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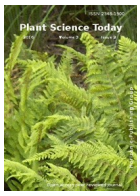

Research Article

Antioxidant response of *Stevia rebaudiana* (Bertoni) Bertoni (Angiosperms; Asteraceae) during developing phase of suspension cell culture

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Abstract

The present study established a protocol for suspension cell culture of *Stevia rebaudiana* and demonstrates the antioxidant enzymes activity during various developing phase of it. Higher concentration of Auxin and Cytokines (3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA) with Ascorbic acid (1 mg L^{-1}) considered as highly suitable growth regulator combination for growth and development of *Stevia rebaudiana* suspension culture. Maximum concentration of stevioside (~70 mg G^{-1} of dry weight of tissue) was obtained on 14th day (exponential phase) which got reduced on 28th day (apoptotic phase) upto ~21 mg G^{-1} of dry weight of tissue. The amount of MDA reduced initially up to stationary growth phase which showed the adaptation of suspension cells in the culture medium and culture environment. Reduction of Chlorophyll showed the enhancing phenolic content with progressive culture period, while enhanced amount of proline was indicating the generation of defense mechanism with depletion of nutrient (with increased culture duration). The amount of SOD got enhanced with elevating the concentration of H_2O_2 as well. The concentration of CAT enhanced upto stationary growth phase of suspension cell while APX concentration showed continuous inhibition up to apoptotic phase from exponential phase.

Keywords
Antioxidants; Single cell suspension culture; stevioside: *Stevia rebaudiana*

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Introduction

Stevia rebaudiana is a budding plant of the Asteraceae family in the field of commercial sweetener market (Sreedhar *et al.*, 2008). Its economic value completely depends upon the secondary metabolite "stevioside" which is 300 to 400 times sweeter than sucrose at the 4% dilution of water (Geuns, 2003). Stevioside is basically used to prevent diabetes without changing the sweetening property of food. Stevioside can't be digested or metabolized by any of the digestive hormone of human, so it promotes the production of insulin, which can control the blood sugar level. Many synthetic drugs are available to cure the diabetes, but 100% positive results of them have not been reported till date (Thiyagarajan and Venkatachalam, 2012). The negative impact of *Stevia* extract had not been reported, so it can be used as an alternative of synthetic drugs or sugar (Thiyagarajan and Venkatachalam, 2012). It is well known that the quantity of any secondary metabolites is always not adequate or constant (Croteau *et al.*, 2000; Dewick, 2002). Various biotechnological methods are applied for enhanced production of secondary metabolites. These methods include organ culture of plant, addition of precursor, elicitation of *in vitro* products, hairy root cultures, genetic manipulation and

bioreactor scaling up production. Among all these techniques, the tissue culture techniques are simpler and have broader criteria. It includes direct *in vitro* shoot regeneration, callus culture, suspension culture, synthetic seeds preparation and somatic embryogenesis. Moreover, the cell suspension cultures are the technique for rapid and uniform production of single cells which are helpful for the production of high quantity of secondary metabolites (Davies and Deroles, 2014). Suspension cell culture also can be used for mass propagation, protoplast isolation and gene transfer. On the other hand plant cell contains a wide variety of defensive systems. These systems (enzymatic and non enzymatic antioxidant system) react with active mode of oxygen and maintain them at low level, so that cells can grow at abnormal culture condition as well (Allen, 1995). The research in the field for the establishment of *S. rebaudiana* cell suspension culture has been done initially (Nabeta *et al.*, 1976; Suzuki *et al.*, 1976; Striedner, 1991; Mathur and Shekhawat, 2013), but the study was limited only up to establishment of suspension cell culture and stevioside quantification. The present study provides a platform for long term culture of suspension cells of *S. rebaudiana*.

Materials and methods

Plant material and callus proliferation

Explants for the establishment of callus culture, were collected from the *in vitro* grown plants of *S. rebaudiana*, from the plant tissue culture laboratory of Department of Bioscience and Biotechnology (Banasthali University, Tonk). The leaves were sliced into 1-2 cm^2 squared section and entrenched on MS basal medium. The medium was an amalgamation of various macro and micro salts with vitamins and essential amino acids. Sucrose (3%) was used as a carbon source for the plants and agar (0.8%) was used to make medium solid by its gelling property. The pH (5.8) of the medium was adjusted just before autoclaving (121 °C and 1.2–1.3 kg/cm^2 pressure for 20 min). MS basal media was enriched with different concentrations of syringe filtered (0.2 µm) Plant Growth Regulators (PGRs) like 6- Benzyl Aminopurine (BAP- 2.0 to 3.0 mg L^{-1}), 2,4 Dichlorophenoxy acetic acid (2, 4-D- 1.0 to 2.0 mg L^{-1}), Kinetin (Kn- 1.0 to 2.0 mg L^{-1}) and α Naphthalaneacetic acid (NAA- 2.0 to 4.0 mg L^{-1}). Three to four discs were relegated in each flask and 5 tentative culture flasks were prepared with each hormonal concentration. Cultured flasks were placed in a thermostatically controlled culture room, which was maintained at temperature 24±2ºC, under 40 W cool white fluorescent lights (Philips, India) and 16 h photoperiod at a photosynthetic photon flux density (PPFD) of 150 µmol^{-2}s^{-1} with 55±5% humidity. Attained calli were further subcultured in interval of 2 weeks.

Establishment of suspension culture

Suspension cells of *S. rebaudiana* were established through 15 to 20 days old friable calli, which were obtained from the media fortified with 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA. Priorily the inoculum was transferred in a 150 ml Erlenmeyer flasks containing 50 ml liquid MS media enriched with 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA. A number of concentrations of ascorbic acid were also added to prevent phenolic emission. The inoculum was up hold at the identical medium for seven days, till the clumps of calli got alienated. Two successive subcultures were done by replacing 40 ml spent media with equal amount of fresh media. Large clumps of callus were removed after each subculture, by using 500 µm pore size nylon sieve. After two subcultures, 10 ml spent media with fine size cells (PCV-0.1ml/5ml culture) were transferred to the 40 ml of fresh media with different amalgamation of GR such as BAP (1mg L^{-1}- 3mg L^{-1}), NAA (2mg L^{-1}- 4mgL^{-1}) and 2,4-D (1mg L^{-1}) with ascorbic acid (1mg L^{-1}- 3mg L^{-1}). Cultures were maintained in an incubator shaker with incessant dark and at agitation of 110 rpm. The temperature of the incubator was maintained at 24±2º C while relative humidity was 60-65%. Packed Cell Volume (PCV) of every flask was recorded on 7th day, 14th day, 21st day and 28th day. PCV was calculated by centrifuging the 5 ml culture at 12,000 rpm for 10 min to estimate the cell growth index in suspension culture (Verma *et al.*, 1976).

Estimation of Physiological parameters

Lipid peroxidation, chlorophyll, H_2O_2 and proline estimation

The Lipid peroxidation level was determined by the method of De Vos *et al.* (1989). It was estimated by examining malonaldehyde (MDA) level using an extinction coefficient of 155 mM^{-1} Cm^{-1}. For this 0.3 g of fresh suspension cells were homogenized in 10 ml of TBA and TCA and absorbance were taken at 532 and 600 nm.

For the estimation of chlorophyll pigments, fresh suspension cells were collected and processed according to the method of Arnon (1949). The absorbance was recorded at 645, 652 and 663 nm incessantly for chlorophyll a, b and total chlorophyll.

Hydrogen peroxide in the well grown separated suspension cells was estimated according to the method of Alexieva *et al.* (2001) by homogenizing the sample in 0.1% TCA and addition of 10mM phosphate buffer (pH 7) with 1 M potassium iodide. Absorbance was taken at 390 nm and H_2O_2 concentration was observed by preparing standard curve of known H_2O_2.

Distorted amount of proline was evaluated by the protocol of Bates *et al.* (1973). Homogenized sample (300 mg) in 5 ml of sulfosalicylic acid was mixed with acid ninhydrin and glacial acetic acid, 1 ml each. Four ml of toluene was added after 1 h

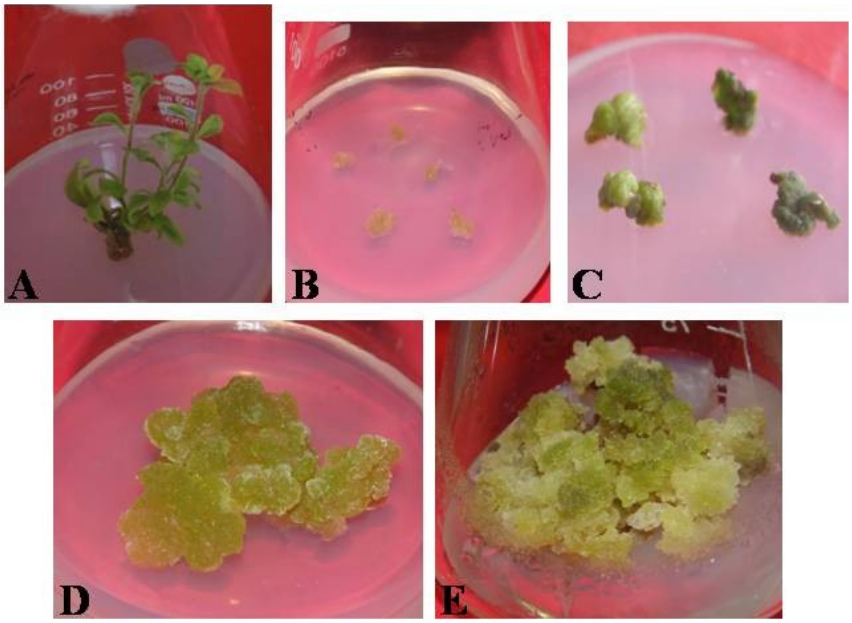

Fig 1: Callus induction from *in vitro* grown plant. **A.** *In vitro* grown plant of *Stevia rebaudiana*. **B.** Implanted leaves on PGR containing MS medium. **C.** leaf generated callus on 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA medium after 7 days. **D. & E** callus after 15th day.

and absorbance of chromatophores was taken at 520 nm.

Estimations of enzymatic activity

Antioxidant enzyme extraction and activity assessment

For enzyme extraction, 0.5 g of fresh suspension cells were homogenized in pre-chilled mortar and pestle with 5 ml of extraction buffer. Extraction buffer was restraining of 1mm EDTA, 0.5% Triton-X and 2% PVP with 1mm Ascorbate in 50 mm phosphate buffer. The homogenate was centrifuged at 14000 rpm for 10 min at 4ºC and the obtained supernatant was used further for estimation of antioxidant enzyme activities. Superoxide dismutase (SOD; EC 1.15.1.1) activity was examined by the method of Beauchamp and Fridovich (1971) at 560 nm, by absorbance of the formazon made by superoxide radical and nitroblue tetrazolium dye. Catalse (CAT; EC 1.11.1.6) activity was assayed by observing the change in absorbance at 240 nm due to hydrogen peroxide by Aebi (1974). Analysis of Ascorbate peroxidase activity (APX; EC 1.11.1.7) was performed by the method of Chen and Asada (1989) by recording the absorbance at 290 nm due to ascorbic acid.

Secondary metabolite analysis

Isolation and estimation of steviol glycoside

Stevioside from the dried (kept in hot air oven for 24 hours at 45º C) suspension cells were isolated by the method described by Talha *et al.* (2012) with slight modification. Dried samples were crushed in powder form and initially defatted with petroleum ether (1g of dry sample in 30 ml of petroleum ether), then extracted with methanol (1 g dried sample in 25 ml of methanol) and kept in the water bath at 60 ºC for 45- 60 min. Pooled extract was cooled and filtered through qualitative filter paper grade 1 (Whatman) filter. Filtered extract was dried, weighed and dissolved in HPLC grade methanol (HI-MEDIA Laboratories Merck Germany).

For quantification of isolated stevioside, Acetonitrile and water (60:40) were used as solvent in isocratic form, for 15 minutes at a flow rate of 1ml min^{-1}. Samples were passed through a C18 reverse phase-packed stainless steel column (4×150-mm diameter) in HPLC (SHIMADZU CLASS-VP) instrument and detected at 254 nm by UV/Visible detector.

$$\text{Quantity of isolated steviol or stevioside (mg/gm of dry weight)} = \frac{\text{area of isolated sample}}{\text{area of standard compound}} \times \frac{\text{amount of standard (mg)}}{\text{dillution in ml}} \times \frac{\text{dilution of isolated sample}}{\text{amount of isolated sample}} \times \frac{\text{potency of standard}}{100} \times \frac{1000}{\text{dry weight of callus}}$$

Results and Discussion

Callus induction and proliferation

Calli were initiated to proliferate from the cut edges of *in vitro* regenerated leaves, after two weeks of implantation (Figure 1). All the combinations showed positive response in terms of callus proliferation, but stability and quality (endurance rate and physical appearance) was not perfect in all calli. Some calli were started to turn brown after some time, while some were growing as yellow from initiation. Medium supplemented with a privileged concentration of BAP (3 mgL^{-1}) and NAA (4 mgL^{-1}) responded superlative (~95%)

Table 1: Effect of different combinations of Cytokines and Auxins on callus induction/ proliferation

S. NO.	PGR concentration (mg L^{-1})				Callogenesis (%)	Nature of callus
	BAP	NAA	KINETIN	2,4-D		
1	2	2	-	-	35.6 ± 0.056	Pale white, friable
2	2	3	-	-	26.6 ± 0.074	Pale white, friable
3	2	4	-	-	50 ± 0.0745	Pale white, friable
4	3	2	-	-	77.8 ± 0.01	Light green, compact
5	3	3	-	-	77.8 ± 0.041	Dark green, friable
6	3	4	-	-	94 ± 0.061	Dark green, friable
7	-	3	1	-	50 ± 0.081	Green, friable
8	-	4	1	-	61.1 ± 0.03	Green, friable
9	-	3	2	-	38.5 ± 0.065	Green, compact
10	-	4	2	-	44.4 ± 0.042	Green, compact
11	2	-	-	1	38.8 ± 0.058	Yellowish, compact
12	2	-	-	2	22.2 ± 0.067	Yellowish, compact
13	3	-	-	1	66.7 ± 0.071	Green, compact
14	3	-	-	2	38.8 ± 0.054	Green, compact
15	-	-	1	1	38.8 ± 0.037	Pale white, compact
16	-	-	1	2	38.8 ± 0.063	Pale white, compact
17	-	-	2	1	22.2 ± 0.055	Yellowish, compact
18	-	-	2	2	38.5 ± 0.071	Yellowish, compact

Results observed after 2 weeks of culture

Data represents mean ± SEM

among all the combination of GR, while Sikdar *et al.* (2012) found poor callusing response in leaf than nodal and inter nodal segment as explant, and showed its best (73.33±6.67%) at 1.0 mg L^{-1} BAP and 3.0 mg L^{-1} NAA. Calli grown in BAP and NAA containing medium were green and friable, however when BAP or NAA combined with 2,4-D or kinetin calli turned into compact. Kinetin with 2,4-D showed the minimum induction ability (22.0% to 38.0%) whereas kinetin with NAA showed fair response (38.0% to 61.0%). 2,4-D was also considered as an excellent growth hormone for the induction and proliferation of *S. rebaudiana* callus (Uddin *et al.* 2006; Janarthanam *et al.* 2010) (data shown in Table 1).

Cell suspension culture

Cell suspension culture is a method to obtain and grow similar type of single cells in liquid medium. Calli clumps in liquid media supplemented with 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA were started slackening within 7 days. Light browning (secretion of phenolic compound) was observed after second subculture. The most favorable growth of *Stevia* suspension culture was recorded on media augmented with BAP (3mg L^{-1}), NAA (4 mg L^{-1}) and minimal amount of ascorbic acid (1 mg L^{-1}) on 14th day by ceiling PCV (0.192 ml pellet/ ml culture), while the lowest PCV (0.1073 ml pellet/ ml culture) was recorded in BAP (1mg L^{-1}) and 2,4-D (1mg L^{-1}) without any trace of ascorbic acid (Data shown in table 2). Present endeavour projected 2,4-D as a bad supporter for the growth of cell suspension, while Sakamoto *et al.* (1993), Meyer and Van Staden (1995) and Mathur and Shekhawat (2013) found supportive role of 2,4-D for suspension culture, biomass accumulation in many plant species as well in *Stevia rebaudiana*. The impact of Auxin and Cytokines were clearly demonstrated on this attempt. Cytokines is well known to instigate the bud breaking while Auxin promotes the cell growth. A combination of both hormones in appropriate concentration gave rise to suspension cells (Figure 2). To prevent from browning, ascorbic acid was added into the medium. Antioxidant property of ascorbic acid plays a vital role to block the leaching of phenolic compound (Verma and Kant 1999).

The suspension cells, which were grown in media augmented with BAP (3mg L^{-1}), NAA (4 mg L^{-1}) and ascorbic acid (1 mg L^{-1}) were further cultured up to 28 days and subjected for enzymatic and non enzymatic biochemical activity and PCV calculation as well.

PCV estimation

The suspension cells were grown for 28 days and a significant increase of PCV was recorded up to 21st day of culture. The highest (0.266 ml pellet/ 5 ml culture) PCV was detected on 21st day of culture. A slight decrease in PCV (0.24 ml pellet/ 5 ml culture) was observed on the 28th day of culture which was not very significant (Figure 4). Physical appearance of suspension cells was completely

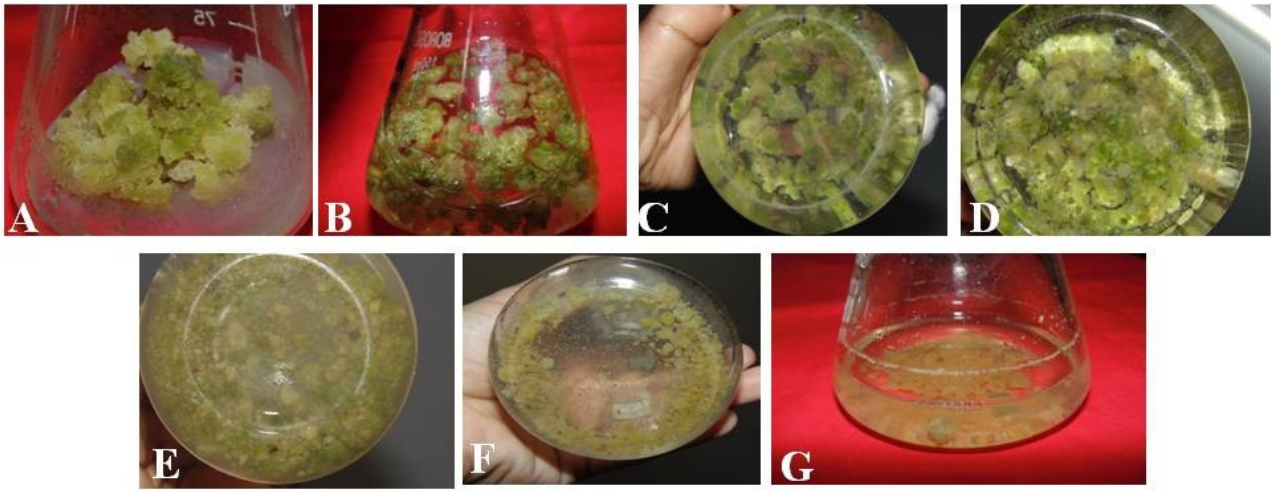

Fig 2. Cell suspension culture of *Stevia rebaudianaa.* **A.** *In vitro* grown callus of *Stevia rebaudiana*. **B.** transfer of callus clumps on liquid medium (3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA). **C.** suspension after first subculture. **D.** suspension after second subculture. **E, F, &G** suspension after 15th day of successive subculture in 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA medium with 1mg L^{-1} Ascorbic acid medium.

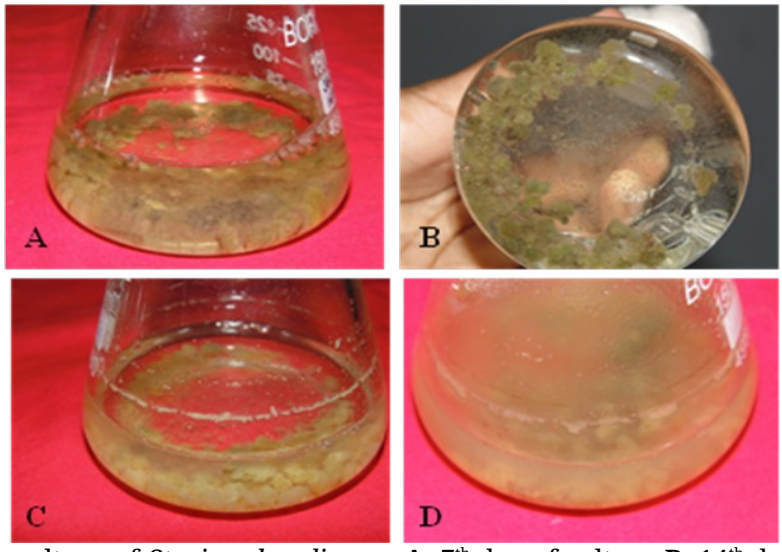

Fig 3. Cell suspension culture of *Stevia rebaudianaa.* **A.** 7th day of culture **B.** 14th day of culture **C.** 21st day of culture **D.** 28th day of culture

different on increasing day of culture. The cells were appeared green on the 7th and 14th day of culture while the culture of 21st day was light yellow with a minor impression of green color. The culture of 28th day was completely turned brown (Figure 3). The finding of present endeavour was shown similarities with the study done by Patil *et al*. (2003) who developed long term suspension cell culture of *Lycopersicum*, He found that the PCV in the *Lycopersicum* cells were stable up to 3 to 5th day, but got a sudden increase on 8th day of culture, which was maintained long term. Mathur and Shekhawat (2013) were also found higher PCV up to 21st day of culture in the 2,4- D harboring medium.

The cells were supposed to be in LOG phase up to 7th day of culture so the Initial PCV was slightly stable. In the Log Phase, cells prepared for segregation and division by using available nutrient in the medium. Cells were further progressed in exponential phase (14th day), in which cells showed a rapid division and also increase in size. This phenomenon supports the finding of increased PCV in suspension cells. In the next phase (stationary phase) (21st day) cells were getting stability and enlarge their size so the PCV again got increased. The final phase of cell growth was accounted as death phase or apoptotic phase in which cell started to get die and depletion of the PCV was clearly observed. Colour of cells were also started to get brown.

Estimation of enzymatic and non enzymatic biochemical activity

The amount of MDA content was recorded high (2.899 µM G^{-1} fresh weight of tissue) on the 7th day of culture which got an unambiguous decrease on 14th to 21st days. The MDA content was significantly elevated on 28th day of culture, when cells reached on death phase (Figure 4). At that stage, the

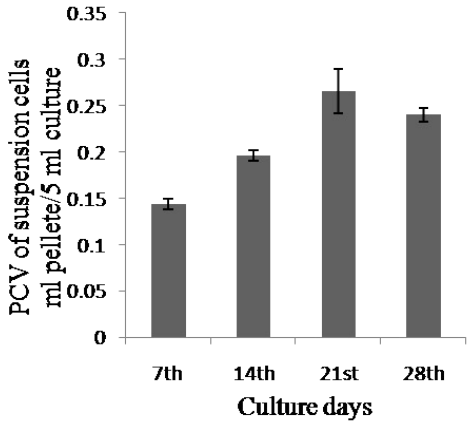

Fig 4: PCV of suspension on experimental time interval

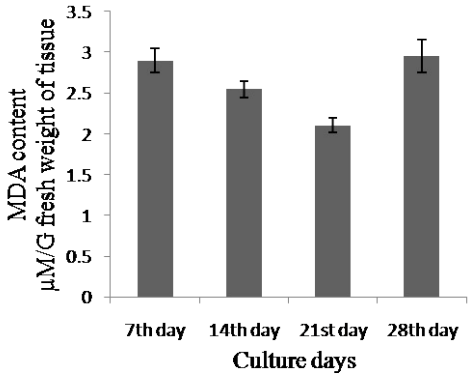

Fig 5: Altered concentration of MDA on different growth phases of suspension cell culture.

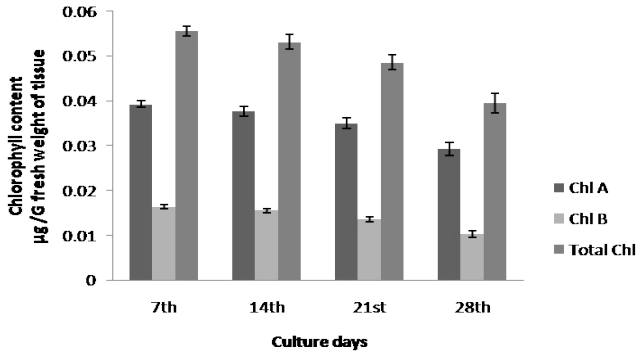

Fig 6: Estimation of chlorophyll on various phase of growth phase of suspension cell culture

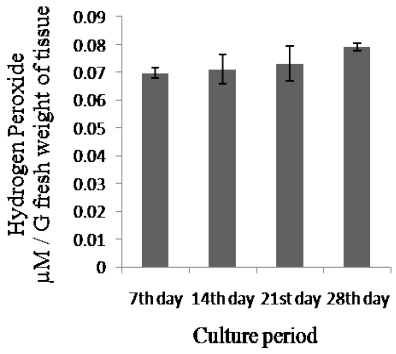

Fig 7: Estimation of Hydrogen peroxide on various growth phases of suspension cell culture

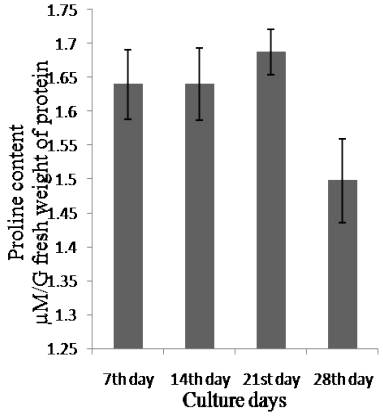

Fig 8: Estimation of Proline on various growth phases of suspension cell culture

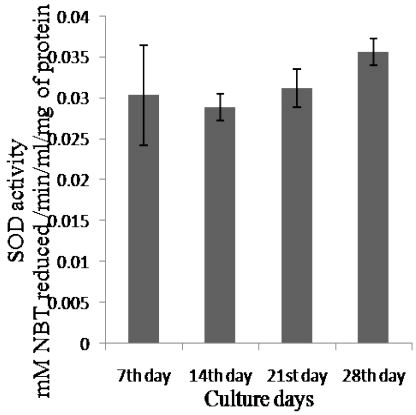

Fig 9: Estimation of SOD activity on various growth phases of suspension cell culture

metabolic activity of cell started to impede which may cause the depletion of phospholipid membrane. MDA is an indicator of the activity of a phospholipid membrane of cells. At the 28th day of culture the nutrient of media also started to get reduce which leads the cell to death. The similar results of alleviated concentration of MDA in coffee suspension cells were also observed by Gomes *et al*. (2006) while Ali *et al*. (2006) found stable concentration up to 9th day of culture in the *Panax ginseng* suspension cells.

Chlorophyll concentration was visibly decreased. The initial days of culture have green color, but with increasing the culture day's suspension cells turned brown on 28th day.

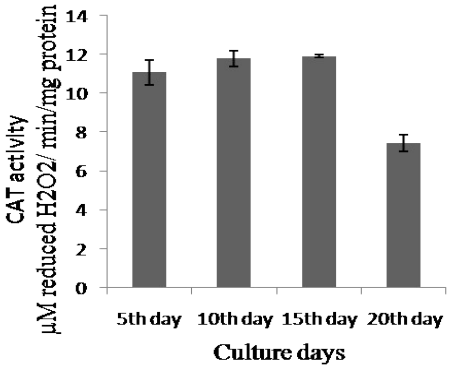

Fig 10: Estimation of CAT activity on various growth phases of suspension cell culture

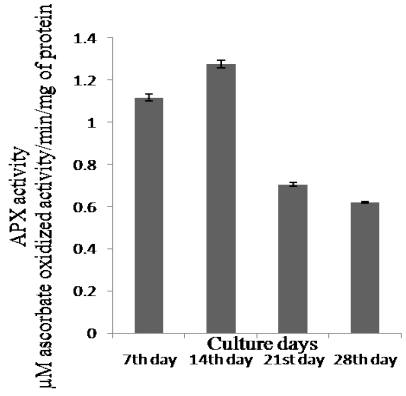

Fig 11: Estimation of APX activity on various growth phases of suspension cell culture

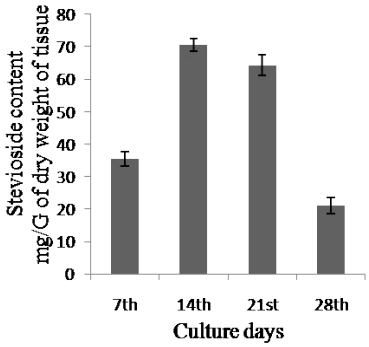

Fig 12: Estimation of Stevioside concentration on various growth phases of suspension cell culture

Spectrophometric calculation also demonstrated the identical results. On the 7th day of culture the total chlorophyll content was recorded 0.055 µg G^{-1} of fresh weight of tissue, which got decreased by 29% and reached upto 0.039 µg G^{-1} of fresh weight of tissue. Phenolic secretion of the suspension cells was controlled by antioxidant activity of Ascorbic acid (Verma and Kant, 1999). With increasing the period of culture ascorbic acid concentration might be depleted, which gave rise to the excess phenolic secretion and turned cell brown. The death phase also might be a reason of reduced chlorophyll content (Figure 6).

The H_2O_2 content was shown a continuous increase with a progressive culture period (Figure 7) while proline concentration got reduced on 28th day with elevation up to 21st day (Figure 8). Hydrogen peroxide plays an important role to trigger the synthesis of defensive compounds in plants at abiotic and biotic stress condition, thus known as the important signaling molecule (Maffei *et al*., 2009; Noreen *et al*., 2009; Kawano, 2003; Barbehenn *et al.*, 2010). Elevated amounts of H_2O_2 illustrates the generation of stress condition in the suspension cells with increasing the time interval of culture. Enhanced period of culture can reduce the essential nutrient for the development of cell and create a stress condition within the cells. Proline is well known as a defensive amino acid of plant. At the time of stress, its level got increased to protect the cell from any toxic condition. Higher proline concentration indicates the nutrition depletion and higher phenolic secretion generated stress condition within the cell. Proline is a well known osmolyte as well as stabilizer for complex II electron transport (Hamilton and Heckathorn, 2001) membranes, 3D structure of proteins (Holmström *et al.*, 2000) and enzymes (Mäkelä *et al.*, 2000). Enhanced amount of proline attributes the enhanced activity of proline biosynthesis enzymes like ornithine aminotransferase and pyrroline-5-carboxylate reductase, as well as inhibition of proline degrading enzymes like proline oxidase and proline dehydrogenase (Kishor *et al.*, 2005).

Antioxidant enzymes also showed alteration in their quantity. H_2O_2 is known as the precursor molecule for activation of gene expression and antioxidant enzymes (Foyer *et al.*, 1997; Morita *et al.*, 1999; Prasad *et al.*, 1994; Hong *et al.*, 2009). Superoxide Dismutase concentration got enhanced with increasing period of culture (Figure 9). SOD is free radicals, produced by O_2 present in the plant during the stress condition, which can be generated through some biotic and abiotic factors. It can be a reductant and an oxidant both and can directly determine the level of O_2 and H_2O_2 in stressed explant. The amount of CAT enhanced with increasing days of the culture period (Figure 10), while an inhibition was observed in the quantity of APX (Figure 11). Slaymaker *et al.* (2002), Yang *et al.* (2008), and Wang *et al.*, (2009) reported that the Catalse and Ascorbate peroxidase are the enzymes to catalyze the H_2O_2 produced in stress plants. The higher concentration of APX might be the reason of exogenously applied ascorbic acid in the suspension cell culture. With increasing the time, the concentration of ascorbic acid got reduced, which showed a reduction in the amount of APX. At the same time CAT showed enhanced activity up to the 21st day of culture but got reduced on 28th day. Although Slaymaker *et al.* (2002), Yang *et al.* (2008), and Wang *et al.* (2009) reported that CAT and APX are responsible for quenching of H_2O_2. Enhanced CAT and APX might the reason of higher demand for H_2O_2 quenching. Reduced amount of CAT and APX suggested that the enhanced production of H_2O_2 leading to cell death is not correlated with the inhibition of H_2O_2 scavenging enzyme (Tenhaken and Rubel, 1997; Rao *et al.* 1997).

Isolation and quantification of Stevioside

The amount of stevioside got increased up to the 21st day of culture, but on the 28th day it got

reduced in suspension cells of *S. rebaudiana* (Figure 12). At this stage suspension cells were reached up to exponential growth phase, which is highly significant for the production of secondary metabolites. Furthermore, H_2O_2 has been reported to increase the secondary metabolite production in plants (Yuan *et al*., 2001, 2002). The present study further strengthens this view. In nature, secondary metabolites are found in very less quantity in plants, but they are quite useful because of their medicinal properties (Croteau *et al.*, 2000; Dewick, 2002). Production from single cell is always a better way to achieve uniform quality, good amount and easier extraction of secondary metabolite (Davis and Deroles, 2014).

Conclusion

S. rebaudiana is well known antidiabetic sweetener plant, with secondary metabolite like stevioside. The production of secondary metabolite production is always limited in the plant in natural environment condition. The suspension cell cultures might be used for the enhanced production of stevioside with scale-up it production upto bioreactor level. The study of antioxidant enzyme can be helpful for the long term culture of *S. rebaudiana* suspension culture. The study can provide a base to understand the culture condition and nutritional demand of suspension cells, so that they can grow and maintain for the long time without inhibition of secondary metabolite. Production of higher amount of stevioside from cell suspension culture might be used commercially. Further research in the field of long term preservation of cell suspension culture is needed.

Authors Contribution

The study was hypothesized by AA and GSS and all the experimental work was executed by SD.

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