. The production of secondary metabolites *in vitro* can be possible through plant cell culture (Barz et al., 1981). The production of solasodine from calli of *Solanum eleagnifolium*, and pyrrolizidine alkaloids from root cultures of *Senecio* sp. have been studied (Nigra et al., 1987). Cephaelain and emetine were isolated from callus cultures of *Cephaelis ipecacuanha* (Jha et al., 1988). The production of azadirachtin and nimbin has been shown to be higher in cultured shoots and roots of *Azadirachta indica* compared to field grown plant (Srividya and Devi 1998).

Plants and/or plant cells *in vitro*, show physiological and morphological responses to microbial, physical or chemical factors which are known as ‘elicitors’. Elicitation has been proved to be effective way to increase secondary metabolite production.

Modulation of plant growth, development and metabolism through application of different plant growth regulators (promoters and retardants) has caught great attention to make a plant commercially more important. Most widely used group of plant growth regulators consist of those which inhibit gibberellin biosynthesis as for example quaternary ammonium compounds pyrimidines, triazoles, and norbornenodiazetines that interfere with biosynthesis of gibberellins and sterols (Izumi *et al.*, 1984). The plant hormones are extremely important agent in the integration of developmental activities. Increase in crop growth rate and total dry matter production due to application of CCC has been reported in rice (Chaudhari *et al.*, 1980) and in *Catharanthus roseus* (Choudhury and Gupta 1996). CCC application in both *in vitro* and field condition reduced plant height of *Catharanthus roseus* L. (Choudhary and Gupta, 1996). In *Catharanthus roseus*, CCC increases biomass and alkaloid production in field growing plants (Choudhury and Gupta, 1999).
Plant growth regulators also found to modulate the expression of several genes in plant. Methyl jasmonate (MeJA) and its free-acid, jasmonic acid, collectively referred to as jasmonates, are important group of growth regulator involved in the activation of AOS gene in tomato (Sivasankar et al., 2000).

**Objectives of the present work:**

- Establishment of efficient micropropagation method of *Stevia rebaudiana* (with special emphasis on the role of CCC).
- Elicitation of phytochemical production especially stevioside, phenol and flavonoid through CCC treatment (both *in vitro* and *in vivo*).
- Investigations on CCC induced higher phytochemicals accumulation especially phenol and flavonoid on antioxidant activity and assessment of cytotoxicity using three animal cell lines.
- Enhancement of biomass (both *in vitro* and *in vivo*) and extension of vegetative phase (*in vivo*) through CCC treatment.
- Investigation on the influence of CCC on the expression level of UGT85C2 gene during stevioside biosynthesis.

**Materials and methods:**

**Collection of plant material:**

*Stevia rebaudiana* Bertoni plants and seeds were procured from medicinal plant nursery of Government Cinchona Factory located at Mungpoo, Darjeeling, West Bengal as gift. Plants were maintained in our departmental garden and seeds were stored in desiccators under standard storage conditions for future uses.

**Choice of explant:**

Axenically grown cotyledonary leaves from germinating seeds were found to be most effective explants among others (nodal segment, apical bud, leaves of field grown plants) tested in this study.
**Preparation of explant:**

Seeds were first surface sterilized with 0.1% (w/v) HgCl$_2$ solution followed by wash in distilled water for several times and then soaked in distilled water for 2-3 hours. Soaked seeds were kept in filter paper inside the petri-plates moistened with water for germination. Regenerated cotyledonary leaves (6-7 days old) were then thoroughly washed in sterile distilled water followed by surface sterilization in 0.1% HgCl$_2$ for 1-1.5 minutes. After that, leaves were rinsed three to four times in sterile double distilled water followed by inoculation in culture media.

**Media and culture conditions:**

Sterile explants were inoculated in sterilized MS (Murashige & Skoog 1962) supplemented with different plant growth regulators like NAA, BA, kinetin and IBA. The culture media contained 3% (w/v) sucrose and solidified with 0.8-1% (w/v) agar-agar. The pH of the media was adjusted to 5.7-5.9 by 0.1(N) NaOH. The gelled media were poured into culture tubes, plugged with non-absorbent cotton and then autoclaved at 121°C under 15 lb/sq. inch pressure for 15 minutes. Then the cultures were maintained at 24 ± 2°C temperature, illuminated with white fluorescent light (2000 lux) under 16/8 hr light/dark and 72% RH condition. Subculturing was routinely made after every four to five weeks of inoculation.

**Callusing, multiple shooting and rooting:**

MS medium (Murashige & Skoog, 1962) supplemented with different plant growth promoters like NAA, BA and kinetin singly or in combinations were used for callusing and multiple shooting. Subsequent rooting of multiple shoots was carried out in MS medium contained different concentrations of IBA.

**CCC priming:**

For rooting and CCC priming, regenerated microshoots were transferred into the MS medium containing different concentrations of IBA and CCC singly or in combinations. Cultures were kept in culture room illuminated with white fluorescent light (2000 lux) under 16/8 hr light/dark
and 72% RH condition. Subculturing was routinely done after every four to five weeks of inoculation.

**Hardening:**
Rooted plantlets were transplanted into 100 ml beaker filled with autoclaved soilrite-soil mixture (1:1) and kept into culture environment till the plantlets resumed growth. After that, the plantlets were transferred to the small pot for full growth followed by final transplantation into the garden. Survival percentage of the plantlets was recorded after one month of final transplantation.

**Investigations on field grown plants:**

**Plant material:**
*Stevia* seedlings were collected from a medicinal plant nursery near Golapbag. One month old seedlings were planted in the pot and nurtured properly for the establishment. After one month of plantation into the pot the established plants were subjected to foliar spray.

**Foliar spray with CCC:**
Foliar application of freshly prepared CCC in 0.2% Tween- 80 (surfactant) was carried out for two consecutive days in early morning. Different concentrations of CCC such as 1.00, 3.00, 5.00 and 10.00 mg/l were prepared. Control plants were sprayed with distilled water containing 0.2% Tween- 80. At the time of CCC application the exposed soil surface of the pot was covered by polythene sheet to prevent contamination of soil with CCC.

**Growth study:**
Changes of growth parameters like plant height, number of leaves, leaf area, number of nodes and branches per plant, internodal length were recorded after one month from the date of foliar application of CCC. Data were collected up to two months in three replicates and the mean values were incorporated in the result.
Estimation of phytochemicals (both in vitro and in vivo) and studies on the antioxidant potential (especially phenol and flavonoid) and cytotoxicity of methanolic extract (in vitro):

1. Amount of soluble carbohydrate was determined following the method of Mc. Cready et al. (1950).

2. Total free amino acid content was determined following the method of Moore and Stein (1948).

3. Total protein was determined following the method of Lowry et al., 1951 using BSA as standard.

4. The chlorophyll content was estimated following Arnon’s (1949) principle.

5. Extraction of nucleic acids (DNA and RNA) was carried out from 100 mg leaf and callus tissue following the method of Cherry (1962). The amount of DNA and RNA in the extract was estimated from a common stock using the method of Choudhuri and Chatterjee (1970).

6. Total phenolic contents were determined according to the method of Slinkerd et al. (1977) with slight modifications using caffeic acid as standard.

7. Total flavonoid content was determined following the method of Kim et al., 2003 using quercetin as authentic sample.

8. DPPH radical scavenging activities of methanolic extracts (500 µg/ml and 1000 µg/ml) from different (control and test sets) calli and leaves were determined following the method of A Kumar and S Chattopadhyay (2007).

\[ \% \text{ Inhibition} = \frac{(\text{Abs. Control} - \text{Abs. Sample})}{\text{Abs. Control}} \times 100\% \]

9. ABTS radical scavenging activities were performed using methanolic extracts (500 µg/ml and 1000 µg/ml) from different (control and test sets) calli and leaves of Stevia rebaudiana following the method of Re et al. (1999).

10. Hydroxyl radical scavenging activity was determined following the method of (Halliwell et al., 1981).
11. The determination of the oxidative DNA damage preventive activity of methanolic extract was performed following the method of Tian *et al.*, (2005) with minor modifications.

12. Cell viability was measured following the MTT bioassay method (Bernas and Dobrucki, 2002).

13. Extraction of stevioside was carried out following the method described in (Ursula Woelwer-Rieck *et al.*, 2010) with slight modifications.

RT-PCR study:

**Plant material:**
Two months old field established micropropagated plants and normal field grown plants (after two months of CCC application) [with and without CCC treated] were selected as plant material for the present work.

**Selection of primers:**
Specific primers for the reference gene (26S rRNA) and target gene (*UGT85C2*) were designed from expressed sequence tags of *Stevia* available at the GenBank (National Center for Biotechnology Information, 2001). Primers design and validity checking were done using QuantPrime Q-PCR primer designing tool (http://www.quantprime.de). The primers were also screened for hairpins, dimer formation, and target specificity by Blast N (Blast, 2011), against the nucleotide databank.

**RNA extraction and purity check:**
Total RNA was extracted from leaf tissue using RNA-Xpress kit (Himedia) following the manufacturer’s instructions. RNA quality analysis and quantification were performed by measuring the absorbance of the extracted RNA sample in a Nano Drop (ND-1000) spectrophotometer (Thermo Scientific).

**cDNA synthesis:**
Synthesis of cDNA was carried using a high-Capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.
**RT-PCR assay:**
Polymerase chain reactions were performed in 96-well microtiter plates using SYBR Green (Invitrogen, Carlsbad, CA, USA) to detect double stranded DNA synthesis. Amplifications were carried out in a 7300 RT-PCR thermocycler (Applied Biosystems, Foster, CA, USA) using the following cycling parameters: 55°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 2 min, 65°C for 30 seconds and 72°C for 30 seconds. The data were collected in the last (extension) phase. The fold Change of the respective gene was calculated by using Livak method (Livak et al., 2001).

**Results and discussion:**
Alteration of plant growth, development and metabolism of *Stevia rebaudiana* through application of plant growth retardant CCC are the thrust areas of this research work. Application of CCC in the MS medium significantly improved the morphology and viability of *in vitro* grown plantlets of *S. rebaudiana*. Cotyledonary leaf was used as explant for the micropropagation. MS medium supplemented with NAA and BA or NAA and kinetin produced vigorous calli. Profuse microshooting was noticed in MS medium contained different combinations BA or kinetin, both singly or in combinations. After that, both calli and microshoots were transferred into fresh MS medium containing different concentrations of CCC. CCC in combinations with IBA was found to be most effective for inducing certain beneficial changes like undesirable stem elongation, profuse rooting, bigger leaf size, increased fresh weight of the plantlets and longer chlorophyll retaining capacity in comparison to MS with only CCC or IBA. CCC treatment significantly reduced internodal length of the microshoots in comparison to MS medium with IBA. Remarkable increase in fresh weight of the microshoots (around 2.3 fold) were recorded in MS medium with 3 mg/l IBA and 3 mg/l CCC combination and 1.4 fold in MS with 3 mg/l CCC over the combination of MS with 3 mg/l IBA. Such increment of the fresh weight was due to formation of larger leaves on CCC application. Moreover, CCC treatment produced visibly stouter *S. rebaudiana* plantlets.

Investigations on the role of CCC on production of different metabolites revealed that, CCC appreciably elevated the metabolite content (both primary and secondary) in calli and microshoots of *S. rebaudiana*. It was noticed that, application of CCC found to increase the
soluble carbohydrate, free amino acids, protein, DNA, RNA, total chlorophyll, phenol and flavonoid content in both calli and leaves in test sets (CCC treated) as compared with that of control sets (CCC non-treated). Phenol and flavonoid content of calli and leaves were studied in details with reference to the role of CCC. Comparative analyses revealed that accumulation of phenol and flavonoid were increased after CCC treatment. Interestingly, CCC induced elevation of phenol and flavonoid content was found to increase the antioxidant potential of the in vitro grown S. rebaudiana. It was observed that, antioxidant activity was increased simultaneously with the increment of phenol and flavonoid contents after CCC treatment. The strong hydroxyl radical scavenging potential of methanolic extract indicated the presence of antioxidant flavonoid. It was noticed that methanolic extract from test set prevented the stand scission to a considerable extent in comparison to that of control set. Moreover, application of CCC didn’t produce any cytotoxic metabolites in S. rebaudiana. Most importantly, application of CCC in MS medium had a positive impact elevation of stevioside content.

It was noted that, CCC appreciably reduced the plant height and intermodal length, increased the total number of leaves, branches per plant and individual leaf area after two months of CCC application over control. Biochemical analysis of field grown plants revealed that application of CCC was also significant enough to induce some beneficial changes in the accumulation of different metabolites in S. rebaudiana. Like in vitro grown calli and microshoots, CCC application was also found to increase soluble carbohydrate, free amino acids, protein, DNA, RNA, total chlorophyll, phenol and flavonoid content in leaf.

Comparative analysis on the effect of CCC on phytochemicals accumulation in normal field grown plants and micropropagated field established plants of S. rebaudiana revealed that influence of CCC was more significant in micropropagated plants as compared with others.

CCC also found to up regulate the expression level of stevioside biosynthesis gene UGT85C2 in S. rebaudiana. Application of CCC found to increase the expression level of UGT85C2 gene in leaf, that was directly associated with elevated stevioside content in S. rebaudiana.
Conclusion:
The achievement of the present work is in accordance with the objectives, outlined at the beginning of the thesis- establishment of efficient micropropagation method of *Stevia rebaudiana* through application of CCC, elicitation of phytochemicals production especially stevioside, phenol and flavonoid through CCC treatment (both *in vitro* and *in vivo*), CCC induced higher increased accumulation of phenol and flavonoid on elevated antioxidant activity and with no significant cytotoxic effect on animal cells, enhancement of biomass (both *in vitro* and *in vivo*) and extension of vegetative phase (*in vivo*) through CCC treatment and CCC induced higher expression level of UGT85C2 gene during stevioside biosynthesis.

This entire piece of work promises to contribute significantly in the area of medicinal plant research. The novelty of the research centers on the implication of CCC in promotion of secondary metabolism in general and stevioside production in particular. These findings have got promising research future and immense commercial scope for pharmaceutical industry.
References:


