ABANT IZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DETERMINATION OF REBAUDIOSIDE-A CONTENT OF IN VITRO PROPAGATED STEVIA (*STEVIA REBAUDIANA* BERTONI)

MASTER OF SCIENCE

AYŞE TUĞÇE İLAL

BOLU, AUGUST 2019

ABANT IZZET BAYSAL UNIVERSITY
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APPROVAL OF THE THESIS

"DETERMINATION OF REBAUDIOSIDE A CONTENT OF IN VITRO PROPAGATED STEVIA (*STEVIA REBAUDIANA* BERTONI)" submitted by **Ayşe Tuğçe İLAL** in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, The Graduate School of Natural and Applied Sciences of BOLU ABANT IZZET BAYSAL UNIVERSITY in 21/08/2018** by

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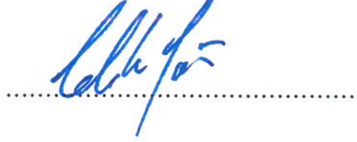
Member Assoc. Prof. Dr. Bahtiyar Buhara Yücesan Bolu Abant İzzet Baysal University	 …………………………………

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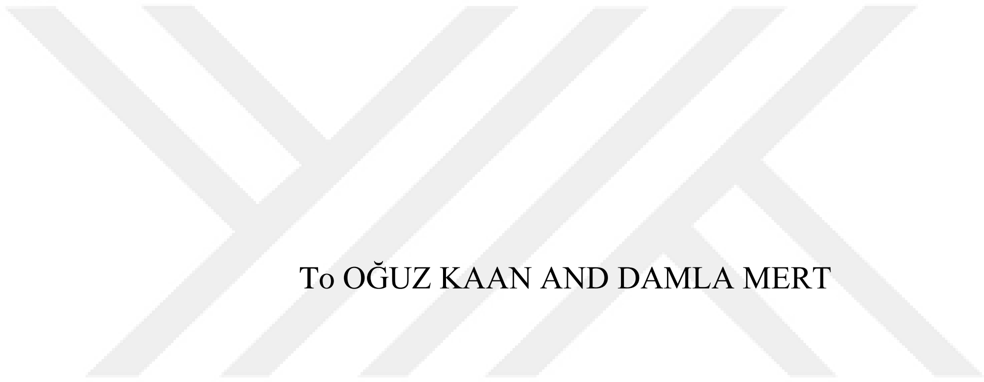

To OĞUZ KAAN AND DAMLA MERT

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

AYSE TUĞÇE İLAL

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ABSTRACT

DETERMINATION OF REBAUDIOSIDE A CONTENT OF IN VITRO PROPAGATED STEVIA (*STEVIA REBAUDIANA* BERTONI)
MSC THESIS
AYSE TUGCE İLAL
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. EKREM GÜREL)

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ABSTRACT

Stevia rebaudiana Bertoni is a perennial shrub which produces zero-calorie diterpeneglycosides in leaves. This study involves two main parts; seed germination and best nutrient combinations for in vitro propagation. In most of our experiments, number of leaves and nodes, shoot and root length, and steviolglycoside content were studied. The seeds of stevia were germinated in vitro under different light densities ranging from 0.8 to 5 klux. Of all the light densities tested, 5.0 klux was found to be the most effective for seed germination. Another experimental results with HPLC analysis showed that the control treatment produced nearly 10 times higher Reb-A than Fe-deficient treated plants. These results suggest that Fe affects growth and Reb A content in stevia plant. According to the results, highest Reb-A was found in stock 5 solution of Murashige and Skoog (St5) deficient medium (2.72%) with the lowest stevioside content (0.86 %) as compared to the other St5 treatments. This is the first study to observe the effects of different concentration of some selected microlements on in vitro culture of stevia plants.

KEYWORDS: *Stevia rebaudiana*, Reb-A, Stevioside, Iron deficiency, Light density, Micro elements

ÖZET

İN VİTRO ÇOĞALTILMIŞ ŞEKEROTU (*STEVIA REBAUDIANA* BERTONI) BİTKİSİNİN REBAUDIOSIDE-A İÇERİĞİNİN BELİRLENMESİ
YÜKSEK LİSANS TEZİ
AYSE TUĞÇE İLAL
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
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(TEZ DANIŞMANI: PROF. DR. EKREM GÜREL)

BOLU, AĞUSTOS - 2019

ABSTRACT

Stevia rebaudiana Bertoni, yapraklarda sıfır kalorili diterpeneglikozitler üreten çok yıllık bir çalı formunda olan bir bitkidir. Bu çalışma iki ana bölümden oluşmaktadır; (i) tohum çimlenmesi için en uygun ışık miktarının, ve (ii) in vitro bitki üretimi için en uygun besin ortamı kombinasyonun belirlenmesidir. Deneylerimizin çoğunda, yaprak ve boğum sayısı, sürgün ve kök uzunluğu ve steviolglikosit içeriği incelenmiştir. Stevia tohumları in vitro olarak 0.8 ila 5.0 klux arasında değişen farklı ışık yoğunluğu altında çimlendirilmiştir. Test edilen tüm ışık yoğunluklarından 5.0 klux'un tohum çimlenmesinde en etkili olduğu bulunmuştur. Diğer bir deneyimizin sonucunda, HPLC analiz işlemini, Fe eksikliğine maruz kalmış bitkilerin 10 kata yakın daha yüksek Reb-A ürettiğini gösterdi. Bu sonuçlar, Stevia bitkisinde Fe'nin büyümeyi ve Reb-A içeriğini etkilediğini ortaya koymaktadır. Sonuçlara göre, St5 eksikliği olan ortamda (% 2.72) en yüksek steviosid içeriği (% 0.86) ile diğer St5 uygulamalarına kıyasla en düşük Reb-A düzeyi gözlenmiştir. Bu çalışma farklı dozlarda mikroelementlerin in vitro kültüre alınmış stevia bitkisinde etkisinin incelenemesi bakımından ilk çalışmadır.

ANAHTAR KELİMELER: *Stevia rebaudiana*, Rebaudiosit A, Steviosit, Demir eksikliği , Işık miktarı, Mikro elementler

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LIST OF ABBREVIATIONS AND SYMBOLS

Chl-A : Chlorophyl-A
Chl-B : Chlorophyl-A
Fe0 : Fe-deficient MS medium
Fe5 :5 Mm Fe in modified MS medium
Fe10 :10 Mm Fe in modified MS medium (control)
Fe20 :20 Mm Fe in modified MS medium
Fe40 :40 Mm Fe in modified MS medium
Fe80 :80 Mm Fe in modified MS medium
GB5 : Gamborg's B5 medium
KC : Knudson C medium
N : Nitsch (N) medium
Reb-A : Rebaudioside A
SG : Steviol glycoside
ST : Stevioside
St5/0 : Defiencient stock-5 solution of MS
St5½ : Half stock-5 solution of MS
St5/1 : MS control
St5×2 : Two-fould stock 5 solution of MS
St5×4 : Four-fould Stock 5 concentrations of MS

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1. INTRODUCTION

In Turkey, 7.000.000 people are diabetes (*Diabetus mellitus*) with a growth trend, and many scientists suggest that 12.000.000 people will be suffered from diabetes in next ten years (TUİK 2012). For the prevailing of the most diabesitic cases, the employment of non-nutritive sweeteners can be considered as an alternative sweetener. *Stevia rebaudiana*, nontoxically zero calories plant, is highly adaptive to wide climatic ranges. In this respect, agricultural facilities on *S. rebaudiana* might be important for a consistent plant production concerning value-added sweetener compounds. *Stevia rebaudiana* is a bushy species belonging to the Asteraceae family that is native to Northeast Paraguay (Soejarto, 2002) and is commonly known as sweetleaf. The plant is known to exhibit medicinal properties that can be used to alleviate symptoms of type-II diabetes and other related diseases. Recently, due to great demand for low-calorie sweeteners, researchers have focused on *in vitro* production of *S. rebaudiana* (Ghanta *et al*., 2007). Although Turkey is one of the rich countries for sugar beet production, subtropical regions including western and southern coastal parts of Turkey might be available for *S. rebaudiana* planting (Amzad-Hossain *et al*., 2010). Domestication of *S. rebaudiana* in Turkey might provide a new insight towards commercialization in terms of great quality production of dry leaf materials and steviol glycoside (SG) have been approved , since 2013 by Turkish Government as a safe sweetener to be used as a food additive; from then, *S. rebaudiana* has a growing trend in public-sense (Yucesan *et al*.,2016).

For *S. rebaudiana* production, there are two critical points to be considered, namely the seed source and the field preference for planting (Felippe and Lucas, 1971). A good seed quality may be difficult to obtain as very often, small germination rates or, even in some cases, no germination at all can be seen, as well as various undesirable genetic variations etc. (Liopa-Tsakalidi *et al*., 2012). Similarly, habitat preference of *S. rebaudiana* as a tropical plant is another obstacle for European breeders (Gardana *et al*., 2003).

Plant tissue culture techniques might provide a new understanding for clonal production of *S. rebaudiana* having a great Reb-A content under *in vitro* conditions (Swanson *et al*., 1992; Bondarev *et al*., 2001, 2003; Sviaram and Mukundan, 2003; Thiyagarajan and Venkatachalam, 2012, Yucesan et al 2016. There have been several reports on *in vitro* regeneration studies for

S. rebaudiana during the last decade. However, either SG contents of the plantlets after being transferred to the field, or further conservation of the source plants underlying the future strategies of *S. rebaudiana* production have been very limited.

1.1 Literature Review

1.1.1 Botany of *S. rebaudiana*

Stevia rebaudiana is perennial species belong in to the Asteraceae (Compositae), commonly available around the region Rio Monday Valley of the Amambay mountain in Northeast Paraguay where it grows at altitudes between 200 and 500 m. The soil preference of the crop is upon to humid, sandy or loamy conditions (Soejarto, 2002). The name of Stevia was given by Cavanilles (Soejarto, 2002). Stevia known as sweet weeds, sweet leaf, sweet herbs and honey leaf is the most valuable tropical natural sweetener perennial herb at Paraguay (Rathore *et al*., 2014). *Stevia rebaudiana* is a 30-60 cm tall, pubescent herbaceous canopy snd perennial rhizomes, also with simple, opposite and narrowly elliptic oblanceolate or linear oblong leaves (Soejarto, 2002; see Figure 1.1). Its chromosome number is 2n=22 chromosome, and chromosomal rearrangements are common this species (Frederico *et al*., 1996; Oliveira *et al*., 2004).

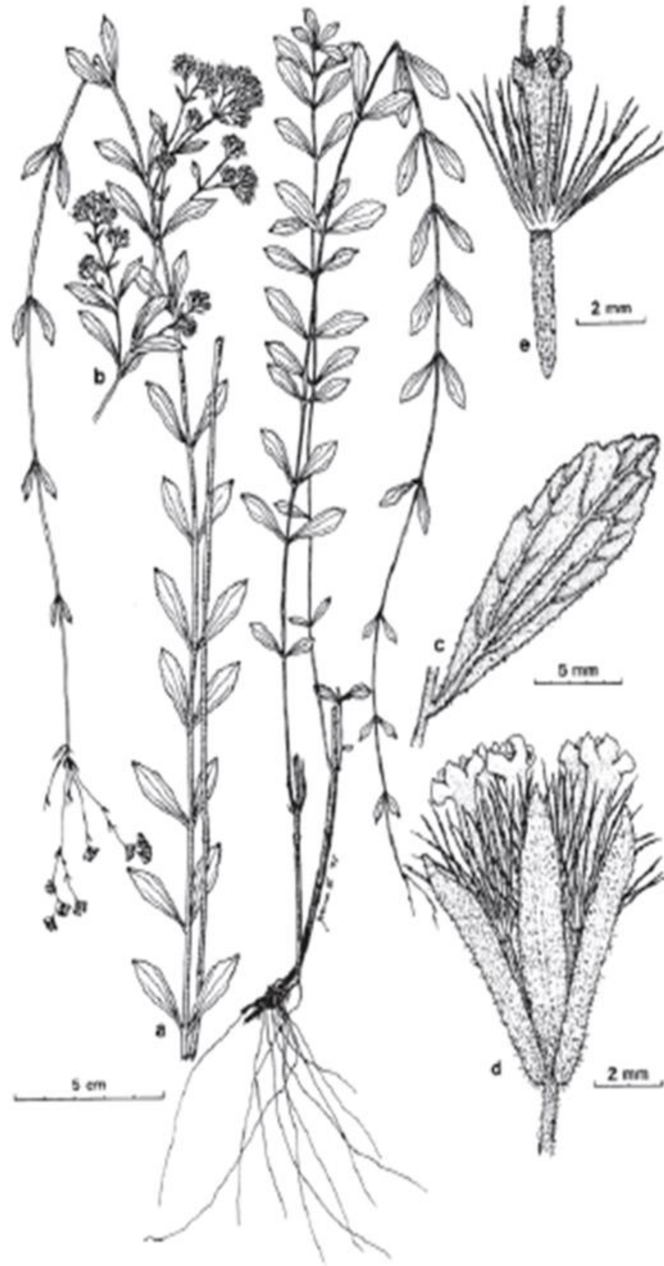


Figure 1.1: Analytical drawing of *Stevia rebaudiana* (transfered from Soejarto, 2002)

1.1.2 Biochemistry of Steviol Glycosides

Stevia has more than 100 compounds. The most commonly available compounds are the ent-kaurene diterponoid glycosides which are mainly stevioside (ST) and rebaudioside A (Reb-A) (Rieck, 2012). Stevioside is the major sweet-tasting glycoside in stevia and has been reported to be 250-300 times sweeter than sucrose (4g/L in water). Stevioside is about 5–15% the weight of dry leaves. Rebadioside A is another sweetetest ent-kaurene glycosides. To date, Reb-A is the second most abundant SG found in the leaves of *S. rebaudiana* which ranges between 25 and 54% ST in the leaves. Also, Reb-A tastes more pleasantly than ST, and thus, it is much better suited for using in food and beverages products. It has been noted that ST and Reb-A, with glucosyl groups of the former replaced in the latter by rhamnosyl groups. This rhamnosyl substitution decreases the sweetness intensity of the SG (Kennelly, 2002). Apart from *S. rebaudiana*, there is only one species, *S. phlebophylla,* containing sweet-tasting SG. In natural source, has been detected in the leaves of *S. rebaudiana* and *Rubus suavissimus*, the roots of the mangrove trees *Brugeiera gymnorhiza*, *Brugeira cylindirica* and in the endosperm of *Cucurbiata maxima* seeds (Ceunen and Geuns, 2013b).

1.2 Literature Review

1.2.1 Biosynthesis of Steviol Glycosides

The biosynthesis of ST and Reb-A was synthesized from kaurene via the mevalonate pathway (MEP pathway). The last step for MEP pathway, the production on isopentenyl diphosphate and dimethyl diphosphate are converted to geranylgeranyl diphosphate (GGDP). All diterpenes SG synthesized from GGDP, first by protonation-initiated cyclization to copalyl diphosphate (CDP) by CDP synthase (CPS). Kaurene is produced from CDP by kaurene synthase (KS). Kaurene enzymes from the gibberellic acid biosynthetic pathway have been identified and characterised from a number of plant species including *Stevia.* Steviol glycoside biosynthesis diverges from gibberellin biosynthesis with the hydroxylation. The aglycone SG has two hydroxyl groups, one attached to the C-19 and the other attached to the C-13. Stevioside is produced from glycosylation of the C-19 carboxyl of steviolbioside. The C-19 is glucosylated after the glucosylation of the C2 of the C13-glucose of steviolmonoside. Rebaudioside A is then synthesized by glycosylation of the C-3 of the C-13-*O*-glucose (Figure 1.2; Brandle and Telmer, 2007; Geuns, 2013).

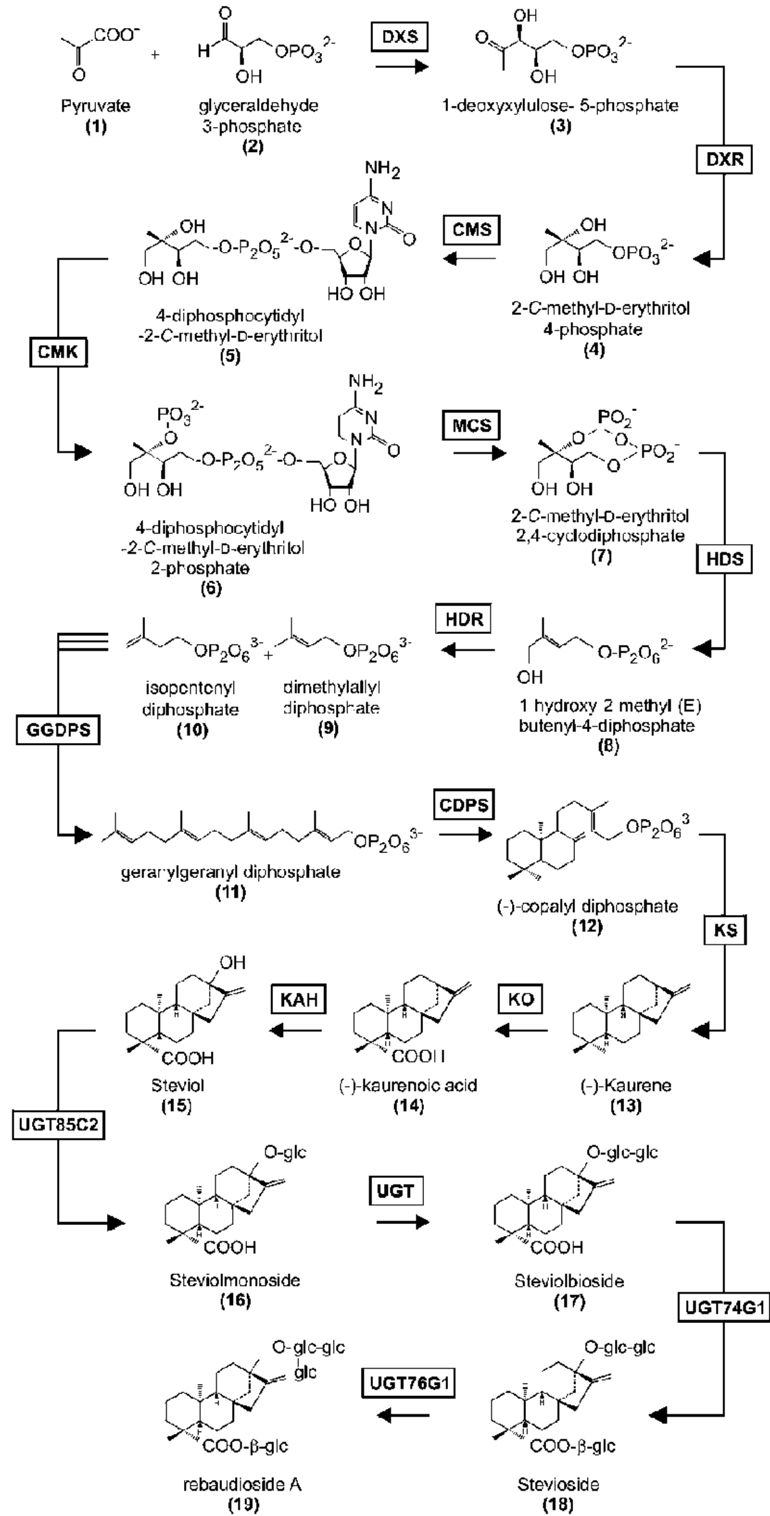


Figure 1.2: The biosynthesis of steviol glycosides via the MEP Pathway (Brandle and Telmer, 2007)

1.2.2 In Vitro Cultivation of *S. rebaudiana*

The seeds of *S. rebaudiana* show weak germination ratio and do not produce steady emergence, that results in a serious variability in terms of plant growth and maturity (Yadav and Guleria, 2012). Fertile seeds are dark black-colored. Therefore, in vitro techniques may be effective for alternative propagation over conventional breeding techniques (Rathore et al., 2013). Plant tissue culture are commonly employed for the largescale propagation of plant materials. Because of continuous deforestation and extensive collection, in vitro conservation of medicinal plant germplasm is important to support chemical analysis and genetic improvement studies (Yucesan et al., 2011). Successful direct organogenesis of stevia was achieved by using different of explant source in comparision to shoot tips, leaves, nodal cuts and axillary meristems (Gantait et al., 2015). According to Singh et al. (2014) reported that nodal segment is the best explant source in comparison to shoot tip and inter nodal segment in terms of multiple shoot induction in stevia. Similarly, Hwang (2006) showed that different plant growth medium containing MS (Murashige and Skoog, 1962), B5 (Gamberg et al., 1968), WPM (Lloyd and McCown, 1980) or SH (Schenk and Hilderbrant, 1972) has various shooting response by time. Most studies suggested that solid full-strength MS medium is very effective growth medium for shoot and root induction (Das et al., 2011; İbrahim et al., 2008; Gantait et al., 2015). Alternatively, Anbazhagan et al. (2010) reported that half strength Nitsch (N6) basal medium for root formation in stevia. On a contrary note, Thiyagorojan and Venkatacham (2012) made a report on MS medium fortified with various concentrations of benzil amino purin (BAP) ranging between 0.5 and 3.0 mg/l and Kinetin(KIN) between 0.5 and 3.0 mg/l, the resuts showed that 1.0 mg/l benzil adenine (BA) was found to be best concentration for high frequency multiple shoot induction.

Plant growth cannot be determined precisely via it is genotype, since it is much influenced by several biotic abiotic factors including light, day length, temperature, nutrient availability. Plant growth identifies three zones (deficiency, adequate and toxic) in the response of growth to increasing tissue concentrations. The transition between deficiency and adequate zones reveals the critical concentration of the nutrient, which may be defined as the minimum tissue content of the nutrient that is correlated with maximal growth or yield. The minerals present in plant tissue culture media culture can be used by the plant cell as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reaction (Bloom, 2006). The media can be divided micro- and macro-elements. Iron (Fe), Copper (Cu), Manganese (Mn),

Zinc (Zn) are considered as micro elements, and Calcium (Ca), Magnesium (Mg) as macro-element. Iron (Fe) constituent of cytochromes and non-heme iron proteins involved in photosynthesis, N_2 fixation and respiration. Zinc (Zn) constituent of alcohol dehydrogenase, glutamic dehydrogenase, carbonic anhydrase etc. Copper (Cu) is part of ascorbic acid oxidase, tyroniase, monoamine oxidase, uriase, cytochrome oxidase phenolaase, laccase and plastocyanin. Calcium (Ca) constituent of the middle lamell a of cell walls and required as a cofactor by some enzymes involved in the hydrolysis of ATP and phospholipids acting as a second messenger in metabolic regulation (Ramage and Williams ,2001). Fe deficiency (lime-induced chlorosis) is a worldwide problem in crop production on calcareous soils. Generally, iron deficiency is observed widely in fruits such as lemon, orange. On the other hand, Fe toxicity (bronzing) is a serious problem in crop production. Waterlogged soil is the second-most severe yield-limiting factor in wetland rice (Schimidt et al., 2012; Marschner, 2012). In Turkey, 73% soil have high concentration of iron. Zn deficiency is also widespread among plants grown in highly weathered acid soils and in calcareous soils. Zn deficiency associated with Fe deficiency (Marschner, 2012). Under Zn deficiency shoot growth is usually more inhibited than root growth. Rye has the highest tolerance to Zn deficiency than cereal species (triticale, barley, bread wheat, oats, durum wheat (Cakmak et al., 1997). Zn toxicity is observed very rarely in soils. Generally, Zn surplus in the soil is usually located next to the mines. Mn deficiency is abundant in plants growing in soils derived from parent material inherently low in Mn and in highly leached tropical soils. Mn deficiency tolerate plants is oat, wheat, soybean and peaches. Cu deficiency is observed in plants growing on soils either low in total Cu. High concentration Cu inhibits root growth before shoot growth (Marschner, 2012). According to Kaçar and Katkat (2007) sugar beet plant is more sensitive Cu concentration than cereal plants. The Ca requirement for optimum growth is much lower in monocotyledons than in dicotyledons. Low Ca concentrations in fleshy fruits increase the losses caused enhanced senescence of the tissue. Ca deficiency-related disorders are widespread, such as tipburn in lettuce, black-heart in celery, blossom end rot in tomato or watermelon and bitter pit in apple (Marschner, 2012; Kaçar and Katkat, 2007). Based on our literature survey ascertained, there is no report on micro-nutrient deficiency and toxicity on *S. rebaudiana* under in vitro conditions.

2. AIM AND SCOPE OF THE STUDY

This study provides a new insight for plant production *S. rebaudiana* via tissue culture techniques to reveal the effects of micro and macronutrients on Reb-A and ST contents. In this view of point, we aimed at;

1) establishing an efficient seed germination protocol,

(2) to find the best nutrient combinations for in vitro propagation of stevia,

(3) understanding the ST and Reb-A biosynthesis in relation to macronutrient and micronutrient variations under in vitro conditions.

3. MATERIALS and METHODS

3.1 Plant Tissue Culture Studies

All tissue culture studies described below were established under in vitro conditions as follows; seed surface sterilization, germination of seeds, preperation of regeneration media, explant choice from source plants, and transferring explants to the regeneration media.

3.1.1 Laboratory Facilities

Surface sterilization, explant preparation, and transfering the explants to the suitable basal growth media were employed in a laminar flow. Culture jars were incubated in a growth chamber equipped with an air conditioner to keep the ambient air at 23 °C, and a humidifier to keep humidity between 55–60%. For the light regime, a 16 hours light and 8 hours dark photoperiod provided by cool-white flouorescent light irradiance (50 µm mol^{-2} s^{-1}) was used in tissue culture experiments.

3.1.2 Seed Surface Sterilization

Seeds were sterilized according to Yucesan et al (2016) in which the seed materials were washed under running tap water in a small beaker for a couple of minutes. Then, 10% (v/v) of commercial bleach containing less than 5% of NaOCl solution were used for the surface sterilization for 10 min at 250 rpm on a magnetic stirrer. After bleach application, seeds were filtered through a fine metal sieve, and soaked in autoclaved distilled water several times.

3.1.3 Preparation of Culture Medium

In vitro studies were carried out with maximum four different basal medium formulations as follows; MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), N (Nitsch 1969) KC (Knudson, 1946) and GB5 (Gamborg et al 1968) supplemented with 3% (w/v) sucrose and 0.6% agar (w/v) (Plant-agar) in Table 3.1

In addition to the media types as mentioned above (Table 3.2), different mineral concentration was also tested in this dissertation. Ferrous sulphate ($FeSO_4.7H_2O$) (ranging between 0.0 and 80 Mm), and finally Stock 5 was used at various strengths up to four-fold higher than its original concentration in MS basal medium (Table 3.2).

Table 3.1: Composition of selected plant tissue culture medium

Media Abbreviation	Schenk and Hilderbrand SH (1972)	Knudson KC	Gamborg et al. GB5 (1968)	Nitsch (1969)	Murashige and Skoog MS (1962)
Macronutrients					
$CaCl_2$	151.00	-	113.23	166.000	332.02
KH_2PO_4	-	250.00	-	68.000	170.00
KNO_3	2500.00	-	250mp0.00	950.000	1900.00
$MgSO_4$	195.05	112.15	121.56	90.270	180.54
NH_4NO_3	-	-	-	720.000	1650.00
$NH_4H_2PO_4$	300.00	-	-	-	-
$Ca(NO_3)_2$	-	241.30	-	-	-
KCl	-	250.00	-	-	-
NH_4NO_3	-	500.00	-	-	-
$(NH_4)_2SO_4$	-	500.00	134.00	-	-
NaH_2PO_4	-	-	130.44	-	-
Micronutrients					
$MnSO_4.H_2O$	10.00	5.68	10.00	18.940	16.90
$ZnSO_4.7H_2O$	1.00	-	2.00	10.000	8.60
$Na_2MoO_4.2H_2O$	0.10	-	0.25	0.250	0.25
KI	1.00	-	0.75	-	0.83
H_3BO_3	5.00	-	3.00	10.000	6.20
$CuSO_4.5H_2O$	0.20	-	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.10	-	0.025	-	0.025
FeNaEDTA	19.80	-	36.70	36.70	36.70
$FeSO_4.7H_2O$	-	25.00	-	-	-
Vitamins and Amino Acids					
myo-Inositol	1000.0	-	100.00	100.00	100.00
Thiamine-HCI	5.0	-	10.00	0.50	0.10
Nicotinic acid	5.0	-	1.00	5.00	0.50
Pyridoxine-HCI	0.5	-	1.00	0.50	0.50
Biotin	-	-	-	0.05	-
Folic acid	-	-	-	0.50	-
Glycine	-	-		2.00	2.00

Table 3.2: The modified MS medium (MMS) contains some inorganic and organic compounds.

Macronutrient Components*	Amount (g/L)
Stock 1	
Ammonium nitrate (NH_4NO_3)	83.5
Potassium nitrate (KNO_3)	95.5
Magnesium sulfate ($MgSO_47H_2O$)	18.5
Stock 2	
Calcium chloride ($CaCI_2.2H_2O$)	44.0
Stock 3	
Potassium dihydrogen phosphate (KH_2PO_4)	17.0
Micronutrient Component	
Stock 4	
Sodium ethylenediamine tetra-acetate (Na-EDTA)	3.73 g
Ferrous sulfate ($FeSO_4.7H_2O$)	2.78 g
Stock 5	
Boric acid (H_3BO_3)	0.620 g
Manganese sulfate ($MnSO_4.4H_2O$)	2.230 g
Zinc sulfate ($ZnSO_4.7H_2O$)	0.860 g
Potassium iodide (KI)	0.083 g
Sodium molybdate (Na_2MoO_4)	0.025 g
Copper sulphate ($CuSO_4.5H_2O$)	0,0025 g
Cobalt chloride ($CoCI_2.5H_2O$)	0,0025 g
Stock 6	
Myo-Inositol	10 g
Nicotinic acid (Niacin, vitamin B3)	0,05 g
Glycine	0,2 g
Pyrodoxine –HCI (Vitamin B6)	0,05 g

*To prepare 1L of MS medium, following stock solutions 20 ml of Stock1, 10 ml of Stock 2, Stock 3, Stock 4 and Stock 5, finally 5 ml of Stock 6 were used.

Table 3.3: The different mineral and stock 5 concentrations represented as iron (F), stock 5 (St5) in our modified MS medium (MS). *Note that in this study, different concentrations of stock-4 and -5 were studied.*

MMS Medium*	MMS Contains Solutions					
	STOCK 1	**STOCK 2**	**STOCK 3**	**STOCK 4**	**STOCK 5**	**STOCK 6**
F0	20 ml	10 ml	10 ml	0 ml	10 ml	5 ml
F5	20 ml	10 ml	10 ml	5 ml	10 ml	5 ml
F10	20 ml	10 ml	10 ml	10 ml	10 ml	5 ml
F20	20 ml	10 ml	10 ml	20 ml	10 ml	5 ml
F40	20 ml	10 ml	10 ml	40 ml	10 ml	5 ml
F80	20 ml	10 ml	10 ml	80 ml	10 ml	5 ml
ST5/0	20 ml	10 ml	10 ml	10 ml	0 ml	5 ml
ST5½	20 ml	10 ml	10 ml	10 ml	5 ml	5 ml
St5/1	20 ml	10 ml	10 ml	10 ml	10 ml	5 ml
St5×2	20 ml	10 ml	10 ml	10 ml	20 ml	5 ml
St5×4	20 ml	10 ml	10 ml	10 ml	40 ml	5 ml

** Fe10: modified MS medium(control); Fe0: iron deficient; Fe5: 5 mM iron; Fe10: 10 mM iron, Fe20: 20 mM iron; Fe40: 40 mM iron, or Fe80: 80 mM, St5/1, modified MS medium, containing St5/0 St5 deficient ; St5½ , half strength St5; St5×2, two-fold; St5×4 four-fold in MS medium*

3.1.4 Effects of Light and Nutrient Concentration on Germination of The Seeds

Seed germination protocol was carried out using two sets of experiments. Firstly, surface sterilized seeds were placed on MS medium under five different light illuminations ranging between 0.8 and 5.0 klux. Each treatment consisted of three plates and each plate was contained 10-15 seeds. These seeds were placed into each petri dish. Surface sterilized Stevia seeds were germinated on Murashige and Skoog (MS) medium. After that, petri dishes were closed and wrapped with paraffin and placed in the incubator with the different light density 0.8 klux, 1.2 klux, 2.0 klux, 3.5 klux and 5.0 klux application for four weeks. Secondly, surface sterilized *S. rebaudiana* seeds were germinated on Murashige and Skoog (MS) medium with (control treatment) or without Fe (Fe0-deficient treatment), and seedlings were kept on these

media for 4 weeks. The number of germinated seeds were counted every weeks and final counting was recorded after four weeks.

3.2 Shoot Regeneration and Rooting of *Stevia rebaudiana*

For in vitro studies, nodal segments (0.5–1.0 cm in length) were used. All tested explants throughout the studies were excised from in vitro germinated seedlings (6 weeks old) under in vitro conditions. The explants excised from in vitro germinated seedlings were transferred to regeneration media as mentioned below. Tissue culture protocols were described below in detail for *S. rebaudiana*. Following germination in vitro, node explants excised from 6 weeks-old seedlings were used in experiments (Figure 3.1). All media formulations and chemicals used in tissue culture experiments were purchased from Duchefa Biochemie (Netherlands). Petri plates (90 × 15 mm) or were used for shoot regeneration. Each treatment used 3 explants; experiments were carried out in triplicate with 3 replicates per treatment.

For rooting and hardening of regenerants, regenerated shoots (approximately 4–5 cm) were cultured for an additional 3 weeks in jar containing full-strength MS medium without PGRs. Each rooting treatment used 6 shoots; experiments were performed in triplicate with a total of 3 replicates per treatment. To remove agar, rooted plantlets (10–12 cm) were washed with distilled water, and then data regarding with shoot/root length, node and leaf number, leaf, shoot, root weight were obtained after 6 weeks of cultivation. Finally, 1gr leaf was used chlorophyll content determination (see below).

Figure 2.1: *Stevia rebaudiana* seedling (6 weeks old)**.**

3.3 Plant Extractions and Analytical Experiments

3.3.1 Steviol Glycoside Extraction

The dried leaf materials were ground to fine powder with a pestle and mortar. About 20 mg dry material was added to a microcentrifuge tube containing 1 ml of 70% (v/v) methanol. After 20 min treatment in an ultrasonic cleaning bath at 50 °C, the tubes were then centrifuged for 10 min, at 12,000 rpm. The supernatant was thoroughly filtrate using a syringe filter (22µm) in HPLC vials.

3.3.2 HPLC Determination

Extraction and HPLC analysis. *Stevia rebaudiana* were extraction according to Yucesan et al (2016). The supernatant was filtered using 0.22-µm PTFE syringe filters prior to HPLC analysis. SG were eluted with an isocratic flow using a mixture of acetonitrile (HPLC grade) and 1% (*w/v*) phosphoric acid buffer mixture at pH 2.6 (70:30 [*v/v*]) for 20 min. Flow rate was 1.0 mL for 10 min with a binary pump (LPG 3400SD; Thermo-Fischer Scientific, Waltham, MA) solvent delivery system, a dual wavelength absorbance detector (MWD-3100 UV-Vis Detector; Thermo-Fischer Scientific) operating at 210 nm and using 356 nm as a reference wavelength, and an auto-sampler (WPS-3000-SL SemiPrep-Autosampler; Thermo-Fisher Scientific) injecting 10 µL of each sample. The special column for SG, name is an Inertsil®ODS-3 (GL Sciences Inc., Tokyo, Japan) This column has 150 × 4.6 mm and 5-µm particle size, was kept warm at 40°C in a column oven system (TCC-3000SD; Thermo-Fischer Scientific) (Yücesan et al., 2016)

Once the final plant extracts were obtained, they were spiked with 100 mg L^{-1} pure SG. Recovery percentages were calculated from five individual extractions and three analytical HPLC runs of each extract. It was used for the confirmation of SG. (Yücesan et al, 2016)

3.3.3 Chlorophyll Content Analysis

The fresh leaves material were collected in 6 weeks. The leaves were crushed small pieces leaves particles with mortar and pestle. Thereafter, 100 mg fresh leaves material was added to a microcentrifuge tube containing 1 ml of 80% (v/v) acetone and mixed them in dark for 1 min (chlorophyll degrade under light). The next step, the tubes were then centrifuged for 10 min, at 12,000 rpm, 4°C. The supernatant phase was thoroughly put using in spectrometric tubes. Finally, the tube measures the absorbance (A) of chlorophyll content using spectrometry (Arnon, 1949)

The chlorophyll concentrations are calculated as follows (80% acetone as a blank control; Arnon 1949)

Ca (mg/g) = (12.7 × A663 - 2.69 × A645) × V/1000 × W (Chlorophyll-a) (Arnon Method 1949)

Cb (mg/g) = (22.9 × A645 - 4.86 × A663) × V/1000 × W (Chlorophyll-b) (Arnon Method 1949)

Ca+b (mg/g) = (8.02 × A663 + 20.20 × A645) × V/1000 × W (Chlorophyll a+b) (Arnon Method 1949)

Where V= Volume of the extract (ml), W=Weight of fresh leaves (g) (Arnon 1949)

3.4 Statistical Analysis

Firstly, we used statistically analyzed using Microsoft Excel (Office 2016) for data. Secondly, One-way analysis of variance was used to calculate statistical significance and the significance of difference among the means was determined using Tukey's test at alpha value of 0.05 by SPSS version 22.0.

4. RESULTS AND DISCUSSIONS

4.1 Seed Germination of *Stevia rebaudiana*

Seed germination protocol was carried out using three sets of experiments. Firstly, surface sterilized seeds were placed on MS medium under five different light illuminations ranging between 0.8 and 5.0 klux (Figure 4.1) Of all light densities tested, 5.0 klux was found to be the most effective for seed germination. However, those of which less than 3.5 klux were found to be the least effective for seed germination with a low germination frequency (~25%; after 4 weeks of sowing).

Surface sterilized *S. rebaudiana* seeds were germinated on MS medium with (control treatment) or without Fe (Fe0-deficient treatment), and seedlings were kept on these media for 4 weeks. It was clearly shown that shoot length in MS was twice higher than Fe0. On the other hand, root growth in Fe0 was 20% higher than the MS. Shoot fresh weight (SFW) in the control treatment was 40% higher than Fe0 treated seedlings. Additionally, average root fresh weight (RFW), mean number of leaves and nodes were similar in both treatments. Chlorophyll contents in the control groups were twice higher than Fe0 treated plants. HPLC analysis showed that MS produced nearly 10 times higher Reb-A and ST than Fe0 plants (Figure 4.2). In Fe0, red pigmentations and chlorosis were observed in young leaves, while the control groups produced green leaves (Figure 4.3 and 4.4)

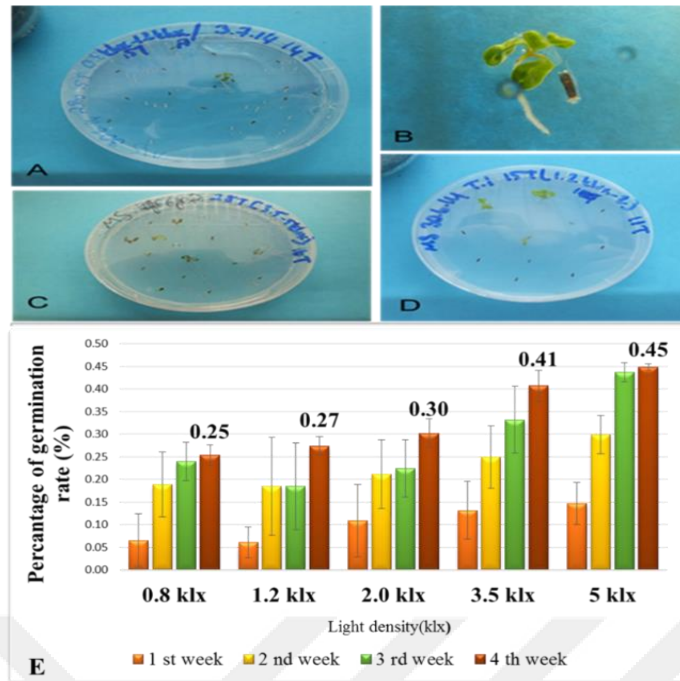


Figure 3.1.: Effect of different light densities on seed germination of *S. rebaudiana* after 2 weeks of germination at 0.8 klx (A, B), 3.5 klx (C), and 1.2 klx (D). Effect of different light densities showed that percentage on seed germination of *S. rebaudiana* (1–4 weeks) (E)

Figure 4.2: With or without iron deficiency on seed germination of *S. rebudiana*. MS-(A), and Fe0 (B)

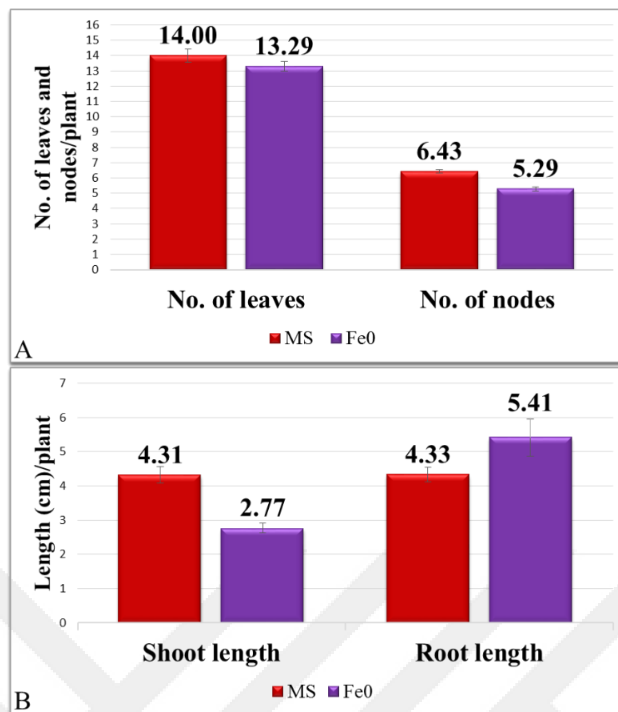


Figure 4.3: Effect of two different media MS and Fe0 on seed germination after four weeks' cultivation. Mean number of leaves and nodes (A), shoot and root length (B).

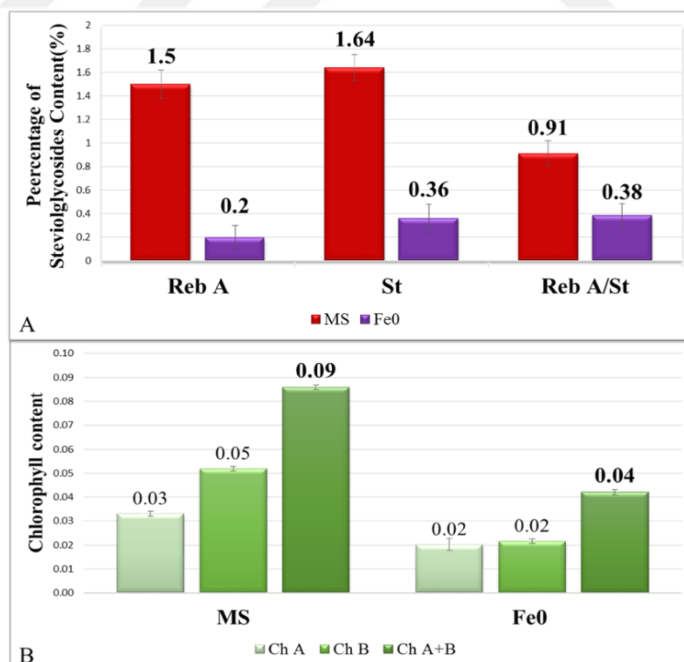


Figure 4.4: Effect of Fe0 on seed germination on percantege of steviol glycoside contents (A) chlorophyll content of the leaves after four weeks'(B) *Ch-A:* Chlorophyl-a, *Ch-B*: Chlorophyl-b or-B; *Reb-A*: rebaudioside A; *ST*: stevioside

4.2 In Vitro Regeneration Studies of *Stevia rebaudiana*

In vitro regeneration of *S. rebaudiana* was carried out using 6 weeks old in vitro germinated seedlings. Nodal explants were placed onto five different basal medium, SH, KC, N, GB5 or MS for a three-week cultivation period. All these media type have different nutrient compositions. This experiment clearly shows that shoot length in MS was twice higher than SH Medium (0.95 cm in SH,1.85 cm in MS), and eight times higher than KC (0.2 cm in KC, 1.85 cm in MS). However, there was no significant difference was observed as compared to N or GB5 medium (Figure 4.5).

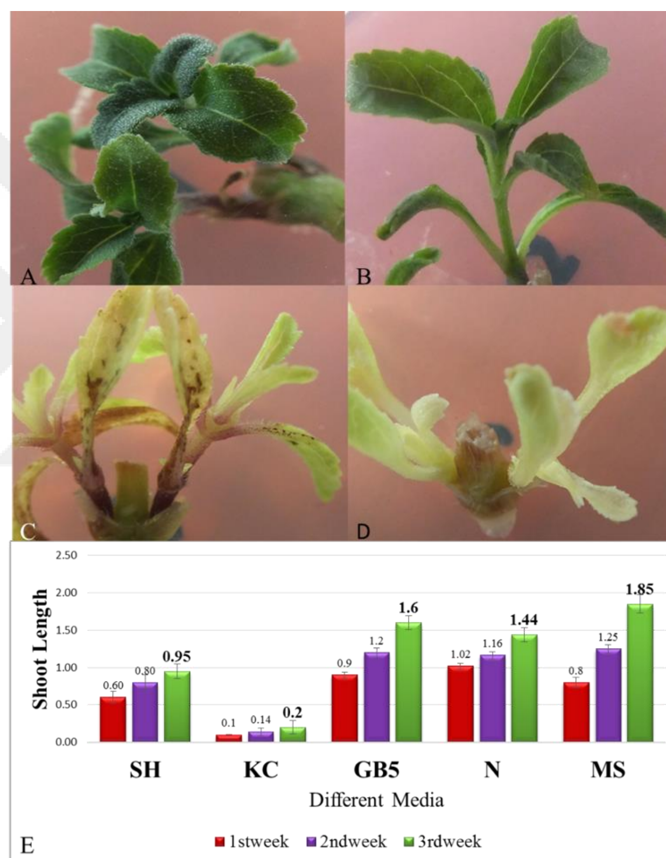


Figure 4.5: Effect of different media on shoot regeneration after three weeks' culture on Nitsch (N) medium (A), MS medium (B), Schenk and Hildebrant (SH) medium (C), and Knudson C medium (D).

4.2.1 Effect of Iron on *Stevia rebaudiana*

Nodal explants were placed on MS and Modified MS medium (MMS) with or without iron concentrations (0, 5, 10, 20, 40 or 80 mM; Fe0, Fe5, Fe10, Fe20, Fe40, Fe80) for six weeks cultivation. Results showed that after 2–3 wk incubation, F0 (Fe- deficient) plants showed very low regeneration, and younger leaves were in yellow colour covered with red pigments as compared to Fe10 medium having green and healthy leaves. On the other hand, Fe80 was toxic for *S. rebaudiana*, plant growth and development was completely reduced (Figure 4.6). For Fe5 plants, leaf veins were green but leaves margins were in yellow colour. For the morphological observations of the leaves cultivated in Fe20, plants displayed similar patterns as seen in MS. It was clearly observed that Fe40 was at critical range, iron overdose in *Stevia* resulted in red pigmentation on the leaf margins (Figure 4.6). After Six weeks' cultivation, Fe0 and Fe80 were not effective on shoot growth, while Fe5 had the highest root length than and other concentrations (Table 4.1). Root, shoot and leaves fresh weight in the MS media were higher than other Fe concentration (Table4.2) Shoot length, node and leaf number in the control treatment was almost similar to the result obtained from Fe20. In spite of these findings, the treatments with varying Fe concentration in MS medium were found to be lower than plane MS medium (Figure 4.7). In this study, root formation was successfully achived on MS medium without PGR. In treatment of Fe20, total chlorophyll content was higher than other treatments (compare 0.20 in Fe20 with 0.10 in MS)

All variying Fe concentrations in Fe10 medium did not affect Reb A contents; however, ST content was lower than MS (see in Figure 4.8 and Table 4.3)

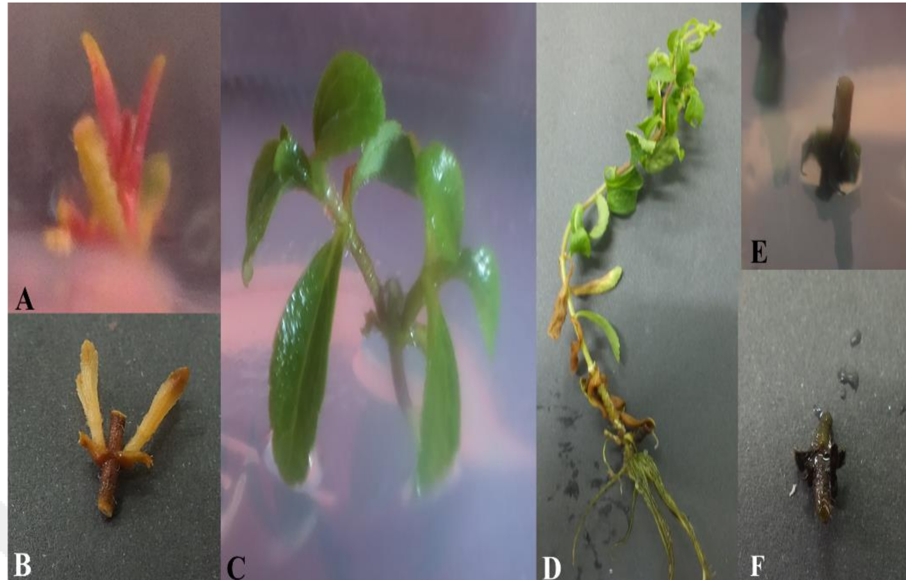


Figure 4.6: Effects of various Fe concentration (Fe0, Fe10 or Fe80) on shoot regeneration after 3- and 6 weeks' cultivation. A) F0 plants 3 wk-, B) 6 wk-old, C) Fe10 plants 3 wk, D) 6 wk-old, E) F80 plants 3 wk-, and F) 6 wk-old plants. *Fe0: iron deficient, Fe10: 10 mM iron, Fe80: 80 mM iron containing MS medium*

Figure 4.7: Effects of modified MS medium with or without iron different concentrations (Fe0, Fe5, Fe10, Fe20 or Fe80) on regeneration from nodal explants of *S. rebaudiana* after 6 weeks. *Fe0: iron deficient; Fe5: 5 mM iron; Fe10: 10 mM iron (control), Fe20: 20 mM iron; Fe40: 40 mM iron, and Fe80: 80 mM iron containing MS medium.*

Table 4.1: Effect of various Fe concentrations (Fe0, Fe5, Fe10, Fe20, Fe40 or Fe80) in MMS medium on shoot length growth recorded during a six-week's regeneration period.

Types of MMS Medium*	Mean shoot length (cm) ± SE						Mean root length (cm) ± SE**
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	
Fe0	0.1±0.4	0.1±0.4	0.1±0.4	0.1±0.4	0.12±0.4	**0.1±0.4**	**0.0**
Fe5	0.9±0.1	2.4±0.2	3.9±0.1	5.9±0.1	8.2±0.1	**9.8±0.1**	**11.0±0.1**
Fe10	1.6±0.1	2.7±0.1	4.9±0.2	6.1±0.2	10.7±0.2	**14.2±0.2**	**7.6±0.1**
Fe20	1.97±0.2	3.3±0.2	4.2±0.2	5.9±0.1	8.8±0.1	**13.2±0.1**	**6.8±0.1**
Fe40	1.3±0.1	1.8±0.2	2.1±0.1	2.4±0.1	3.2±0.2	**3.9±0.2**	**0.0**
Fe80	0.0	0.0	0.0	0.0	0.0	**0.0**	**0.0**

* *Fe10: modified MS medium(control); Fe0: iron deficient; Fe5: 5 mM iron; Fe10: 10 mM iron, Fe20: 20 mM iron; Fe40: 40 mM iron, or Fe80: 80 mM,* ***root length of the regenerants were scored after 6 weeks of cultivation.*

Table 4.2: Effect of various Fe concentrations (Fe0, Fe5, Fe10, Fe20, Fe40 or Fe80) on mean number of nodes and leaf numbers recorded during a six-week's

Types of MMS Medium	Mean node number ± (SE)						Mean leaf number ± (SE)					
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk
Fe0	0	0	0	0	0	0	2±0.5	2.0±0.5	2±0.5	2±0.5	2±0.5	2±0.5
Fe5	2±0.0	3±0.0	5±0.0	7±0.0	9±0.0	10±0.0	3±0.5	7±0.8	10±1.0	12±1.0	14±1.0	16±1.0
Fe10	2±0.0	4±0.0	6±0.0	7±0.0	9±0.0	12±0.0	4±0.5	8±0.8	14±1.1	16±1.3	20±1.3	22±1.3
Fe20	2±0.0	4±0.0	5±0.0	6±0.0	8±0.0	12±0.0	4±0.5	8±0.8	14±1.2	18±1.3	22±1.4	24±1.4
Fe40	2±0.0	2±0.0	3±0.0	4±0.0	5±0.0	6±0.0	2±0.5	3±0.5	4±0.87	6±0.87	8±1.00	10±1.0
Fe80	0	0	0	0	0	0	0	0	0	0	0	0

** Fe0: iron deficient; Fe5: 5 mM iron; Fe10: 10 mM iron, Fe20: 20 mM iron; Fe40: 40 mM iron, or Fe80: 80 mM*

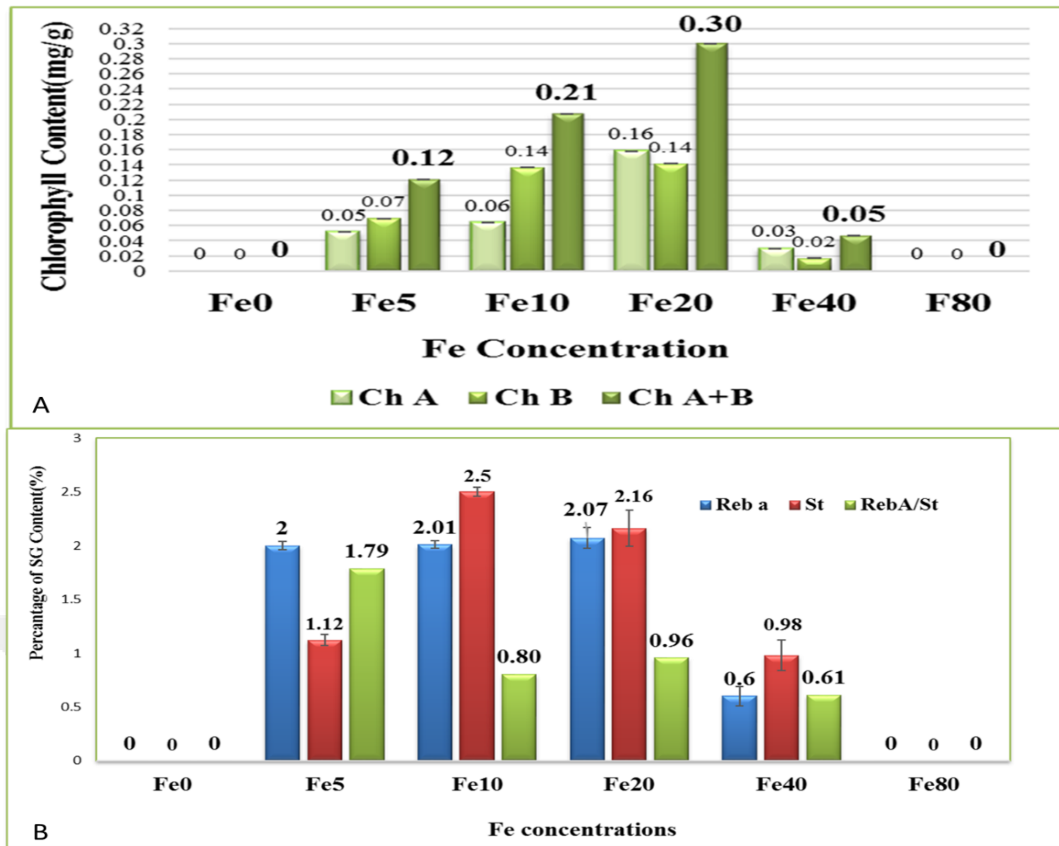


Figure 4.8: Effect of MS and iron modified MS medium on chlorophyll content (mg/g) (A), % of Reb-A and ST content (B). Note: iron concentrations as follows Fe0, no iron; Fe5, 5 mM iron; Fe20, 20 mM iron; Fe40, 40 mM iron, and Fe80, 80 mM in modified MS medium

Table 4.3: Root, shoot and leaf fresh weight of 6 weeks old regenerants produced from different levels of iron-containing MS medium, and steviol glycoside content (% DW) under in vitro conditions (ND: not detected; ±: SD).

Fe Concentration*	Root Fresh Weight (RW) (mg)	Shoot Fresh Weight (SW) (mg)	Leaf Fresh Weight (LW) (mg)	Reb-A** (%)	ST** (%)
Fe0	N.D	N.D	N.D	N.D	N.D.
Fe5	297.04±14.42	166.13±22.77	227.94±45.38	2.0	1.1
Fe10	434.31±81.04	344.08±67.12	532.20±77.07	2.0	2.5
Fe20	333.15±9.42	238.25±2.78	325.57±86.89	2.1	2.2
Fe40	N.D	126.88±4.76	252.43±34.58	0.6	0.8
Fe80	N.D	N.D	N.D	N.D	N.D

** Fe0, no iron; Fe5, 5 mM iron; Fe20, 20 mM iron; Fe40, 40 mM iron, and Fe80, 80 mM in modified MS medium; ** Reb-A, rebaudioside A; ST, stevioside*

4.2.2 Effect of Stock 5 (St5) on In Vitro Regeneration of *Stevia rebaudiana*

In this study, nodal explants showed a similar response in terms of shoot length at different St5 concentrations after three weeks of cultivation. However, in ST5/0 medium, shoot length was shorter than St5/1 and other concentrations of modified St5/1 medium (St5/0, St5½, MS, St5×2 or St5×4). In st5 deficient medium (St5/0), leaves had black spots, while in St5×2, wherein stock 5 solution was half strength, leaves were in normal appearance. In doubled St5 (ST5×2), leaves were in convulate, while all leaves were abnormal in four-fold high St5 (St5×4 in). It was also shown that root length decreased gradually depending on increasing concentrations of St5/1. As seen in Table 4.3, St5½ had the highest root length (10.7 cm). Increasing ST5 concentration up to four fold decreased the root length from 10.7 to 0.6 cm. Additionally, root fresh weight was lower than other St5 modified treatments. The results revealed that St5½ , MS plane, St5×2 was not effective shoot length, similarly St5/0 and St5×4 decreased the shoot length from 11.8 to 8.2 cm (Table 4.4). The more root fresh weight was observed on MS medium (316.40 mg), while the less root fresh weight was detected in St x4 (27.55 mg). Further, in St5½, the leaf weight was the highest (566.20 mg) as compared to the other treatments (Table 4.6). It was shown that two and four-fould St5 concentrations negatively affected chlorophyll content in the leaves. However, there was no difference among St5/0, St5½ and St5/1 medium. In terms of Reb-A content, the highest Reb-A was found in St5/0 medium (2.72%) with the lowest stevioside content (0.86 %) in as compared to the other St5 treatments.

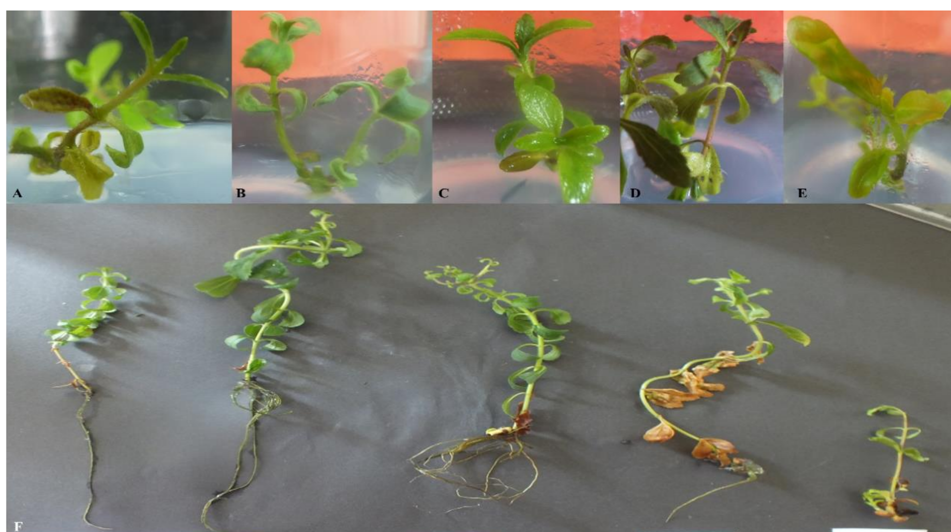
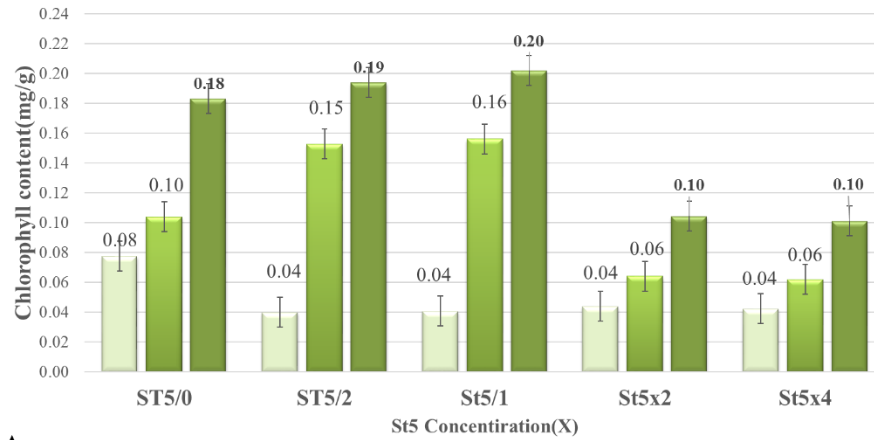


Figure 4.9: Effect of MS and modified MS medium with or without ST5 concentrations on regeneration after 6 weeks' cultivation St5/0 A), St5/2 B), St5/1 C), St5×2 D) St5×4 E



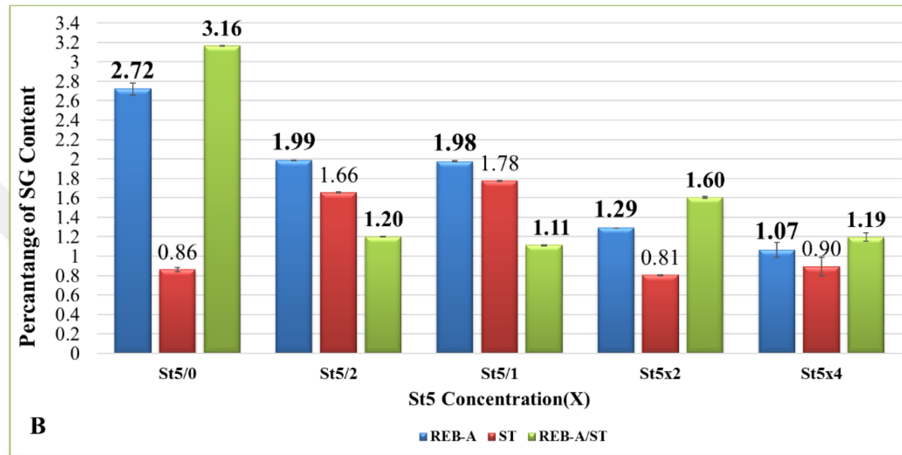


Figure 5: Effect of various St5 concentrations (St5/0, St5½ , St5/1, St5×2, and St5×4) in modified MS medium on chlorophyll content (A), and content of Reb-A ST) content (B).

Table 4.4: Effect of various St5 concentrations (St5/0, St5/2, St5/1, St5×2, St5×4) in modified in MS medium on shoot length growth recorded during a six-week's regeneration period.

Medium*	Mean node number ± (SE)						Leaf Number ± (SE)					
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk
St5/0	1±0.0	3±0.0	5±0.0	7±0.0	8±0.0	8±0.0	3±0.4	5±0.4	9±0.4	13±0.4	15±0.4	19±0.4
St5½	2±0.0	4±0.0	6±±0.0	8±0.0	10±0.0	10±0.0	3±0.4	6±0.4	9±0.7	11±1.0	17±1.0	21±1.0
St5/1	2±0.0	4±0.0	5±0.0	7±0.0	10±0.0	11±0.0	4±0.4	8±0.4	12±0.7	16±1.0	20±1.0	22±1.0
St5×2	2±0.0	3±0.0	5±0.0	9±0.0	10±0.0	12±0.0	4±0.4	8±0.4	12±0.8	16±1.0	22±1.0	22±1.0
St5×4	2±0.0	3±0.0	4±0.0	5±0.0	6±0.0	6±0.0	4±0.4	8±0.4	11±0.7	12±1.0	12±1.0	13±1.0

**root length of the regenerants was scored after 6 weeks of cultivation.*

Table 4.5: Effect of various St5 concentration (ST5/0, ST5½ , , ST5×2, ST5×4) on regeneration from nodal explants of *S. rebaudiana* at 1-6 weeks node number and leaf number.

Medium	Mean Shoot length (cm) ± SE						Mean root length (cm) ± SE*
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	
St5/0	1.3±0.1	2.1±0.1	5.2±0.1	6.7±0.1	9.2±0.1	11.8±0.1	**6.2±0.1**
St5½	1.7±0.1	3.2±0.2	5.4±0.2	6.8±0.2	10.5±0.2	14.4±0.2	**10.7±0.1**
St5/1	1.8±0.1	3.1±0.2	4.8±0.2	7.1±0.2	10.3±0.2	14.5±0.2	**7.6±0.1**
St5×2	2.0±0.2	3.5±0.2	4.6±0.2	6.9±0.2	10.2±0.2	14.6±0.2	3.8±0.1
St5×4	1.3±0.1	2.9±0.2	4.7±0.2	5.4±0.3	6.4±0.3	8.2±0.2	**0.6**±0.0

**St5/1, modified MS medium, containing St5/0 St5 deficient ; St5½ , half strength St5; St5×2, two-fold; St5×4 four-fold in MS medium*

Table 4.6: Biomass (mg) of root, shoot and leaf fresh weight detected in different St5 concentrations (six weeks old) grown under in vitro conditions and steviol glycoside content in leaves (ND: not detected; ±: SD).

*St5 Concentrations**	*Root Fresh Weight (mg)*	*Shoot Fresh Weight (mg)*	*Leaf Fresh Weight (mg)*	*Reb-A (%)*	*ST (%)*
St5/0	135.60±11.40	245.60±68.99	242.70±14.24	2.71	0.86
St5½	220.20±32.52	379.70±42.20	566.20±27.70	2.01	1.66
St5/1	316.40±15.60	360.10±71.52	480.93±66.42	1.99	2.27
St5×2	251.60±33.97	284.40±29.89	294.00±41.68	1.29	0.85
St5×4	27.55±24.72	249.40±19.39	126.80±10.40	0.85	0.90

**St5/1 ,modified MS medium, containing St5/0 St5 deficient ; St5½ , half strength St5; St5×2, two-fold; St5×4 four-fold in MS medium*

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 Seed Germination of *Stevia rebaudiana*

Stevia seeds has a very low percentage of germination. There has been few report on stevia seed germination concerning effect of light and Fe deficiency. To begin with, this study reports plant regeneration protocol for *S. rebaudiana* species as well as an assessment of ST and Reb-A production in the leaves of in vitro plants. Two sets of experiments were carried out in the experiments of *S. rebaudiana*. Firstly, different densities of light were compared using MS medium (Figure 4.1). Secondly, Fe deficiency was compared using MS medium. For both sets of experiments, Sterilized seeds were germinated on MS and seedlings were kept on these media for 3-4 weeks.

First experiment studied the effciency of stevia seed germination under different light densities (Figure 4.1), it was clear that seeds placed on MS medium under different light density was successfull for germination. When overall means of Stevia seeds under light density were taken into account, it was evident that increasing light density, from 0.8 to 5.0 klux steadily increased percentage of seed germination. 5.0 klux was the most effective light density for the seed germination, which produced mean ~25% seed germination rate. Our result was supported by some earlier studies (Carneiro et al.,1997; Goettemoller and Ching, 1999, Abdullateef and Osman 2011) in which they reported that germination was depended on testa color and light condition, light wavelength For example, black colour seeds had higher germination rate than tan colour ones and those seeds had also higher germination under light conditions than that of the dark. The germination rate in *S. rebaudiana* seeds was enhanced with the effects of both white (400-700nm) and red light more than the darkness (Abdullateef and Osman 2011). Acoording Sakaguchi and Kan (1982), they reported that growing *S. rebaudiana* from seed normally has a very low germination success (0-10%) thus, *S. rebaudiana* is usually not being propagated by seeds. Stem cutting has been selected as a powerfull tool of *S. rebaudiana* propagation. In our study, maxium germination rate was 45% at 5.0 klux density in contrast to earlier reports.It was clearly found that

light is an important factor responsible for *S. rebaudiana* seed germination and seedling growth.

In the second study in which the role of Fe ions was checked, the results showed that Fe ions significantly induced chlorophyll content, and subsequently increased the content of SG and Reb A. In young leaves of plants and especially in the last leaves, yellowing between vessels is a sign of iron deficiency (Kacar and Katkat 2010). Brown necrosis may occur in some plant leaves (rice,corn etc)(Marschenner 2012). However, chlorosis is observed in ornamental plants and fruit trees (cherry, peaches etc.). In Our study, F0 medium has red pigmentations and chlorosis were observed in young leaves, while the control groups produced green leaves (Figure 4.3). While overall means of seed germination in MS and Fe0 medium, it was shown that increasing concentrations of Fe (from 0 to 10 mM) steadily increased the shoot length, shoot weight, root weight; however, the highest root length was produced in Fe0 medium only. In literature, there has been no any report addressing nutrient effects of germination of *S. rebaudiana* as mentioned in this study.

In conclusion, the seed germination experiments results show that light density and Fe concentrations were important for seed germination. Due to the fact that seed quality is a critical point for *S. rebaudiana* researchers, optimisation of seedling production through seeds require well-equipped light sources for large-scale planlet production (Yücesan et al. 2015).

5.2 In Vitro Regeneration Studies in *Stevia rebaudiana*

First experiment employed the nodal type of explants cultured on SH, KC, N, GB5 and MS medium for a three-week cultivation period. These medium formulations were evaluated for regeneration efficiencies. (Table 3.1) For example, The SH mineral formulations contains almost 50% less calsium than MS medium. As a result that on the leaves yellow, brown spots surrounded by a sharp brown outlined edge. The Knudson C mineral formulations absent FeNaEDTA and microelements than MS medium. This media grown young and old leaves were look like bleached. The Nitsch, GB5 mineral formulations do not have not significant difference in MS medium as a result of no significance morphological data. This experiment clearly shown that shoot length in MS was twice higher than SH Medium, eight times higher than KC, approximately same as N and GB5 (Table 4.1) According to Gantait et al. (2015) the comparative effect of MS, B5, WPW and SH media in stevia where they found that our results supported MS Medium was the best to offer most favourable condition in vitro regeneration.

There has been no report on the effects of the concentrations of Fe concerning several morpholocigal parameters and SG (ST and Reb A) profiling of *S. rebaudiana*. Therefore, MS medium was selected as a typical basal medium throughout the studies conducted for Stevia species involved in this study. Normally, Fe combinations (0, 5 mM, 10 mM, 20 mM, 40 mM or 80 mM), St5 (/0, ½ , /1, ×2, or ×4) and was optimized to achieve a high efficiency in vitro regeneration of *S. rebaudiana*.

As the economic importance of *S. rebaudiana*, there are only few studies associated with nutrient supply and SG accumulation. Based on litetature survey ascertained, visual symptoms of nutrient deficiency in *S. rebaudiana* include such changes in leaf colour from dark green to yellow or pale green. sometimes mottled or with dark necrotic spots and impairtment of overall growth (Ceunen and Geuns, 2013a; Ramesh et al., 2006; Ibrahim et al., 2008). These deficiencies reduced SG levels within the leaves, with the most severe decreases generally observed in Ca, K and S deficiencies. On the other hand, addition of B and Zn increased SG levels significantly. In this study, we showed, for the first time, new data related to the some nutrient to SG Stevia. Our findings showed that Fe deficiency in *S. rebaudiana* accounts for such

changes in leaf colour. The highest SG was 20 mM Fe (2.0% Reb A, Reb A/ ST 0.96). The highest concentrations Fe resulted in lowest SG content in leaves. Iron has a critical role for synthesis of hemoglobin. Higher amount of iron in Stevia leaves is benefical hemoglobin level in the body. Besides, stevia leaves could also be use to prepare various sweet preparations for combinating iron deficiencies as anemia which is the major nutrional disorder of devoloping conturies (Abou-Arab *et al* 2010, Uçar *et al* 2017). Our result of experiment show that Fe40 was at critical range with a red pigmentation resulting no root development. Morever, Fe80 was toxic, stevia nodes were highly necrotic. In the future, this data show that very important, design of stevia medium for in vitro culture depending on iron elements.

Our result supported that stimulary effect of higher level of iron in basal medium have been reported sweet orange (Citrus sinensis L.) Best medium formulated for the micropropagation of the sweet orange MS medium supplement with NH_4NO_3 and Fe (Niedz and Evens 2007). In our studies with tobacco leaf discs, iron was removed from both shoot-forming and non-shoot-forming media earlier than any other mineral (Ramage and Williams, 2002). Over 50% of iron was taken up by shoot-forming explants in the first 4d of culture. Similarly, 75% of the exogenous iron was removed from nonshoot-forming media and therefore its consumption does not appear to be related to morphogenesis (Ramage and Williams 2002).

The st5 deficiency was black spot leaves. The highest percantage of Reb A (2.72) and Reb A/ ST (3.16) was st5 deficient. According to Kalpana et al. (2010) and Jain et al. (2009), the amount of micronutrient copper in MS medium has a positive effect on in vitro morphogenesis and total biomass in stevia. These findings can play an important role in field studies as optimum copper levels that can be maintained in field conditions to produce high yielding *S. rebaudiana* variety which has high stevioside content. According to Javed *et al.,* (2010) best medium formulated for the commercial and massive micropropagation of the *S. rebaudiana* was MS medium supplemented with 3 mg/L BAP, 60 g/L sucrose, 9 ml/L Fe-EDTA and 27.5 µg/L CuSO4. According to Das *et al* (2005), their experiment showed that the amount of Zn in stevia plants gradually decreased the plant growth. Their experiments also showed that the interaction effect between P and Zn did not show any positive effect on

phosphorus and zinc availability in the soil as well as stevioside concentration in the stevia leaves.

In conclusion, minerals are essential component of plant tissue culture media, limited number of studies have been made of direct effect of mineral nutrients on stevia growth and development. Optimization of individual micronutrients in the culture medium have been reported in Chili peppers, Jatropha and Paspalum (Jain *et al* 2012). In addition, optimized level of micronutrients also improved the growth of tissue culture resulting in higher level of biomass and more chlorophyll content in their leaves. Morever, in this present study, micronutrients of modified MS medium resulted that chlorophyll content and plant biomass can be variable depending on the concentration of Stock-5 solution in MS medium. It might negatively influence the accumulation of SG in the leaves.

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