

AN APPROACH FOR IMPROVING STEVIOL GLYCOSIDES AND PRODUCTIVITY OF *stevia rebaudiana* *In vitro*

ROBA M. ISMAIL¹, WAFAA, E. GRAD² AND MONA, I. MASSOUD²

1. Agric. Genetic Engineering Research Institute, Agric. Research Center, Giza, Egypt

2. Sugar Crops Research Institute, Agric. Research Center, El-Sabahia, Alex. Egypt

S *tevia rebaudiana* is an important source of active natural compounds, especially steviol glycosides that are present mainly in the leaves (Modi and Kumar, 2018). Recently, stevia leaves has attracted economic interest as a new promising sweet-tasting calorie-free botanical food stuffs on the world market for their content of protein, antioxidant compounds, vitamins ,minerals, pigments, inorganic constituents and steviol glycosides that used as a sugar substitute or dietary supplements (Singh *et al.*, 2019). It has become well known not only for its sweetness but also for offering to biological properties and therapeutic benefits, where have shown activity in cancer prevention, as well as anti-diabetic, anti-obesity, anti-hyperglycemic, anti-hypertensive, anti-inflammatory, antibacterial or immunomodulatory, anti-diarrheal, and diuretic effect (Tadhani and Subhash 2006; Barriocanal *et al.*, 2008 and Bernal *et al.*, 2011). The leaves contain several steviol glycosides, which differ in chemical structure characteristics and sweetening properties up to 250-300 times sweetening potency that of sucrose. The major constituent being stevioside and rebaudioside A are constituting about

50% of the total glycosides and other minor amounts of rebaudiosides B, C, D, E, F, dulcoside A and steviolbioside (Bergs *et al.*, 2012 and Prakash *et al.*, 2014).

Hence, there is a need to replace sugar with low-calorie stevia sweeteners as a natural plant source which will improve of the products nutritional and the cultivation of *S. rebaudiana* Bertoni has successfully expanded to several countries (Sicžlabur *et al.*, 2013 and Singh *et al.*, 2019). Therefore, *in vitro* propagation or plant tissue culture appears as an alternative technique for rapid multiplication of stevia within a short period of time and can be improved a quality and quantity of planting (Das *et al.*, 2005). Also, a large number of genetically similar or genetic improvement and disease-free plantlets can be obtained with higher levels of the sweetening compounds, larger foliar mass, and resistance to drought through direct organogenesis of *in vitro* propagation (Dhananjay and Deshpande, 2005).

Stevia rebaudiana can be propagated by two ways seeds and stem cuttings. But, seeds propagation have many problems like very small in size, low fer-

tility, self-incompatibility, poor germination and heterogeneity, which subsequently change the level and structure of steviol glycoside (Sivaram and Mukundan, 2003 and Abdullateef *et al.*, 2012). Also, propagation by stem cutting has many problems like low frequency of shoot occurrence and physical damage of the mother plant (Yadav *et al.*, 2011). Therefore, *in vitro* culture was perfect useful tool for *S. rebaudiana* mass clonal propagation (Ramírez-Mosqueda and Iglesias-Andreu, 2015). Plant tissue culture as an important tool for the continuous production of active compounds including secondary metabolites and engineered molecules (Kadhim *et al.*, 2014). Similarly, in order to obtain homogeneous and highly vigorous plants in a short time, plant scientists are concentrating on developing optimal protocols for stevia species with industrial and medicinal importance. This can reduce the time, costs, rapid production, and scalability compared to those offered by alternative sources. The morphogenesis response seems to be highly dependent plant growth regulators used in the media, which is again cultivar and genotypic specific.

However, many studies examining the development of optimum protocols for the propagation in a short period (Depuydt and Hardtke, 2011; Razak *et al.*, 2014 and Blinstrubienè *et al.*, 2020) and total phenolic content and antioxidant activities (Roberts and Renwick, 2008), of *S. rebaudiana* *in vitro*. Also, some studies are focusing on the steviol glycosides content under the optimal light *in vitro* grown *S.*

rebaudiana plants (Bondarev *et al.*, 2001; and Ladygin *et al.*, 2008) to improve physiology and medicinal properties in leaves. As the Joint FAO/WHO Expert Committee on Food Additive (JECFA) and Food and Drug Administration have approved rebaudioside-A with purity >95% and accepted daily intake (ADI) of stevioside up to 5.0 mg kg⁻¹ body weight. Further research and development need to be carried out to improved stevia varieties with higher yield and quality through plant breeding methods and biotechnological approaches. The purpose of this research was established an efficient regeneration protocol for enhance of steviol glycoside compounds as quality with improving productivity of stevia yield and evaluate the possible suitability of this technique to be applied to rapid production of selected plants.

MATERIAL AND METHODS

Explant source

Stevia rebaudiana plant namely Spanti was obtained from Sugar Crops Research Institute, Agricultural research center, (ARC). Under aseptic conditions in laminar airflow cabinet at Gene Transfer Laboratory (GTL), Agriculture Genetic Engineering Research Institute (AGERI), healthy young plants were collected from green house and washed under running tap water for 15 minutes. Thereafter, the explants were sterilized with 70 % (v/v) ethanol alcohol for a few second and then by 5% Clorox for 5 min. Explants were washed with sterile distilled water three

times, followed by 0.1% (w/v) mercuric chloride (HgCl_2) for another 5 minutes and rinsed with sterile distilled water three times.

***In vitro* studies**

The young leaves and hypocotyl as explants were cut into pieces of 2x2 cm then cultured on different MS medium on three stages to select the best media for callus, shoot and root formation. These stages were as follows:

1. Callus induction stage

The explants were cultured on MS medium (Murashige and Skoog, 1962) containing different concentration and combination of plant growth regulators (PGRs) Naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-Benzylaminopurine (BAP) as specified in Table (1). All media were with sucrose (30 gm/l) and solidified with agar (8 gm/l) then adjusted pH value to 5.75 ± 0.05 before autoclaving at 121°C for 20 min. Parameters callus biomass was investigated as described by Nosov (2011) on intervals for period of 30 days after culturing and incubating in growth chamber at $27 \pm 2^\circ\text{C}$. The experiments were performed in triplicate.

2. Differentiation shoot stage

Callus was transferred on MS medium containing different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 or 3 mg/l)

3. Root formation stage

Differentiated shoots were transferred to MS supplemented with 1.0 mg/l of indole-3-butyric acid (IBA).

Effects of light and dark on productivity and quality callus

From the above biomass, the best media were selected to be applied to get healthy callus with steviol glycosides. The cultures were divided into two groups Group was maintained under 18hrs light (18 hrs light/6 dark) and other group under dark conditions.

Extraction of steviol glycosides

One gram of callus from each culture medium was macerated in three times 20 ml of MeoH 80% using shaker (Type U850. US). Each maceration stage was constant for 30 minutes for complete extraction. Macerates were filtered and collected then completed to known volume with 80% MeoH. The extract was passed through active charcoal for clarifying and kept until further analysis.

Evaluate of total steviol glycoside

Steviol glycosides were analyzed according to (Vanek *et al.*, 2001) using high-performance liquid chromatography (Agilent 1200, Agilent, Germany). The extract of steviol glycosides was separated on a reversed phase an Zorbax (NH_2) column (4.6x250 mm, 5 μm) with a mobile phase consisting of acetonitrile: water (84 : 70 v/v), pH 2.7 adjusted with H_3PO_4 , at a flow rate 2 ml/min with ambient temperature (25°C). Steviol glycosides were de-

tected at the wavelength of UV detector, 210 nm and compared with those obtained for pure standards of stevioside (ST), rebaudioside A (RA) and steviolbioside (stb) by means of retention time.

Acclimatization stage

Initially rooted plantlets were well washed in distilled water to remove the agar after pulled out gradually from the media. After sufficient roots were development, plantlets were subjected to hardening using two strategies, i.e. plastic pots with glass cover or Hoagland solution technique (hydroponic culture).

Hydroponic culture

As described by Hoagland and Arnon (1950), plants were transferred to Hoagland solution culture (company name). Topsin (0.5gm/l) was the fungicide that used to prevent fungal disses and air pump used for gas exchange.

Adaptation in soil

It is important to mentioned two important points, the first one that stevia plants which transplanted to a pots covered either with glass or plastic cover to keep humidity, and the second that acclimatization stage placed in growth room and then acclimatized plants transfer to green house after three weeks. *Stevia* plantlets were acclimatized by culturing plantlets in pots contain beat moss, sand and perlite (1:1:1). Topsin was added immediately after planting and regularly irrigated with topsine. Disper root® in a concentration of 0.5gm/l was used to stimulate root development. Plants were covered either by plastic phytacone or

glass jars and incubated at growth room at 28°C for one month.

Statistical Analysis

The treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Production of stevia is performed by stem cuttings which requires high laboratory potential or seeds that have a weak germination rate which limit the large-scale cultivation of stevia (Goette-moeller and Ching, 1999). Therefore, tissue culture is an alternative way for solving the limitation of stevia propagation. *In vitro* micro propagation from shoot tip and leaf explant has been reported (Uddin *et al.*, 2006). Murmu *et al.*, (2016) developed an appropriate and faster protocol for *Stevia rebaudiana* Bertoni clonal propagation through callus and shoot culture. Taak *et al.*, (2020) optimize the best PGRs combination and concentration for shoot-induction, shoot-elongation and root-induction for stevia plant.

Callus formation

Two explants i.e., young leaves and hypocotyl segments of stevia were examined for their callus formation and proliferation ability on five different media No.1-5 (Table.1) Results showed that there were significant differences among all media with control; except medium contain only 2,4-D which young leaf explants failed to form any callus, while, the maximum callus initiation (100%) obtained from young leaf explants on 0.5

mg/l NAA. The lowest percentage of callus initiation (zero and 47.5%) obtained from hormone free medium and medium with 0.5 mg/l 2,4-D and 1.5 mg/l NAA, respectively (Fig.1a). Mathur and Shekawat (2013) reported that development of callus didn't occur on medium without growth regulators (PGRs). While, Keshvari, *et al.*, (2018) showed that control without PGRs, had little calli which indicating the high reaction of stevia explants for callus production under aseptic *in vitro* conditions. In the case of callus developed from hypocotyl explants, the best callus formation (92.5%), was on media containing 2,4-D and NAA at concentration of 1.0 and 2.0 mg/l respectively. However, MS medium with only 2,4-D gave the lowest percentage of callus culture. (Fig.1b). Callus induction from hypocotyl explant revealed low response (30%) on the higher concentrations of 2,4-D (2 mg/l) and in the absence of NAA.

On the other hand to enhance callus formation from young leaf explants three other media were examined (No. 6-8, Table.1) in addition to the previous five media. Results showed that the young leaf explants couldn't develop callus in the absence of NAA. Medium containing 1.0 mg/l NAA revealed 95% regeneration efficiency and highest weight of callus (0.35gm) followed by medium with 1mg /l BAP and 1 mg /l 2,4-D (0.16 gm) after 4 weeks. Statistical analysis confirmed significant differences among all media under study (Fig. 2). Metry *et al.*, (2003) were established *in vitro* propagation system for *S. rebaudiana* using nodal cutting

as an explant and MS medium supplemented with different combinations of PGRs. They found that callus formation obtained in the presence of auxin either NAA with BA or IAA with BA. Abd El-Motaleb *et al.*, (2015) cultured leaves and nodal segment explants of *S. rebaudiana* plant on MS medium supplemented with different concentrations of 2,4-D (0.0, 0.5, 1.0, 2.0 and 4.0 mg/l), they found that the highest amount of callus was found on medium with 2.0 mg/l 2,4-D while, medium with 4.0 mg/l 2,4-D given the poorest callus, indicating that the higher concentration was not appropriate for callus initiation. Murmu *et al.* (2016) found that the best calluses induction on medium contain 2,4-D and NAA (100%) was (0.5 +1.5 mg/l) when using young, leaf and nodal segments explant. Taak *et al.*, (2020) found that callus formation and maximum callus induction was achieved on medium containing 2 mg/l 2,4-D and 1 mg/l Kinetin with leaf disc explants as 80% of callus formation was achieved with leaf segments comparing to 60% with nodal, they also found that leaf discs were the most efficient for callus formation comparing with shoot tips segments.

Differentiation shoot stage

Obtained callus from different explants were cultured on different concentration of BAP in order to differentiate into shoots. Callus developed from hypocotyl failed to differentiate, while callus resulting from young leaf explants succeeded in differentiate and giving multiple shoots. Medium with 2.5 mg/l BAP re-

vealed highest percentage of callus differentiation (24%) among all treatments (Fig. 3), it was also the best in terms of the number of branches resulting from the callus. This data is agreement with the result has been previously obtained by Murmu *et al.*, (2016) who found that optimal stevia shoot initiation was observed on MS medium supplemented with (0.5-2.0 mg/l) BAP.

Root formation

The obtained shoots were then transferred to MS medium with 1.0 mg/l IBA for root formation, all shoots were produced roots. Metry *et al.*, (2003) found that MS medium supplemented with 0.01 mg/l IBA with 10 mg/l GA3 and STS was the best medium for stevia root formation. Verma *et al.*, (2011) found that higher concentration of IBA (2.0 mg/l) gave highest root formation (69.76%) and early root initiation (7.1 days). Noordin *et al.*, (2012) illustrated that increasing of IBA concentration gradually decreased root induction as 0.25 mg/l IBA showed the best effect in promoting root formation comparing with different concentrations of IBA ranging from 0, 0.25, 0.5, 1.0 and 1.25 mg/l. Our results is consistent with the results of Abdul Razak *et al.*, (2014) and Murmu *et al.*, (2016) that showed 1.0 mg/l IBA was the superior concentration for root induction in stevia plant. Moreover, Attaya (2017) showed that the optimum root growth root growth with (76.6 %) root formation was observed using 1.0 mg/l IBA on half strength MS medium.

Regeneration steps start from the callus formation until reaching the root formation stage was illustrated in Fig. (4).

Identification and quantitative analysis of steviol glycoside compounds by HPLC in stevia leaves extract.

In this investigation, the different concentrations and combination of NAA, 2,4-D and BAP have positive effect on total biomass and steviol glycoside content in *S. rebaudiana cv. Spanti in vitro*. The combination with NAA, or BAP and 2,4-D effects in MS medium on steviol glycoside compounds in stevia callus under *in vitro* condition determined with HPLC analysis. The concentration of steviol glycosides were calculated as percent of total area while the concentration of stevioside (ST) and rebaudioside A (RA) were calculated as weigh percent of the callus. Results showed that the steviol glycosides were affected by both type and concentration of hormone in the medium. It can be concluded that steviol glycoside in supplemented medium were higher than that in mother leaves or MS free hormone (Fig. 5 and Table 2). The average ST and RA values in mother leaves were lower than in other media. This result is in agreement with Hsing *et al.*, (1983), who reported that stevioside content in callus was 16.24% which it was two and four times higher than that in the leaf and flowers of the same plant. However, the steviol glycoside content in callus cultures might even be twice as high as in stevia leaves (Luwańska *et al.*, 2015 and Pandey *et al.*, 2016). According to Nazishb *et al.*, (2013), the addition of BA alone into the

MS-medium influences the steviosides content (30.89 $\mu\text{g/g-DW}$ to 63.77 $\mu\text{g/g-DW}$). The range of stevioside content was from 8.38 to 49.21 mg /g the highest was in callus cultured on MS medium supplemented with 1 mg/l NAA followed by MS medium supplemented with 0.5 mg/l BAP and 1.5 mg/l 2,4-D and similar to that of MS medium supplemented with 1 mg/l BAP and 1 mg/l 2,4-D (Fig. 5). Such increase may be attributed to the combination of phytohormones *in vitro* culture. The studies of Bondarev *et al.*, (2003), Luwańska *et al.*, (2015) and Pazuki *et al.*, (2019) indicated that the presence of PGRs improve and regulate biochemical processes in plant, provide accurate growth and cell division *in vitro* culture and influenced on the amount of steviol glycoside (SvGls) profile when the addition of combination of these PGRs in media. Rebaudioside A as a high potency sweetener has a better quality of sweetness than stevioside which has a bitter aftertaste character that makes it difficult to use in many foods. Stevioside is 140 times sweeter than sucrose, while rebaudioside is 240 times sweeter (Prakash *et al.*, 2008). It can be noticed that the a higher RA content (24.22 mg/gm) was obtained in MS medium containing of 1 mg/l BAP and 1 mg/l 2,4-D. The biosynthesis of steviol glycosides in stevia cell cultures depends to greater extent on genotypic of cell strain and composition or concentration of components of the nutrient medium (Bondarev *et al.*, 2019 and Blinstrubienė *et al.*, 2020). It can be concluded that the differential of steviol glycoside concentration under all applied

conditions may be related to metabolic shift to the methylerythritol phosphate pathway (MEP) in order to synthesize the chlorophyll phytophilic chain and then production of steviol glycosides, starting from the induction of ent-kaurenoic acid hydroxylase (KAH) activity in green stevia roots (Libik-Konieczny *et al.*, 2020). Blinstrubienė *et al.*, (2020) recorded variations in the steviol glycosides, bioactive compounds and antioxidant activity of the cellular compounds of callus from the leaf and stem explants when they studied that addition 2.0 μM NAA and 5.0 μM proline in media on their yield and quality characteristics. The literature also provides *In vitro* plant tissue culture offers a probability of generating plant material containing a great level of stable selected secondary metabolites as SGs (Ramakrishna and Ravishankar 2011; Hussain *et al.*, 2012 and Konieczny *et al.*, 2020). Also, an increase in RA/ST ratio was summarized in Fig. (5). Tabulated data indicated that the RA / ST ratio was increased with increasing BAP concentration from 0.5 to 1mg/l in MS medium to 0.856 and 1.33 respectively and the lowest rate (0.12) was recorded in mother leaves while MS medium containing 1mg/l NAA was recorded 0.435. Development of *S. rebaudiana* variety with a higher of RA and a reduced stevioside is the main aim of plant breeders for the improvement and utilization of this source of natural sweeteners. Therefore, it can be selected to investigate the effects of photoperiod on steviol glycoside compounds in MS medium containing 1mg/l BA and 1mg/l 2,4-D and MS medium containing 1mg/l NAA.

Effect of photoperiod (light: dark) on steviol glycoside compounds

As the objective of the present study was to improve the quality of leaves, it was necessary to study the effect of photoperiod for 18 hrs light and 24 hrs dark on steviol glycoside compounds. From the suggested combination media two were selected to be exposed to 18 hrs light and 24 hrs dark photoperiod. Differences in steviol glycosides percent have been shown in Table (2) and Figs. (6 and 7). Light encouraged on production of high amount and different steviol glycosides component in callus. MS medium supplemented with 1 mg/l BAP and 1mg / l 2,4-D was more appropriate medium than MS for stevia callus production. Stevioside concentration as area % was considerably higher in MS medium supplemented with 1mg/l NAA under dark (86.76%), compared to the MS medium containing 1 mg/l BAP and 1mg / l 2,4-D (52.94%). Medium under light observed increase in area % of rebaudioside C , A and E than in under dark which showed more intense lingering sweetness than sucrose (Tao and Cho, 2020). Similar patterns of steviol glycoside increase for steviolbioside RA, and RE were found in MS medium supplemented with 1 mg/l BAP and 1mg / l 2,4-D (Fig.7) than 1mg/l NAA (Fig. 6). Light with an intensity of 8 Wt m⁻² at 25 C and about 70% air humidity was shown to activate the SGs in callus about two times higher of SGs (Yoneda *et al.*, 2018 and Bondarev *et al.*, 2019). Libik-Konieczny *et al.*, (2020) stated that

culture conditions and the type of stress factor led to changes in the morphology and an increase in oxidative stress manifested as an enhancement in endogenous hydrogen peroxide concentration in the cultured samples under light or in the medium with the highest osmotic potential as well as the increase in the expression level of ent-kaurenoic acid hydroxylase, responsible for the redirection of metabolic route to steviol glycoside biosynthesis pathway. The approved measure of sweetness quality is the ratio of RA/ ST. Under the photoperiod, this media gave high RA /ST ratio in light (1.33) than in dark (0.94). Medium MS containing BAP at 1 mg/l and 2,4-D at 1 mg/l under 18 hrs light gave good results in composition and rates of steviol glycoside. Furthermore, the sweetness intensity rate was found to be about 1.43 times sweeter than MS medium containing NAA at 1mg/l as sucrose equivalent that it had the highest weight of callus.

3-Acclimatization stage

Two methods have been tested, and the rooted shoots were transferred into aquarium containing Hoagland solution and/or in pots containing soil. The plantlets that acclimatized under Hoagland solution couldn't survive. On the other hand, the acclimatization under soil condition was successes as the plantlets survived with a percentage of 64%. It was observed that the thickness of stem played a role in acclimatization success. Results showed that the glass cover is better than plastic one as all plantlets under plastic

cover are died, while, the plantlets under glass cover survived (Fig. 8). Metry *et al.*, (2003) illustrated that the percentage of stevia survived plantlets ranged from 20 to 80% and sand-peat moss was the best potting medium at 50% (v/v). Verma *et al.*, (2011), optimize a protocol for *Stevia rebaudiana* Bertoni regeneration and acclimatization. They found that transfer plantlets to glass jar with polypropylene (PP) caps were better than plastic pots with polythene cover, as plantlets with glass jar appropriate relative humidity with high success during acclimatization. Razak *et al.*, (2014) illustrate that during acclimatization, transparent polythene can improve the survival plants as it control the humidity and can help in adaptation of the plants into new environment. They also showed that the plastic covering was essential for the successful plant acclimatization.

Conclusion

An efficient regeneration protocol for *S. Rebaudiana* (Spanti) was established in this study. From the aforementioned results, it can be concluded that young leaves as explants, for callus induction, were better than hypocotyl. BAP with concentration 2.5 mg/l was best condition for stevia regeneration and 1.0 mg/l IBA was the best for successful rooting. Medium supplemented with 2,4-D and BAP gave good results in steviol glycoside components and increased in rebaudioside A content with high rate RA/ST. MS media supplement with NAA(1mg/l) was the best for callus weight, acclimatization and adaptation in soil.

SUMMARY

Nowadays, *Stevia rebaudiana* leaves have attracted economic interest as a natural non-nutritive, non-toxic, high-intensity sweeteners as well as its therapeutic benefits. The aim of the study was established an efficient regeneration protocol to improve stevia's sweetness with enhancing productivity of stevia leaves as well as to evaluate the possible suitability of this technique to be applied to rapid production. The young leaves and hypocotyl of *Stevia rebaudiana* namely Spanti explants were cultured on a MS medium supplemented with different concentrations and combination of NAA, 2,4-D and BAP as plant growth regulators. The results showed that leaf explants obtained highest callus formation (100%) on MS medium supplemented with 0.5 mg/l NAA while callus formation from hypocotyl explants was (92.5%) on MS medium contains 1.0 mg/l 2,4-D and 2.0 mg/l NAA. Callus derived hypocotyl failed to differentiate, while callus resulting from young leaf explants were differentiated into shoots on MS medium supplemented with 2.5 mg/l BAP. MS containing 1 mg/l BAP and 1 mg/l 2,4D showed best results in steviol glycoside components and high rebaudioside A (RA) (24.22 mg/g callus) yield comparing with other used media under inoculation for 18 hrs light. Also this media gave the high rate of RA over stevioside (1.33) as an indicator of leaves quality with the sweetness intensity rate 1.43 times sweeter than MS medium containing 1mg/l NAA as sucrose equivalent

under the same experimental conditions. Medium with 1.0 mg/l IBA was suitable for rooting stevia shoots. Plantlets were acclimatized by 64% in the soil contain peat moss, sand and perlite (1:1:1) under controlled chamber at 28°C for one month which transferred to the green house.

Key words: Non-nutritive, callus formation, hypocotyl explants, steviol glycoside.

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Table (1): Different concentration and combination of PGRs for callus induction.

Medium No.	MS	NAA mg/l	BAP mg/l	2,4-D mg/l
Control	+	-	-	-
1	+	0.5	-	-
2	+	-	-	2.0
3	+	1.0	-	1.0
4	+	2.0	-	1.0
5	+	1.5	-	0.5
6	+	1.0	-	-
7	+	-	1.0	1.0
8	+	-	0.5	1.5

Table (2): Steviol glycoside compounds identified by HPLC in stevia callus and fresh leaves.

Compounds Concentration as Area%	Fresh leaves	Media				
		MS	MS + 1mg/l NAA		MS + 1 mg/l BA+ 1mg /l 2,4-D	
			Light	Light	Dark	Light
Steviolbio- side	-	5.86	-	-	11.43	0
Stevioside	45.09	91.03	36.91	86.76	18.54	52.94
Rebaudi- oside C	33.00	-	40.05	-	0	22.39
Rebaudi- oside A	3.11	-	9.03	5.61	28.13	2.40
Rebaudi- oside E	4.60	-	4.51	6.64	22.81	-
Unkown coumpounds	3.86	3.860	2.12	-	15.37	-

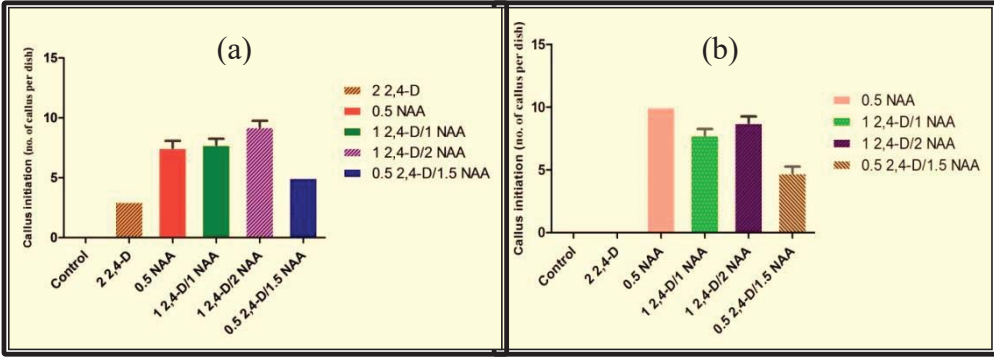


Fig. (1): Callus initiation from different plant growth regulators (a): leaf explants (b): hypocotyl explants.

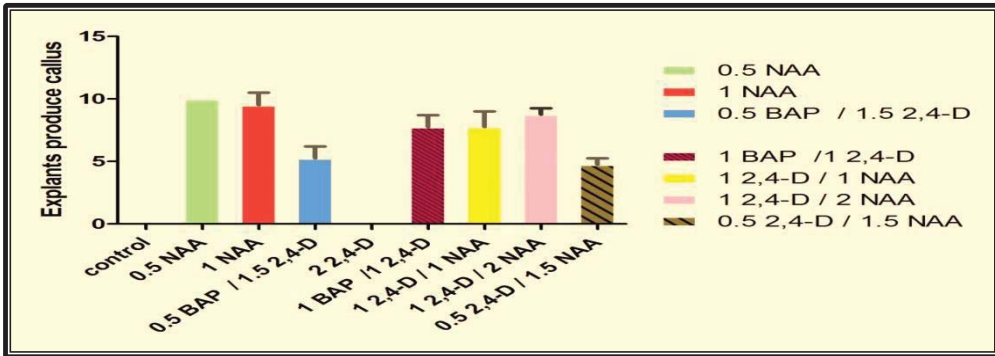


Fig. (2): Callus initiation using different growth regulators and young leaf explant.

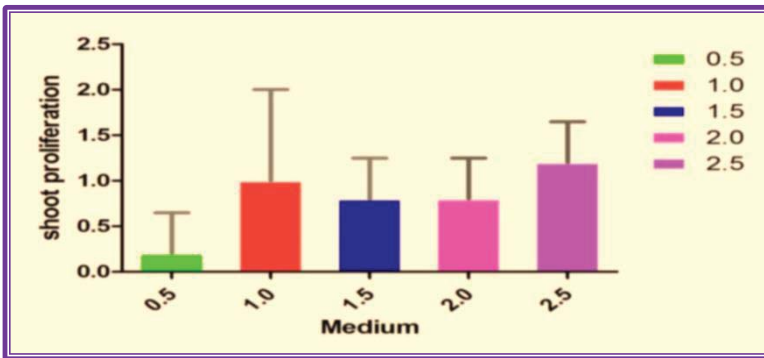


Fig. (3): Effect of BAP (mg/l) on shoot proliferation from calli explant regenerated from young leaf.

Stevia rebaudiana In vitro

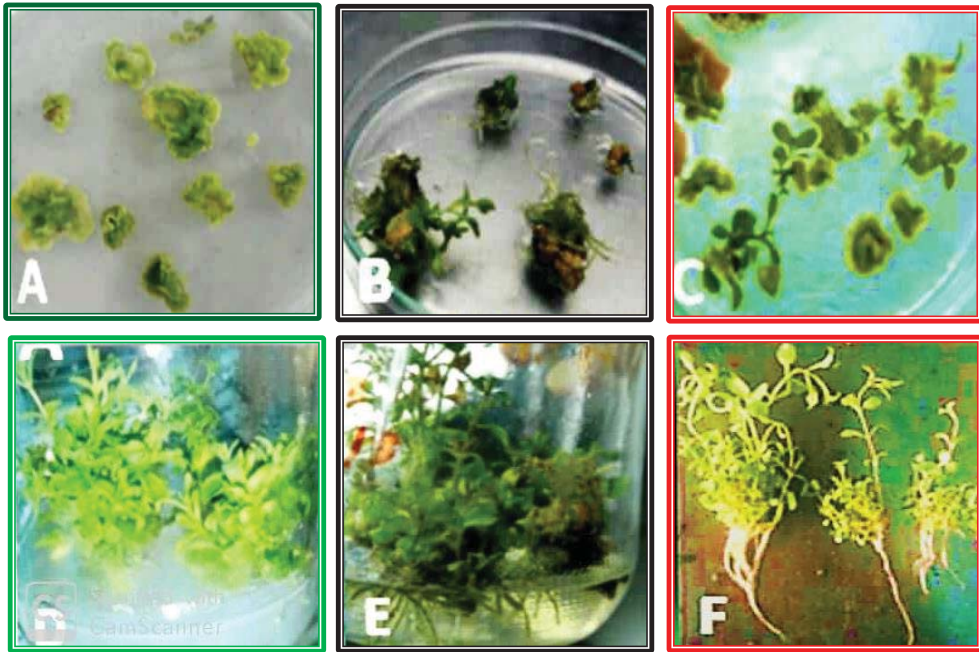


Fig. (4): Regeneration of *Stevia rebaudiana*.

- A: Callus production after 3 weeks of leaf explants on MS medium with 1 mg/l NAA
- B & C: Differentiated callus into shoot formation
- D: Shoots regeneration from calli explant on MS supplemented with 2.5 mg/l BAP,
- E: Root formation on MS with 1.0 mg/l IBA,
- F: Will rooted stevia plants

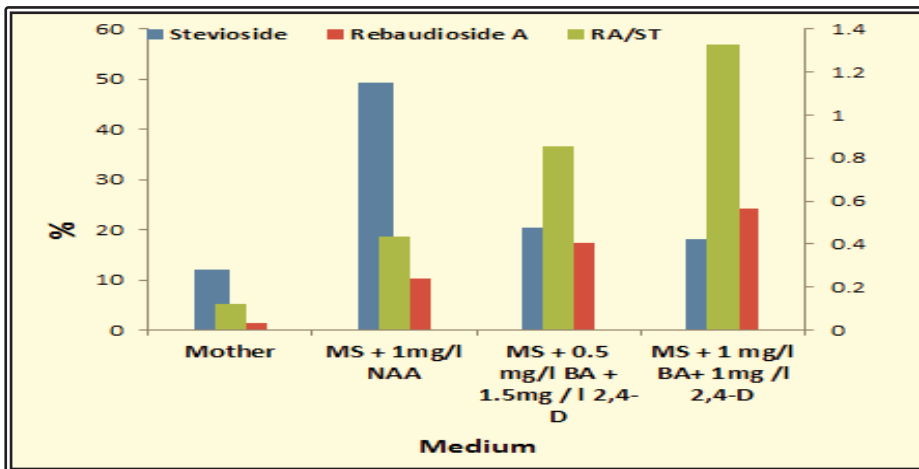


Fig. (5): The Effect of NAA, BAP and 2,4-D in MS medium on the content of stevioside(ST), rebaudioside A(RA) and RA/ST rate compounds.

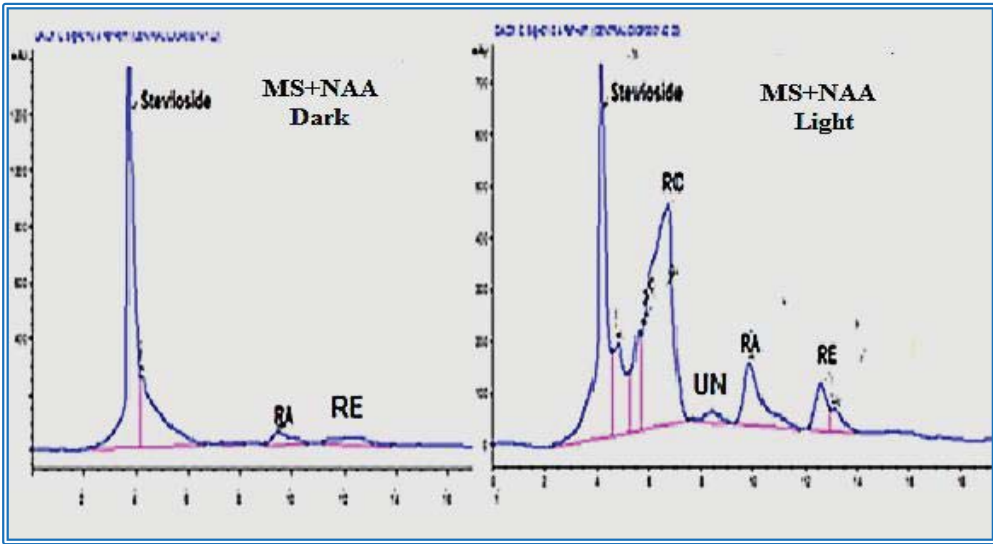


Fig (6): HPLC analysis of the steviol glycoside compounds in cultured callus on MS containing naphthaleneacetic acid (NAA 1mg/l).

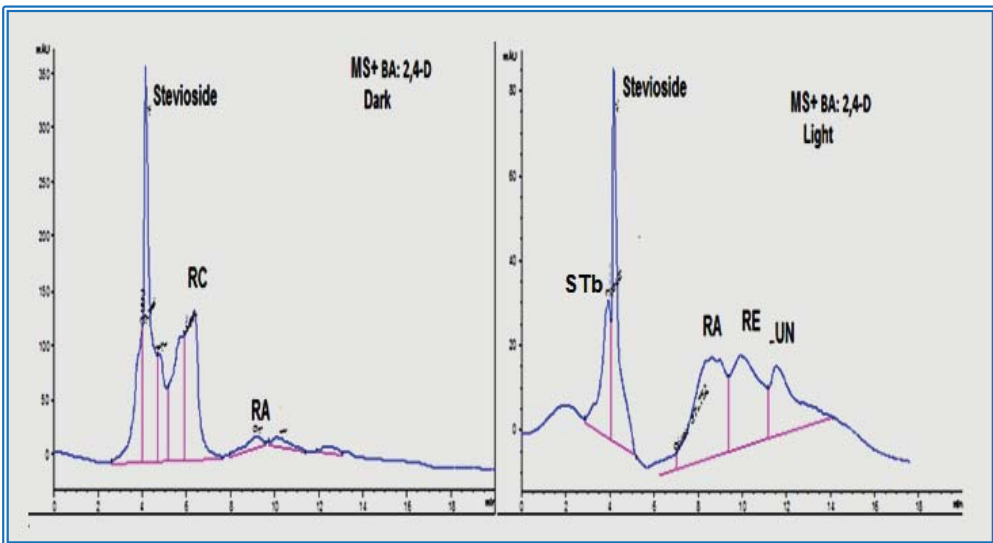


Fig .(7): HPLC analysis of the steviol glycoside compounds in cultured callus on MS containing 1mg/l benzylaminopurine (BAP):1mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D).



Fig. (8): Acclimatization stages for stevia plantlets under soil condition.
A: stevia plantlets with glass cover under growth room conditions,
B&C: stevia plantlets will survive when transplant to green house after three weeks.
D&E&F: well acclimatized stevia plant in green house.