ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

IN VITRO PROPAGATION OF STEVIA[*STEVIA REBAUDIANA* (BERT.)] AND DEVELOPMENT OF A VARIETY WITH A HIGH STEVIOL GLYCOSIDE CONTENT WHICH CAN BE GROWN AT A COMMERCIAL LEVEL IN TURKEY

DOCTOR OF PHILOSOPHY

REFIK BÜYÜKGÖÇMEN

BOLU, MAY 2016

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APPROVAL OF THE THESIS

In Vitro Propagation of Stevia[*Stevia rebaudiana* (Bert.)] and Development of a Variety with a High Glycoside Content which Can Be Grown at a Commercial Level in Turkey submitted by **Refik Büyükgöçmen** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in **Department of Biology, Abant Izzet Baysal University** by,

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Director, **Graduate School of Natural and Applied Sciences**

I dedicate this thesis to my wife; Sema Büyükgöçmen , my little daughter; Şevval Büyükgöçmen, my mother; Satife Büyükgöçmen, my father; Metin Büyükgöçmen and my brother; Teoman Büyükgöçmen and my sister; Pelin Şirin for their love, prayers, and encouragement.

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.sults that are not original to this work.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.

Refik BÜYÜKGÖÇMEN

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ABSTRACT

IN VITRO PROPAGATION OF STEVIA[*STEVIA REBAUDIANA* (BERT.)] AND DEVELOPMENT OF A VARIETY WITH A HIGH STEVIOL GLYCOSIDE CONTENT WHICH CAN BE GROWN AT A COMMERCIAL LEVEL IN TURKEY

PHD THESIS

REFIK BÜYÜKGÖÇMEN

ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

(SUPERVISOR: PROF. DR. EKREM GÜREL)

(CO-SUPERVISOR: ASSOC. PROF. DR. BUHARA YÜCESAN)

BOLU, MAY 2016

This study involves two main parts; in vitro propagation and field cultivation. In vitro propagation studies were conducted at the Biotechnology Laboratory of Abant İzzet Baysal University, Department of Biology, Bolu and cultivation experiments were conducted at the Research field of Polisan Kimya A.Ş. in Yalova and Balıkesir and private farm in Adana owned by Tevfik Ramazanoğlu during two successive plantation periods. In all of our experiments, plant height, crop dry leaf yield and stevioside contents were studied. The experiments were planted according to randomized block design in factorial restriction (RBD) with four replications. At laboratory stage; propagation of stevia via conventional and tissue culture techniques were compared. The seeds of two stevia genotypes (GF and CB) were germinated in vitro under different light regimes ranging from 1000 to 5000 lux. Higher germination rates were achieved between 2000-3000 lux with a frequency of 50% germination within two days. According to the results, the highest plant height and leaf yield were determined in Adana at 2014 spring plantation. The highest stevioside and RebA ratios in dry leaves were obtained in Yalova region at 2nd harvest.

KEYWORDS: In vitro propagation**,** *Stevia rebaudiana* B, Cultivation, Stevioside

ÖZET

STEVIA[*STEVIA REBAUDIANA* (BERT.)] BİTKİSİNİN IN VITRO ÇOĞALTIMI VE TÜRKİYE'DE TİCARİ OLARAK YETİŞTİRİLEBİLECEK YÜKSEK STEVIOL GLYCOSIDE İÇEREN ÇEŞİT GELİŞTİRİLMESİ

DOKTORA TEZI

REFIK BÜYÜKGÖÇMEN

ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ

BIYOLOJI ANABILIM DALI

(TEZ DANIŞMANI: PROF. DR. EKREM GÜREL)

(İKİNCİ DANIŞMAN: ASSOC. PROF. DR. BUHARA YÜCESAN)

BOLU, MAYIS - 2016

Bu çalışma 2 kısımdan oluşmaktadır; in vitro çoğaltım ve yetiştirme. İn vitro çoğaltım çalışmaları Bolu Abant İzzet Baysal Üniversitesi, Biyoloji Anabilim Dalı Biyoteknoloji Laboratuvarı'nda yapılmıştır ve yetiştirme denemeleri; Polisan Kimya A.Ş.'nin Yalova ve Balıkesir deneme tarlaları ile Adana bölgesinde Tevfik Ramazanoğlu'na ait özel çiftlik arazisinde iki farklı dikim zamanı uygulanarak yürütülmüştür. Araştırmaların tümünde, bitki boyu, bitki kuru yaprak verimi ve steviosid içerikleri gibi özellikler çalışılmıştır. Araştırmada bitkiler Tesadüf Blokları Deneme Deseninde ve dört tekerrürlü faktöriyel tasarım (RBD) şekline göre dikilmiştir. Laboratuvar aşamasında; geleneksel ve doku kültürü teknikleri aracılığı ile stevia bitkisinin çoğaltımı karşılaştırılmıştır. İki stevia genotipine (GF ve CB) ait tohumlar 1000-5000 lux arası farklı ışık rejimleri altında çimlendirilmiştir. En yüksek çimlenme oranı, iki gün içinde %50 çimlenme frekansı ile 2000-3000 lux arasında elde edilmiştir. Diğer yandan elde edilen sonuçlara göre, en yüksek bitki boyu ve yaprak verimi Adana'daki 2014 ilkbahar plantasyonunda elde edilmiştir. Kuru yaprakta ise, en yüksek steviosid ve Reb-A oranı Yalova bölgesinde 2. hasatta elde edilmiştir.

ANAHTAR KELİMELER: In vitro çoğaltım**,** *Stevia rebaudiana* B, *Stevia rebaudiana* tarımı, Stevioside

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LIST OF ABBREVIATIONS AND SYMBOLS

%	: Percentage
°C	: Celcius
cm	: Centimeter
DAS	: Days After Sowing
da	: Decares
ha	: Hectar
kg	: Kilogram
m	: Meter
Max.	: Maximum
Min.	: Minimum
K_2O	: Potassium
N	: Nitrojen
Ave.	: Average
P_2O_5	: Phosphorus
pH	: pH

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Additionally, I wish to express my deepest gratitude to my wife and daughter who always being so encouraging and supportive.

1. INTRODUCTION

1.1 In vitro Propagation

Stevia is a natural sweetener extracted from the leaves of the *Stevia rebaudiana*. Its sweet taste derives from a group of compounds known as steviol glycosides, which can be up to 300 times as sweet as cane sugar (Metivier and Viana, 1979b). The key advantage of *S. rebaudiana* is that it is a natural and zero-calorie intensive sweetener. Biotechnological approaches seem critical for effective plant propagation and high leaf yield. That's not only for the domestication of stevia plants with a uniform steviol glycoside composition, but also cost effective refinery processing for the particular sweeteners.

In this respect, an efficient micropropagation protocol was developed for a possible commercial implementation using Murashige and Skoog (1962) basal medium (MS) only.

1.2 Field Cultivation Studies

Stevia has a temperamental nature when the plants are first set out and that is often reflected in its sluggish growth. After the first 30-35 days, it picks up growth depending upon the prevailing weather conditions. Branching and tillering are also much more profuse (Shock, 1982). Stevia's growth pattern can be divided into four stages: germination, grand growth period, flowering, and seed maturity. The first stage includes germination and establishment, the second vegetative growth, the third floral bud initiation to pollination and fertilization, and the fourth seed growth and filling. The temperature is very important duration of sowing to seed emergence and 24ºC is considered optimal for seed germination (Goettemoeller and Ching, 1999).

In all over the world, stevia has been successfully grown apparently under variety of geographic locations, although it originated in the highland regions of northeastern Paraguay, San Pedro occur between 23 and 24º S latitude (Shock, 1982), and 54 and 56º E longitude (Alvarez, 1984; Bertonha et al. , 1984; Montei ro,

1986). It is this extreme versatility that holds importance for this plant. For instance, stevia is grown as a perennial crop in subtropical regions including parts of the United States, while grown as an annual crop in mid to high latitude regions (Goettemoeller and Ching, 1999). Many results indicate that agronomic yield mainly depend on the genetic characters of the plant and the phenotypic expression, which ultimately depends governed by the climatic and environmental factors (Ermakov and Kotechetov, 1996; Metivier and Viana, 1979a).

Like most of plants, the growth and flowering of Stevia are affected by radiation, day length, temperature, soil water, and by wind in exposed places. Chen et al. (1978). Tateo et al. (1999) in early 1976, was studied the seasonal variation in stevioside content and had opined that environmental and agronomic factors have more influence on stevioside production than the growth habit.

1.3 Literature Review

Stevia rebaudiana Bertoni, belonging to the family Asteraceae, is a perennial shrub which grows up to 1 meter. In 1899, Swiss botanist Moisés Santiago Bertoni, while conducting research in eastern Paraguay, first described the plant and the sweet taste in detail. The leaves of stevia are the source of the diterpene glycosides, viz. stevioside and rebaudioside, which are estimated to be 100–300 times sweeter than sucrose (Ishima and Katayama, 1976; Tanaka, 1982). It is very valuble plant due to its adaptability to wide climatic range, the high-sweet content, and its significant contribution to the welfare of human life. Being calorie free, this plant can be a very good and effective solution for complex diabetic problems and obesity in humans.

The management of diabetes is a global problem until now and successful treatment has not been yet discovered. The last three decades, it has been estimated that diabetes would affect approximately 57 million people. The worldwide demand for high potency sweeteners, particularly natural sweeteners, is expected to increase in the years to come.

Stevia has been cultivated in continental China, Korea, Brazil, Taiwan, Thailand and Malaysia. The seeds of stevia show a very low germination percentage

(Felippe, et al., 1971; Felippe and Lucas, 1971; Monteiro, 1980; Toffler and Orio, 1981). Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Tamura et al., 1984; Nakamura and Tamura, 1985). Vegetative propagation is too limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982).

The use of *in vitro* culture technology is, therefore, a suitable alternative method for large scale plant production within a short period of time. The micropropagation of plants through shoot tip or axillary bud culture allows recovery of genetically stable and true to type progeny. There are few reports on in vitro clonal propagation of stevia plants using leaf, nodal, internodal segments and shoot tip explants. Bespalhok and Hattori (1997) obtained only embryogenic callus from floret explants of *S. rebaudiana*. In vitro plant regeneration from shoot tip explants of *S. rebaudiana* was reported by Patil et al. (1996), Uddin et al. (2006) and Debnath (2008). Sivaram and Mukundan (2003) obtained maximum number of shoot buds (7.9 shoots/explant) from nodal explants on MS medium supplemented with 8.87 -µM BAP and 5.71 -µM IAA.

A number of complex factors include the genetic make-up of the plant, chemical factors (nutrient medium components/water, macro and micro elements, sugars and plant growth regulators gelling agent and also physical growth factors (Pierik, 1988) determined the *in vitro* growth and development of plant. Therefore, a consistent plant production in terms of similar natural compounds is of great importance in value-added plant species.

1.3.1 In vitro Propagation

Stevia rebaudiana is a perennial, photoperiod sensitive, entomophilous, and self-incompatible bushy shrub. Due to its high intensive sweeteners derived from ent-kaurene diterpenoid glycosides (steviol glycosides) in the leaves, it has been of great value in the global market of intensive sweeteners, since the consumers look for the natural alternative ingredients in their diets. Steviol glycosides offer a source of sweetness without any calorie, toxicity, and carcinogenic effect in human health

(Kinghorn 2002). In the US and Europe the use of pure steviol glycosides (>95%) is allowed for human consumption as a non-medical ingredient up to 4 mg/kg of body weight/day (Wölwer-Rieck et al. 2012).

According to the market reports, global sales of stevia-derived sweeteners forecast with an increase from 3500 tonnes in 2013 to around 10,000 tonnes in 2017 representing a growing trend of 186%. Moreover, the market for steviol glycosides is expected to reach a value of US$ 275 million, having almost double value as compared to the present level (Leaderhead Food Research and Mintel, 2013). Of those natural sweeteners, rebaudioside-A (Reb-A) and stevioside (ST) are predominantly found in stevia plants (Reb-A and ST, 2–6%, 5–10% of dry weight, respectively). Generally, total steviol glycoside content ranges from 4–20% of dry leaf weight depending on the varieties. Therefore, a plurality of studies is required to meet the need of agricultural facilities on the stevia domestication with high steviol glycoside content.

However, in this respect, *S. rebaudiana* shows very poor seed germination percentage (Goettemoeller and Ching 1999), and efficiency of clonal propagation via stem-cuttings is restricted by the number of source plants that they need particular season for high survival rate (Tamura et al. 1984; Khalil et al. 2014). In vitro propagation has immense potentiality for the conventional true-to-type propagation and breeding procedures for many industrial plants. Based on our literature survey ascertained for *S. rebudiana*, in vitro regeneration studies have been mainly focused on direct and/or indirect organogenesis in the last decade (Mitra and Pal 2007; Sreedhar et al. 2008; Ahmad et al. 2011; Das et al. 2011; Preethi et al. 2011; Manthur and Shekhawat 2013; Khalil et al. 2014; Singh et al. 2014; Ganait et al. 2015; Ramirez-Mosqueda and Iglesias-Andreu 2015). However, genetic variability of the cultivars, different explant sources, cultivation periods, and media formulations significantly affect regenerant production.

On the other hand, tissue cultures studies incorporated with field trials are yet a major challenge in commercial production. Therefore, in this study we aimed at establishment of an efficient and a rapid clonal propagation protocol from nodal

segments. In addition to regeneration studies, field experiments testing a wide range of steviol glycoside profile in the plant samples derived from node-explants and seed germination was investigated for the first time in different vegetative periods.

S. rebaudiana is a perennial, photoperiod sensitive, entomophilous, and self-incompatible with tiny white flowers. It is propagated through cuttings and seed germination; however, stem cutting needs large input stock plants and labor, and the achievement of the technique is season-dependent (Khalil et al. 2014). In addition, seeds of this plant are smaller in size and germination frequency is very low. Because of these limitations ascertained, stevia propagation efforts have been largely focused on in vitro clonal propagation studies.

There are plenty of in vitro regeneration studies conducted in *S. rebaudiana* the last decade concerning mainly direct and/or indirect organogenesis (Hwang, 2006; Reis et al., 2011; Preethi et al., 2011; Lata et al., 2013; Gantait et al., 2015). However, genetic variability of the cultivars, different explant sources, cultivation periods, and media formulations significantly affect regenerant production. In the present study, i) steviol glycoside profiling of the plantlets either derived from nodal explants or seeds before and after transferring to the field, and ii) their agronomical parameters in field studies will be assessed (i.e. shoot length, leaf area).

1.3.1.1 Glycosides in *S. rebaudiana*

In leaf tissues of stevia, there are eight diterpene glycosides with sweetening properties have been identified. The sweetest compounds are (the two main glycosides) stevioside, traditionally 5 to 10% of the dry weight of the leaves, and rebaudioside-A (Reb-A), 2 to 4%. These are synthesized, at least in the initial stages, using the same pathway as gibberellic acid, an important plant hormone (Singh and Rao, 2005).

The same pathway using for other glycosides such as rebaudioside-B, rebaudioside-C (1 to 2%), rebaudioside-D, rebaudioside-E, rebaudioside-F, dulcoside-A, dulcoside-C and steviolbioside, as well as flavonoid glycosides, coumarins, cinnamic acids, phenylpropanoids and some essential oils are also other related compounds including minor glycosides (Erik et al., 1956; Erich et al., 1961;

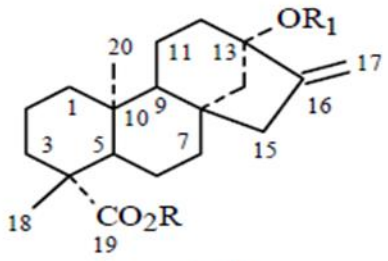
Harry et al., 1956; Hiroshi et al., 1976; Masur et al., 1977; Yohei and Masataka, 1978; Rajbhandari and Roberts, 1983; Makapugay et al., 1984; Crammer and Ikan, 1986; Kinghorn, 1987; Tsanava et al., 1989; Shaffert and Chebotar, 1994; Putieva and Saatov, 1997; Dzyuba, 1998; Dacome et al., 2005; Sekaran et al., 2007) (Table 1; Figure 1).

The stevioside, rebaudioside-A, rebaudioside-B, rebaudioside-C, rebaudioside-D, rebaudioside-E, dulcoside-A and steviolbioside are 250 to 300, 350 to 450, 300 to 350, 50 to 120, 200 to 300, 520 to 300, 50 to 120 and 100 to 125 times sweeter than sucrose, respectively (Crammer and Ikan, 1986; Yadav et al., 2011). (Figure 1)

Table 1. Percentage of major biochemical constituents of stevia.

Components	Value (g/100 g dry leaf weight)
Carbohydrates	35.2
Proteins	12.0–20.42
Lipids	2.7–4.34
Ash	13.12
Stevioside	4–14%
Rebaudioside A	2–4%
Rebaudioside C	1–2%
Dulcoside A	0.4–0.7%
Rebaudioside D,E,F; steviolbioside; rubusoside	>0.4%

(Yadav and Guleria, 2012)



1 - 9

1: R = β-Glc, R_1 = β-Glc^2-β-Glc

2: R = H, R_1 = β-Glc^2-β-Glc

3: R = β-Glc, R_1 = Glc^2-β-Glc
|3
β-Glc

4: R = H, R_1 = β-Glc^2-β-Glc
|3
β-Glc

5: R = β-Glc, R_1 = β-Glc^2-β-Rh
|3
β-Glc

6: R = β-Glc^2-β-Glc, R_1 = β-Glc^2-β-Glc
|3
β-Glc

7: R = β-Glc^2-β-Glc, R_1 = β-Glc^2-β-Glc

8: R = β-Glc, R_1 = β-Glc^2-β-Xyl
|3
β-Glc

9: R = β-Glc, R_1 = β-Glc^2-β-Rh

(Kovylyaeva, et all, 2007)

Figure 1. *Stevia rebaudiana* glycosides. Their sweetness is due to the glycosides stevioside (1), steviolbioside (2), Rebaudiosides A (3), Reb-B (4), Reb-C (5), Reb-D (6), Reb-E (7), Reb-F (8), and dulcoside A (9)

1.3.1.2 Rebaudioside-A

Rebaudioside-A is of particular interest because it has the most desirable flavour profile (DuBois, 2000). Stevioside traditionally makes up the majority of the sweeteners (60 to 70% of the total glycosides content) and is assessed as being 110 to 270 times sweeter than sugar. It is also responsible for the bitter aftertaste, sometimes reported as a ‘‘licorice’’ taste. As well as sweetness, stevioside may have a lingering effect or certain degree of pungency, which is not appreciated by the majority of people, and reduces its acceptability.

In total sweetener, Rebaudioside-A is usually present as 30 to 40% of it and has the sweetest taste, assessed as 180to 400 times sweeter than sugar, with no bitter aftertaste (licorice taste or lingering effect). The ratio of rebaudioside-A to stevioside is the accepted measure of sweetness quality; the more rebaudioside-A, the better the quality (Yadav et al., 2011) (Table 2). The yield of sweetening compounds in leaf tissue can vary according to the method of propagation (Tamura et al., 1984a), day

length (Metivier and Viana, 1979) and agronomic practices (Shock, 1982). Unlike many low-calorie sweeteners, stevioside is stable at high temperatures (100°C) and over a range of pH values (Kinghorn and Soejarto, 1985). It is also non-calorific, non-fermentable and does not darken upon cooking (Crammer and Ikan, 1986).

Table 2. Quantitative composition of rebaudiosides in various samples of *S. rebaudiana* leaves.

Collection site	Rebaudioside contents, % (per 100 g dry leaves)		
	Stevioside	Rebaudioside A	Rebaudioside C
Russia (Voronezh Oblast')	5.8	1.2	0.5
Ukraine (Crimea)	4.8	1.3	0.3
South Korea	5.5 [13, 14]	2.5 [13, 14]	1.4 [13, 14]
China	6.6 [13, 14]	3.7 [13, 14]	2.1 [13, 14]
Paraguay	4.6 [13, 14]	1.9 [13, 14]	0.9 [13, 14]
	9.1 [4]	3.8 [4]	0.6 [4]
Japan	7.7 [13, 14]	1.9 [13, 14]	0.9 [13, 14]
Canada	5.0 [12, 15]	0.3 [12, 15]	0.1 [12, 15]
Viet Nam	15.5 [12, 15]	3.8 [12, 15]	1.4 [12, 15]

(transfer from Kovylyaeva et al., 2007)

1.3.1.3 Glycoside Content in Different Plant Parts

Individual tissues of stevia appear to differ significantly in the stevioside content declining in order: leaves > shoots > roots > flowers (Sekaran et al., (2007). The fact that the highest stevioside content is found in the leaves suggests that they serve as the main tissue for both synthesis and primary accumulation of stevioside compounds.

1.3.1.4 Importance of Stevia Tissue Culture

To exploit industrial application of stevia, its large scale production is needed. Seeds of stevia show a very low germination percentage (Felippe and Lucas, 1971; Monteiro, 1980). Since the world population is increasing rapidly, there is extreme pressure on the available cultivatable land to produce food and fulfill the needs. Therefore, for other uses such as production of pharmaceuticals can help to use the available land effectively.

The development of micropropagation methods for a number of medicinal plant species has been already reported and needs to be adopted (Naik, 1998). A wide range of secondary metabolites which are used as pharmaceuticals, agro-chemicals, flavors, fragrances, colors, biopesticides and food additives, plants are valuable source. Over 80% of the approximately 30,000 known natural products are of plant origin (Phillipson, 1990; Balandrin and Klocke, 1988).

Through plant tissue culture, the totipotent characteristics of plant can be used for the in vitro regeneration of plant. The great enthusiasms of biotechnologists are seen in the potential use of cell culture in the production of valuable secondary products. Plant tissue culture is a noble approach to obtain their substances in large scale. In the present scenario, it is an effective procedure for converting less medicinally important plant metabolites to a valuable product. Many companies in India and abroad are moving in this direction.

Stevia plants do not metabolize in the human body (Randi, 1980), making stevia safe for those who need to control their blood sugar level. With the current demand for food supplements having low carbohydrate, minimum calorie and low sugar content, the stevia plant and its extracts have proven to be the ultimate choice. The health benefits of stevia are evident from the fact that they are approved as a dietary supplement by the Food and Drug Administration (FDA).

In addition to its non-caloric sweetening properties, *S. rebaudiana* has many medicinally important properties. It is used for the treatment of various conditions such as cancer (Yasukawa et al., 2002), diabetes (Lailerd et al., 2004), obesity, cavities, hypertension (Dyrskog et al., 2005), fatigue, de-pression, and yeast infection. It possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, anti-fungal, anti-viral, anti-inflammatory, anti-bacterial properties and increases urination. It has been found to be non toxic, non addictive, non carcinogenic, non mutagenic, non teratogenic and is devoid of genotoxic effect. It does not affect blood sugar level, hence safe for diabetics (Alan, 2002; Mogra and Dashora, 2009). Hence, it is of large industrial and therapeutical value.

1.4 Field Cultivation Studies

1.4.1 Agricultural Importance of Stevia

Many different characteristics of stevia that make it a potentially valuable agricultural species (Table 3), although there are few reasons, e.g. day length sensitivity/short day plant (Lester, 1999; Valio and Rocha, 1966), sensitive to water logging, low to moderately resistant to drought (Jia, 1984), poor early growth (Borie, 2000), heavy weed competition at early stages (Andolfi et al., 2002), sensitivity to frost, poor seed germination (Barathi, 2003; Carneiro et al., 1997; Duke, 1993; Shock, 1982), short period of germinantive power (Marcavillaca, 1985), poor tolerance to high soil pH (Shock, 1982), self incompatible (Chalapathi et al., 1997b), asynchronous seed maturity, generally limiting its agronomic utility.

Mainly ten different steviol glycosides are responsible for its sweetness in different plant parts. That is great importance for both understanding the peculiarities of diterpenoid glycoside production and for adoption of mass scale production techniques. Most of studies conducted so far could suggest few management approaches for improving production requirements. Stevia had made significant agricultural impact in countries such as Japan, China and USA.

Table 3. Agronomically important characters of stevia.

Serial Number	Characters
1	Wide climatic adaptability
2	Perennial in nature (Andolfi et al., 2002), unique regeneration capacity after frost injury (Singh and Kaul, 2005)
3	Leaf, the economic part
4	Vegetative propagation is possible (Chalapathi et al., 1997b)
5	3–4 harvests per year (Donalisio et al., 1982)
6	Intercropping during the initial growing period
7	Easy propagation through seeds, stem cuttings, and division of roots (Singh and Kaul, 2005).

(Transferred from Ramesh et al, 2006)

In many countries stevia leaves are used for sweetening, as is, or dried and pulverized, or soaked in water; the liquor is used for sweetening. Some European and

Asian companies produce beverage, chocolate, pudding and chewing gum from Stevia. The herbage contains 0.12– 0.16% essential oil, which is up to 0.43% in the inflorescence (Kinghorn and Soejarto, 1985).

1.4.2 Cultivation

The first cultivation of stevia has been reported as early as BC 1500 in South America by native Guarani tribe (Misra, 2011). But real cultivation has been started in early 1970s in Japan (Crammer and Ikan, 1986). At that stage of cultivation, stevia crop exhibited much more vigor than in natural populations (Shock, 1982), a fact suggesting that with appropriate crop management practices.

After decade, commercial cultivation has extended in Japan, Southeast Asia, and United States (Fors, 1995; Sakaguchi and Kan, 1982), but it is being cultivated in some semitropical areas in the world. As the plant does not survive winter climate, it is cultivated in Europe as a leaf crop under greenhouse conditions (European Commission, 1999).

There have been studies on development of modern techniques of cultivation, propagation through tissue culture, and selection (Acuna et al., 1997; Akita et al., 1994; Ashwini, 1996; Ferreira and Handro, 1988a,b,c; Filho et al., 1992; Flachsland et al., 1996; Handro et al., 1993; Huang et al., 1995; Kornilova and Kashnikova, 1996; Nepovim and Vanek, 1998b; Patil et al. , 1996 ; Sivaram and Mukundan, 2002; Tam ura et al., 1984b).

1.4.3 Seed and Germination

Generally, the only problem in cultivation of stevia using seed is low germination and there is always requirement to improve the germination rate by several methods. Sometimes potting media played an important role in enhancing the germination rate of seed and better potting medium can be used for successful establishment of seedling of any plant (Figure 2)(Kumar, 2013) Winter seeds sown in cold weather showed poor germination (Shock, 1982). Some researchers e.g. Alvarez et al. (1994) reported that just after harvest, it was impossible to sow the seeds

immediately, and concluded that the seeds should be kept in sealed tight containers in the refrigerator at 4°C, since it loses viability at room temperature.

Further, studies indicated that germination was best at 25°C (Felippe and Randi, 1984; Randi and Felippe, 1981) and at this temperature, 63.2% of maximum germination occurred after 101.4 h (Takahashi et al., 1996). Cabanillas and Diaz (1996) had reported the performance of seeds under different temperature and light conditions at Argentina. No viable seed treatment to enhance seed germination has been reported elsewhere. In another research about seed germination; the final observation regarding germination per cent showed that maximum germination (67.5%) was recorded in soil medium followed by the combination of soil and rice husk (57.4%). The minimum germination was recorded in vermiculite (41.1%). The germination in sand (48.4%) was better than vermiculite (Kumar, 2013).

Stevia seeds are small size and it is a general practice to raise nurseries. It is propagated through either seeds or cuttings. Seeds are germinated in the glasshouse in spring and the plants (usually 6–7 weeks old) are transplanted into the field (Lester, 1999). In the temperate latitudes, the production cycle for annual crops starts with the 6–7 weeks old plants grown from seed. Seeds were stored for 11 months at 4°C or at ambient temperature and humidity (Cabanillas and Diaz, 1999).

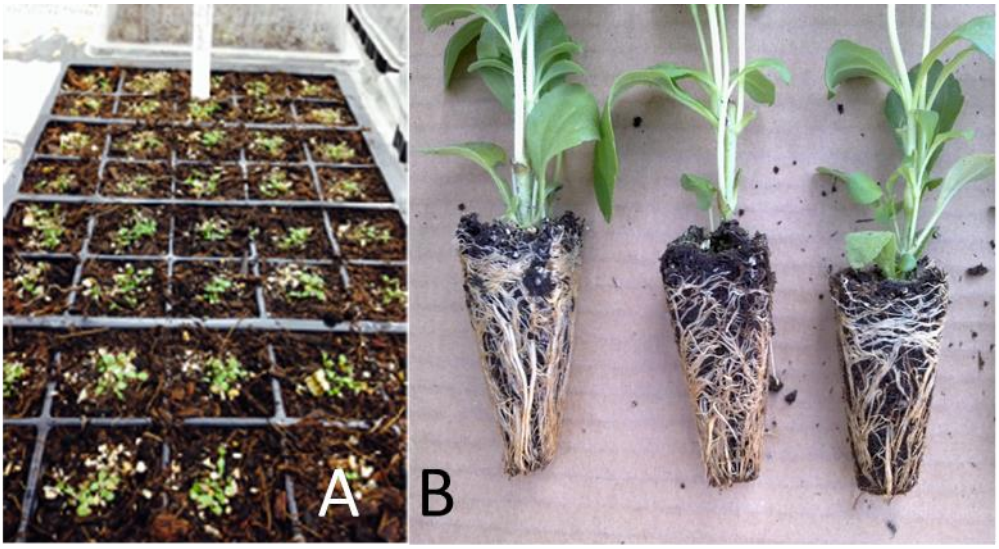


Figure 2. Germination of the seeds. Two weeks after sowing in plastic trays (a); well-rooted 4 months old seedlings (b).

The data reveals that germination was started from third day in all media compositions except vermiculite but in vermiculite the germination was started from the fifth day. Except vermiculite, it is clear from the observations that very steady and fast germination was recorded in all media. In the case of vermiculite very poor germination was observed on fifth day of the sowing of seeds but after fifth day the germination per cent increased significantly over previous days in comparison to other media (Kumar, 2013).

1.4.4 Crop Density

For stevia; crop density is closely depending on the environment. Crop density should consider the root spread. Crop density is a parameter decided by the crop spread above ground so as not to interfere with the development of the adjoining plants. (Figure 3)

Figure 3. Crop density (40 × 25 cm)

Some experments indicated that higher growth and yield, when low plant density was adopted (60x20 cm), while dry leaf yield was higher in denser planting (60x10 cm) (Murayama et al, 1980). In contrast, Lee et al. (1980) had reported that plant height, number of branches, and number of nodes were unaffected by planting density (50–70 cm between and 10–30 cm within rows), but dry leaf yield per plant decreased with increasing plant density. In accordance to the above, Dona lisio et al. (1982) had recommended a plant population of 80,000 ‑ 100,000 plants ha^{-1}. Reduction in row to row spacing was also attempted. A spacing of 50 × 20 cm (Filho et al., 1997a) or 45 × 22.5 cm (Chalapathi, 1996) performed well but still narrow spacing of 25 × 25 cm was also tried by Angkapr Adipta et al. (1986b), however, this is not advisable considering the root spread of the crop. Basuki (1990) tried a very high density to manage weeds however, this would result in poor crop growth due to intense light competition and the leaf/stem ratio will decline. Leaf yield was found to increase up to 1.1 lakh plants ha^{-1} for the first year of production (Brandle et al., 1998). Under Palampur conditions, 50,000 plants ha-1 were maintained at a spacing of 45 x 45 cm (Singh and Kaul, 2005). The highest stevia yield was obtained at 70 x 25 cm spacing at Abkhazia (Gvasaliya et al., 1990). Therefore, it is advisable to carry out experiments in each planting zone to establish adequate plant population density for that particular area (Ramesh, 1990).

1.4.5 Harvest

The optimum time of harvest depends on the cultivar and growing season. Leaves are harvested about 4 months after planting by cutting the plants at about 5–10 cm above the soil level (Donalisio et al., 1982). This must be at the maximum crop biomass stage, otherwise yield can be lower than normal (Shuping and Shizhen, 1995). Since the crop is highly sensitive to low temperature, in cold areas, crop may be harvested before or at onset of winter (Columbus, 1997). During flowering, stevioside dissipates from leaves (Bian, 1981; Hoyle, 1992), thus, leaves should be harvested at the time of the flower emergence (Dwivedi, 1999) or before flowering (Barathi, 2003).

1.4.6 Yield

Stevia is a semi-perennial species, which can be maintained up to 5–6 years, with 2 or 3 harvests per year. Earlier, Bridel and Lavielle (1931a,b,c) and Metivier and Viana (1979a) reported a stevioside yield of 60–65 and 72 g kg^{-1} dry leaf, respectively. In terms of economic biomass productivity, the dry leaf yield in the natural habitat, Paraguay, was between 1500 and 2500 kg ha^{-1} (Kumar et al., 2006) under dry land conditions and around 4300 kg ha^{-1} per year at irrigation conditions (Jordan Moleiro, 1984). Leaf yield of 3000 kg ha^{-1} with a stevioside concentration of 105 mg g^{-1} equivalent to 66.2 ton ha^{-1} of sugar was obtained at Canada (Brandle and Rosa, 1992).

In another research in Japan, 1 or 2 harvests per year is possible with a dry leaf yield of 3000 and 3500 kg ha^{-1} in the first year, 4000–4500 kg ha^{-1} in the second, 4000 – 6000 kg ha^{-1} in third, diminishing to 4000 kg ha^{-1} in the fourth year (Taiariol, 2004). In late transplantation; crop grown for single cut, harvesting is done after 3–4 months of transplanting and continues till flowering begins, because the maximum sweetener in the leaves appears in flowering stage. Perennial crop may continue up to 4 years, once planted in the same field. Life span of the crop is reported to be 7–8 years and herb yield increases up to 4 years. Maximum amount of leaves are produced in the third or fourth year. Under agro-climatic conditions of Palampur, first harvest is taken at 90–110 days after transplanting during June or

July. Subsequently, second harvest is taken after 60–75 days of the first harvest in early September at the time of flower bud initiation.

Maximum amount of leaves are produced in the third or fourth year. Flowering of the plant should be avoided and pinching of the apical bud should be done to enhance bushy growth of the plant with side branches. In the first year, average fresh biomass yield of 15–20 ton ha^{-1} was obtained out of two harvests and increased in subsequent years up to 20–30 ton ha^{-1}. An average dried leaf yield of 17, 20, 23, and 25 q ha^{-1} could be produced from this total biomass yield in the first, second, third, and fourth years, respectively (Singh and Kaul, 2005).

1.5 Botanical Description

Stevia rebaudiana (Bert.) is one of the 950 genera of the Asteraceae family (Lester, 1999; Soejarto et al., 1983) and in the Stevia genus, there are more than 200 species. Soejarto et al. (1983) had proved that *S. rebaudiana* gave the sweetest essence. It is a perennial herb with an extensive root system and brittle stems producing small, elliptic leaves (Shock, 1982).

In Paraguay, *Stevia* is normally described as perennial herb in its natural habitat, though under some environmental conditions and management situations it behaves as an annual or mixture of plants of both types. The cultivated plants reported to be more vigorous. Since leaves are the principal sweet bearing parts of the plant, the proportion of leaf to whole plant, the leaf weight ratio is important. High ratios of leaf/stem are desirable in cultivated *Stevia* because of the low stevioside concentrations (<5 mg g^{-1}) in stem tissue (Ramesh et al., 2006). Stevia grows to about 50–60 cm tall (Brandle and Rosa, 1992; Lester, 1999), 100 cm (Shock, 1982), or up to 120 cm (Dwivedi, 1999).

The authorities divided the growth pattern of stevia into four stages: germination, growth period, flowering, and seed maturity. The first stage includes germination and establishment, the second vegetative growth, the third floral bud initiation to pollination and fertilization, and the fourth seed growth and filling. The duration of sowing to seed emergence is related to the temperature, and 24°C is considered optimal for seed germination (Goettemoeller and Ching, 1999).

Stevia root is fibrous, filiform, and perennial, forming abundant stock (Schmeling, 1967) that is hardly ramified and does not deepen, distributing itself to the land surface; and is the only part that does not contain stevioside (Vargas, 1980; Zaid an et al. , 1980). Sunk (as quoted by Taiari ol, 2004) described that the fine roots congregate around the soil surface and thicker roots in the deepest zones.

The stem is annual, subligneous, more or less pubescent, with tendency to decline, and more or less graft (Sakagu chi and Kan, 1982).

Stevia's first photosynthetic organs are formed after germination from the two cotyledons in the seed. Those organs are rounded in shape. Stevia has an alternate leaf arrangement and herbaceous growth habit with flowers arranged in indeterminate heads. Leaves are small, lanceolate, oblong, serrate, and sweet (Dwivedi, 1999). For stevia, the leaf area index (LAI) at 80 days after sowing (DAS) was 4.83 (Fronza and Folegatti, 2003). It is fairly obvious that the amount of intercepted light principally depends on the leaf surface area of the crop, and is usually expressed as leaf area index.

Stevia is self-incompatible (Chalapathi et al., 1997b; Miyagawa et al., 1986) and probably insect pollinated plant (Oddone, 1997). The flowers are small and white (Dwivedi, 1999) with a pale purple throat. The tiny white florets are perfect, borne in small corymbs of two to six florets. Corymbs are arranged in loose panicles (Goettemoeller and Ching, 1999). A plant takes more than a month for producing all its flowers (Taiariol, 2004).

In stevia, viable seeds percentage is very poor [Shock (1982), Duke (1993), and Carneiro et al. (1997)]. Seeds are contained in slender achenes, about 3 mm in length. Each achene has about 20 persistent pappus bristles. Reproduction in the wild is mainly through seed, but seed viability is poor and highly variable (Lester, 1999). Seeds have very little endosperm and are dispersed in the wind via hairy pappus.

Stevia has 10 glycosides out of which stevioside and rebaudioside A are important. Plant organs contained different amounts of the sweet glycoside, stevioside, which declined in the following order; leaves, flowers, stems, seeds, and roots. The sweetness in the leaves are two times higher than that in inflorescence (Dwivedi, 1999). The highest amount of steviosides was found in the upper young

actively growing shoot sections, whereas lowest senescent shoot sections exhibited the lowest amount of such compounds. During ontogeny, a gradual increase in the stevioside concentration was observed in both mature Stevia leaves and stems, and this process lasted up to the budding phase and the onset of flowering (Bondarev et al., 2003b).

2. AIM AND SCOPE OF THIS STUDY

The aims of the study were to develop an efficient and reproducible in vitro propagation system for *S. rebaudiana*, using a local stevia genotype and determine the adaptation possibilities by conducting field experiments in Turkey. Chromatographic techniques were employed to determine the steviol glycoside compounds (diterpenoid glycosides) along with the process development of the stevia plants from the laboratory to the field. Pre-breeding programs were adopted and all stevia genotypes were characterized in respect to agronomic as well as sweetener parameters which might be considered as an alternative source of sugar or other calori-free sweeteners for further breeding studies.

3. MATERIALS AND METHODS

3.1 In Vitro Propagation

3.1.1 Plant Samples and Seed Germination

Seeds of *stevia rebaudiana* was provided by an agricultural company (Polisan Tarım, Istanbul, Turkey) in 2013, and kept at +4 °C until use. Germination was done in plastic trays (12×9 cells) containing compost:soil:perlit:vermiculate mixture at a ratio of 10:10:1:1, in which 5 seeds per cell were sown. In order to keep humidity at high level (>80%), each trays were covered with a transparent lid. One week after germination, the germinated seedlings were transferred individually to a new tray, each cell containing one seedling only. Additionally, seeds were germinated also under in vitro conditions. To achieve this, the seeds were surface disinfected in 0.1% (w/v) mercuric chloride for 3 min, and rinsed immediately with autoclaved distilled water 5–6 times. Then, the seeds were germinated in disposable Petri plates (90 × 15 mm) containing 20 mL Murashige and Skoog (MS) medium (Murashige and Skoog 1962). MS medium including 30 g/L sucrose was prepared and pH was adjusted to 5.7 before adding 8 g/L plant agar, and then, autoclaved at 121 °C for 20 min.

3.1.2 Plantlet Production, Rooting and Hardening off

For clonal propagation, nodal segments were excised from two-week-old seedlings germinated in vitro, and placed on MS medium for 3 weeks in order to produce stock plants throughout the experiment. In this respect, the nodal explants (3 mm) were cultured in glass culture tubes with transparent lid (20 × 150 mm; Sigma-Aldrich, USA) containing 10 mL of MS medium supplemented with different concentrations (0.1, 0.5, 1.0 or 2.0 mg/L) of kinetin (KIN) or 6-Benzylaminopurine (BAP) or hormone-free MS medium for 3 weeks. The explants were placed in the medium in upright position with the lower half of the explant dipped inside the agar. For each experiment, 10 explants (1 node/culture tube) were used, and the experiments were repeated three times. Following the shoot formation, all node-derived shoots (3–4 cm in length) were transferred to the rooting medium in glass

jars (330 mL) containing 30 mL of MS medium supplemented with or without 0.25 or 0.50 mg/L of IAA, IBA or NAA. All media used for both shoot regeneration and rooting were supplemented with 30 g/L sucrose and 8 g/L plant agar. For each treatment, 4 shoots per jar (Magenta B-cap, Sigma-Aldrich, USA) with 10 replicates were used, and experiments were repeated three times. Then, the shoots were maintained in their respective treatments for 4 weeks.

All regenerants (~14.0 cm in length) produced in vitro conditions were subsequently transferred to the plastic trays containing a mixture of substrate (Kekkila C1, Finland), perlite and vermiculate at a ratio of 10:2:1. Afterward, each tray was placed into portable greenhouses (50×70×140 cm) covered with transparent polytene shelters in a plant growth room.

The chemicals used in tissue culture experiments were purchased from Duchefa Biochemie, Netherland. Plant cultures were maintained in a plant growth chamber at 23.0±0.5 °C under a 16-h photoperiod provided by cool-white fluorescent light with an irradiance of 35 μmol photons m^{-2} s^{-1} at 65% humidity. Hardening off was done in portable greenhouses placed in a another growth room equipped with an average light irradiance of 50 μmol photons m^{-2} s^{-1} (cool-white fluorescent) at 60–70% humidity at 27.0±2.5 °C, prior to transferring to the field in Yalova, Turkey.

3.1.3 Field Experiments

Field experiments were carried out in Yalova province, Turkey, for 16 weeks. Nine-week-old (a period from shoot induction to the hardening off) "regenerants" produced in vitro and seed-derived "seedlings" in pots were planted in the field as 10 plants per m^2 in two separate plots, with a total area of 500 m2. The roots of regenerants and seedlings were dipped into fungicide (Quadris: Azoxystrobin methyl and Tachigaren 70 WP: Hymexazol) against root diseases for 3 seconds, before being drilled into the soil. Drip irrigation system was used throughout the field experiments, and weed control was done by hand weeding. Neither fertilizer nor herbicide and pesticide were used in this study. Steviol glycoside content was recorded at late vegetative stage (at 9^{th} week of plantation) and flowering stage (at 13^{th} week of plantation). All plants were harvested after 16 weeks of plantation.

3.1.4 Extraction and HPLC Analysis

The leaf materials collected from 20 randomly selected plants (seedlings or regenerants) from each plot were used for the quantification of steviol glycosides. For the extraction, heat-dried (40 °C) leaf samples were ground to fine powder with a pestle and mortar, and added 1 mL of 70% (v/v) methanol to 20 mg of each sample in micro-centrifuge tube (2 mL size). After ultrasonication at 50 °C for 15 min, the tubes were immediately centrifuged at 10,000 rpm for 10 min. The supernatant was filtered using 0.22 µm PTFE syringe filters prior to HPLC analysis. Steviol glycosides (SG) were eluted with an isocratic flow using acetonitrile (HPLC grade) and 1% (w/v) phosphoric acid buffer mixture at pH 2.6 (70:30) for 20 min. Flow rate was 1.0 mL for 10 min with a binary pump (LPG 3400SD, Thermo, USA) solvent delivery system, a dual wavelength absorbance detector (MWD-3100 UV-Vis Detector) operating at 210 nm, and 356 nm was used as a reference wavelength, and with an auto-sampler (WPS-3000-SL SemiPrep-Autosampler) injecting 10 µL of each sample. The column, Inertsil®ODS-3 (GL Sciences Inc., Japan), 150 × 4.6 mm in length and 5 µm particle size, was kept warm at 40 °C in a column oven system (TCC-3000SD).

Recovery method was employed in a way that once the final plant extracts were obtained, they were spiked with the 100 mg/L pure steviol glycosides. Recovery percentages were calculated from 5 individual extractions, and 3 analytical HPLC runs of each extract. Moreover, a fraction collector system (AFC-3000 Ultimate Fraction Collector) was used for the confirmation of SGs. Further confirmation was employed by using a thin layer chromatography system. Steviol glycoside contents were determined on the basis of their molar absorptivity at 210 nm using Rebaudioside A for calibration. In this study, the six individual SG including rebaudiosides (Reb-A, -C, -D and –F), stevioside (ST) and dulcoside A (Dul) were analyzed. All standard SGs were purchased from Chromadex®, USA.

3.2 Field Experiments

3.2.1 Plant Materials

Two different seed/seedling sources were used in field experiments. One of them from China and other was from Grow Fide (Antalya, Turkey) seedling material. Grow Fide did not have specific variety but mixed Central American seeds (Figure 4).

In our experiment, the seedlings were supplied by Grow Fide and our own materials which propagated by in vitro culture at Abant izzet Baysal University. The samples were planted randomized complete block, factorial design (RBD) with four replications. Four plot each of them has gross area about 250m^2 were planted and observation were recorded from that area per plot. The yield parameters recorded were dry weight of leaves (kg ha^{-1}), stevioside content (%), Reb A, Reb C content (%) and stevioside yield (kg ha^{-1}).

Figure 4. Spring 2013 plantation/Yalova (a), Grow Fide seedlings (b).

3.2.2 Growing Conditions and Collection of In Vitro Plant Material

Stevia plants were planted in 3 different locations of Turkey; Yalova, Adana and Balıkesir/İvrindi (Appendix, Map 1, Map 2). All locations were assumed to fit with stevia cultivation.

Yalova city is located in northwestern Turkey, near the eastern coast of the Sea of Marmara. Coordinates are 40˚39′ 20″ N, 29˚16′ 30″ E. Adana city is in southern Turkey and a major agricultural, industry and commercial center. It is the fifth most populous city in Turkey. The city is situated on the Seyhan River, 30 kilometres (19 miles) inland from the Mediterranean Sea, in south-central Anatolia.

Coordinates are 37˚0′ N, 35˚19.28′ E. Balıkesir/İvrindi is a town and district of Balıkesir in the Marmara region of Turkey. Coordinates are 39˚35′ N, 27˚29′ E.

3.2.3 Climatic Data 2013/2014

Long term climate data and detailed temperature and rain statistics were given (at appendix Table 4, Table 5 and Table 6) (Figure 5, Figure 6, and Figure 7). In Yalova region 2013 rainfall was about 23% less than long year average but 2014 was 11% above (Figure 5).

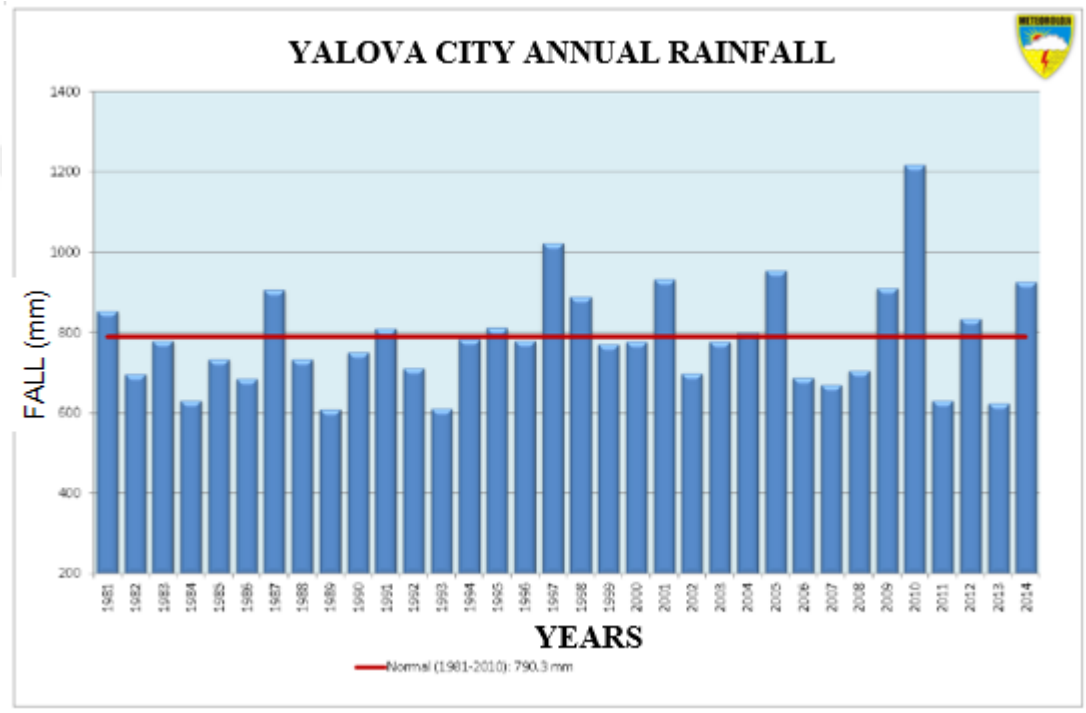


Figure 5. Yalova city 1981-2014 average rainfall.

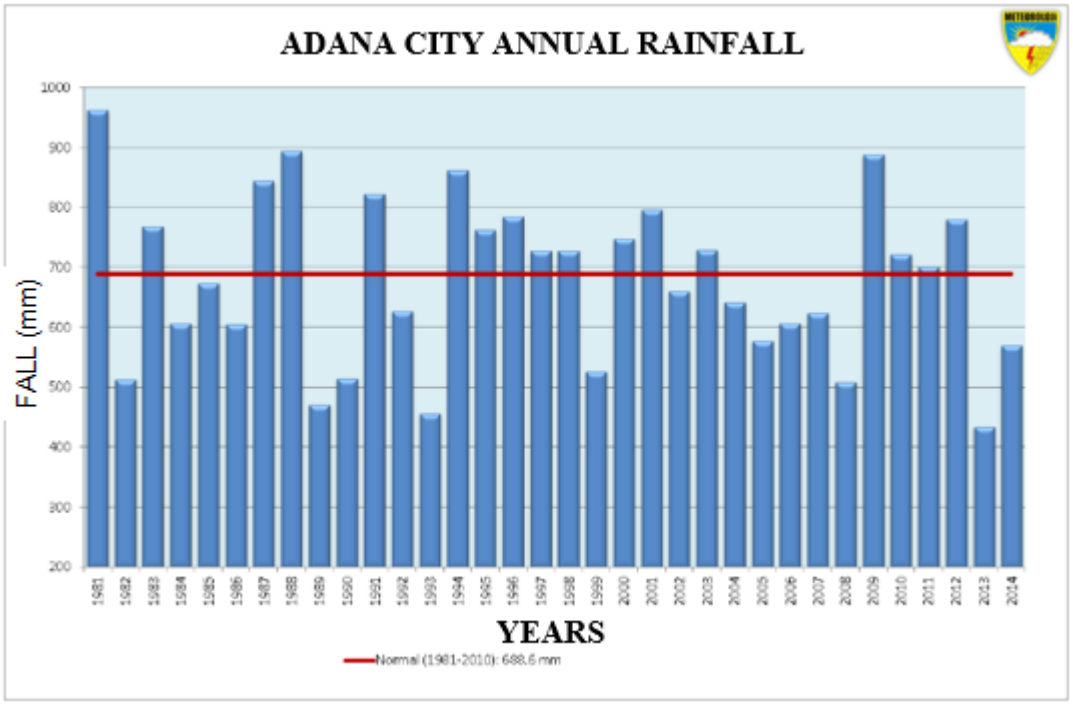


Figure 6. Adana city 1981-2014 average rainfall

In Adana region both 2013 and 2014 rainfalls were 12-28% less than long years average (Figure 6).

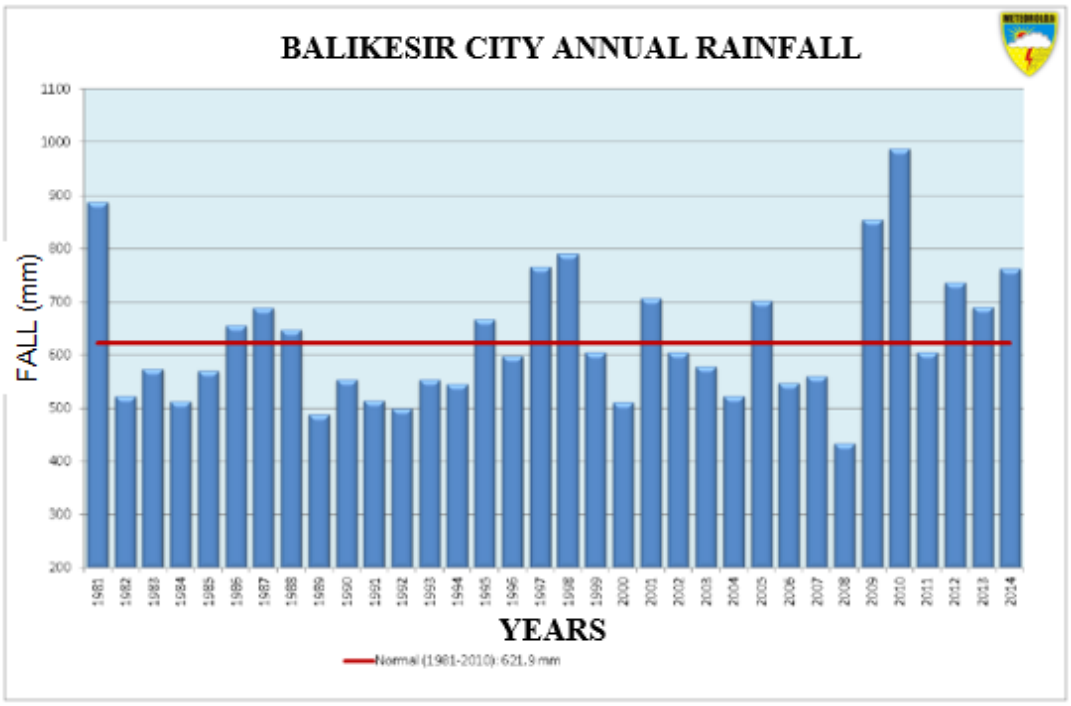


Figure 7. Balıkesir city 1981-2014 average rainfall

In Balıkesir region both 2013 and2014 rainfalls were between 8-11% more than average of many years (Figure 7). Additionally, in 2014, Turkey average temperature

was 14,9 ºC and that was 1,4 ºC upper than 1981-2010 years average temperature 13,5 ºC.

3.2.4 Soil Conditions

Stevia can be grown in a wide range of soils but has poor tolerance to salinity and therefore it should not be grown in saline soils (Chalapathi et al., 1997b). The crop grows well on a wide range of soils given a consistent supply of moisture and adequate drainage. a grows naturally on infertile, sandy acid soils with shallow water tables (Ramesh at al, 2006). Soil analysis of three locations is given at appendix Table 7, Table 8, Table 9.

3.2.5 Plantations

3.2.5.1 Spring Planting 2013

In the first season the experimental crop of *S. rebaudiana* was raised during Feb-April 2013. Grow Fide seedlings directly planted to Yalova location. During each year, nurseries were raised by sowing seeds of the mixed Grow Fide stock materials. Grow Fide material is not a registered commercial variety. Seeds were sown in February and almost 60-day-old seedlings were transplanted on 10th May 2013 at a density of 90,000 plants per hectare at spacing of 40cm×25cm. Four plot each of them has gross area 250m^2 (25×10m) were planted and observation were recorded from that area per plot. In each plot, get 4 samples and each sample contain 10 plants. Before plantation all seedlings prepared by fungicides (Quadris: Azoxystrobin methyl and Tachigaren 70 WP: Hymexazol) against to root diseases like Alternaria spp. Irrigation system was a “drip irrigation” system. First irrigation was given right after transplanting and another after 2 days. Subsequent watering was done when it was needed and after drought season fortnightly up to a depth of 5 cm till fall rains. Removal of weeds was done manually. Two hand weeding and hoeing were done to manage the weeds.

First harvest was taken at flower bud initiation on July 3rd when the crops were almost 45–50cm height (Figure 8). Second harvest was taken on 2nd of September as the first harvest parameters. Finally, the last harvest was taken on 27th

of October flowering in the crop was avoided by pinching of the apical bud to enhance growth of side branches. After harvesting, sun-dried, powdered and stored in airtight containers in a cool place for estimation of stevioside content. Total dry leaf yield was about 310 kg da^{-1} for first year spring plantation. Stevia glycosides analysis performed by Chormadex Company, USA.

Figure 8. Spring 2013 plantation (a), Grow fide seedlings (b), Harvest 2013 Yalova (c).

Vigorous crop regeneration was observed during onset of spring March, 31.2014(last week of March) from the underground root crowns. (Figure 9)

Figure 9. Sprouting

3.2.5.2 Winter Planting 2013

As seen in spring plantation materials, winter plantation materials supplied by Grow Fide. Grow fide materials were planted in Adana, Yalova and İvrindi locations (Figure 10).

Adana; seeds were sown in mid of October and almost 76-days-old seedlings were transplanted on 26th December 2013 at a density of 35,000 plants per hectare at spacing of 70cm x 40cm.

Yalova; seeds were sown in mid of October and almost 76-days-old seedlings were transplanted on 7thJanuary 2013 at a density of 90,000 plants per hectare at spacing of 40cm x 25cm,

Balıkesir/İvrindi; seeds were sown in mid of October and almost 76-days-old seedlings were transplanted on 8th December 2013 at a density of 90,000 plants per hectare at spacing of 40cm x 25cm. Four plot each of them has gross area 250m^2 (25x10m) total 1000 m^2 were planted and observation were recorded from that area per plot. In each plot, get 4 samples and each samples contain 10 plants.

Before plantation, all seedlings were prepared by fungicides (Quadris: Azoxystrobin methyl and Tachigaren 70 WP: Hymexazol) against to root diseases like Alternaria spp. Irrigation system was “drip irrigation” for all locations. First irrigations was given immediately after transplanting and another after 2-3 days.

Subsequent watering was done when it was needed (1-2 times in a week) up to a depth of 5-7cm. Removal of weeds was done manually. Two hand weeding and hoeing were done to manage the weeds. With the onset of winter in November, growth of the plants ceased except Adana location.

Figure 10. İvrindi location.

The crop was exposed to frost/cold wheather-susceptible and withered due to frost injury in December, January and February. Dead biomass was removed from all the plots during the end of February/March 2014.

3.2.5.3 Spring Planting 2014

As previous plantations: Materials were supplied by Grow Fide but additionally in vitro materials by Abant Izzet Baysal University. All materials planted in Adana, Yalova and İvrindi locations (Figure 11, Figure 12 and Figure 13)

Seeds were sown at the end of February and 75-days-old seedlings at a density of 90,000 plants per hectare at spacing of 40cm x 25cm.). Four plot each of them has gross area about 250m^2 (25x10m) were planted and observation were recorded from that area per plot. In each plot, get 4 samples and each samples contain 10 plants. In all locations, as previous seasons, before plantation, all seedlings prepared by fungicides (Quadris: Azoxystrobin methyl and Tachigaren 70 WP: Hymexazol) against to root diseases like Alternaria spp. Irrigation system was a

"drip irrigation" system. First irrigation was given immediately after transplanting and another after 2 days. Subsequent watering was done when it was needed.

Adana; seeds were sown at the end of February and 75-days-old seedlings were transplanted in May 2nd 2014 at a density of 90,000 plants per hectare at spacing of 40cm x 25cm,

Figure 11. Spring 2014 plantation/Adana

Figure 12. Spring 2014 plantation/Yalova

Yalova; seeds were sown at the end of February and 75-days-old seedlings were transplanted in May 1st 2014 at a density of 90,000 plants per hectare at spacing of 40cm x 25cm, Balıkesir/İvrindi; seeds were sown in mid of October and 76-days-old seedlings were transplanted in May 4th 2014 at a density of 90,000 plants per hectare at spacing of 40cm x 25cm.

Figure 13. Spring 2014 plantation-İvrindi/Balıkesir

In the second season, the experimental crop of *S. rebaudiana* was planted in May 1st, 2014 in Yalova, May 2nd in Adana and May 5th in Balıkesir regions. Grow Fide seedlings directly were planted to Yalova, Adana and Balıkesir locations. During each year, nurseries were raised by sowing seeds of mixed Grow Fide stock materials. Plantation was at a density of 90,000 plants per hectare at spacing of 40cm x 25cm for all regions in 2014. Four plot each of them has gross area 250m^2 (25x10m) were planted and observation were recorded from that area per plot. In each plot, get 4 samples and each sample contain 10 plants.

In 2014 season, tissue culture in-vitro seedlings which produced at Izzet Baysal University planted in June 10th in Yalova. Like 2013 season; before plantation all seedlings prepared by fungicides (Quadris: Azoxystrobin methyl and Tachigaren 70 WP: Hymexazol) against to root diseases like Alternaria spp. Irrigation system was a "drip irrigation" system. First irrigation was given immediately after transplanting and another after 2 days. Subsequent watering was done when it was needed and after drought season fortnightly up to a depth of 5 cm till fall rains. Removal of weeds was done manually. Two hand weeding and hoeing were done to manage the weeds.

Yalova; seeds were sown in mid of February and almost 75-days-old seedlings were transplanted on May 1st 2014 at a density of 90,000 plants per hectare at spacing of 40cm x 25cm. (Figure 14)

Figure 14. Yalova spring 2014 plantation

In Adana and Balıkesir; seeds were sown in mid of February as Yalova and almost 75-78-days-old seedlings were transplanted on May 2nd 2014 in Adana, 5th in Balıkesir at a density of 90,000 plants per hectare at spacing of 40cm x 25cm. (Figure 15)

Figure 15. A,B- Adana, C- Balıkesir Spring 2014 plantation

In vitro plants were prepared at Abant İzzet Baysal University during 2013 winter and 2014 spring. Those seedlings were transplanted on June 10th, 2014 in Yalova at a density of 90,000 plants per hectare at spacing of 40cm x 25cm (Figure 16, Figure 17)

Figure 16. In vitro plants. Spring 2014 plantation.

Figure 17. In vitro plants in the field (spring 2014 plantation).

In Yalova, first harvest (2013 spring plantation) was done at flower bud initiation on 23th June, 2014 when the crops were almost 45-50cm height. Leaf drying (Figure 18). Second harvest was done on 16th August 2014. The third harvest was done on 24th September 2014. For 2014 spring plantation; first harvest was done on 30th June, 2014, Second was done on 19th September, 2014 and the third and the last one was done 11th November 2014.

In Balıkesir region, same as Yalova region first harvest was taken at flower bud initiation on 24-25th June, 2014 when the crops were almost 50cm height. Second harvest was taken on 18th September 2014.

Different than the other 2 regions in Adana region, first and unique harvest was taken at flower bud initiation on 26th September, 2014 when the crops were

almost 90-100cm height. For 2013 winter plantation; first harvest was taken on 14th June 2014, second was taken on 19th September, 2014.

In vitro plants were taken at flower bud initiation on 10th September, 2014 when the crops were almost 45-50cm height (Figure 19, Figure 20).

Flowering in the crop was avoided by pinching of the apical bud to enhance growth of side branches. After harvesting, sun-dried, powdered and stored in airtight containers in a cool place for estimation of stevioside content. Total dry leaf yield was about 250-310 kg da^{-1} for the 2nd year spring plantation. Stevia glycosides analysis was performed by Institut Prof. Kurz GmbH, Germany.

Figure 18. Yalova. Spring 2013 plants, first harvest leaves were dried.

Figure 19. In vitro plants spring 2014 first/unique harvest.

Figure 20. In vitro plants spring 2014 first/unique harvest.

3.3 Data Analysis

3.3.1 Invitro Propagation

All tissue culture data were analysed statistically by using SPSS, Version 17.0 (SPSS Inc., Chicago, IL, USA). ANOVA was used to determine statistical significance, and significance of the differences between the means ±SE (standard error) values were calculated using Duncan's multiple range test at $p < 0.05$. Similarly, HPLC data are expressed as percentage against dry mass.

3.3.2 Field Cultivation Studies

Experiment was a factorial experiment and carried out using Completely Randomized Block Design (CRBD) with 3 replications. Analysis of variance and the Waller-Duncan K-ratio t-test (Waller and Duncan, 1969) were used to determine significant differences among factors. SAS/PC statistical program was used for all computations (SAS Inc. 1996).

4. RESULTS AND DISCUSSIONS

4.1 RESULTS

4.1.1 In Vitro Regeneration

Table 4 shows the effect of MS medium in combination with or without growth regulators on shoot regeneration. Based on the results, all treatments, irrespective of their medium combinations, produced 2 shoots from nodal explants (segments) after 3 weeks of cultivation (Figure 21).

Only two parameters, shoot length and callus appearance, varied depending on the medium composition. For example, the highest shoot length (3.1 cm) was observed on MS medium containing 1.0 mg/L KIN, while it was 2.6 cm at 0.1 mg/L.

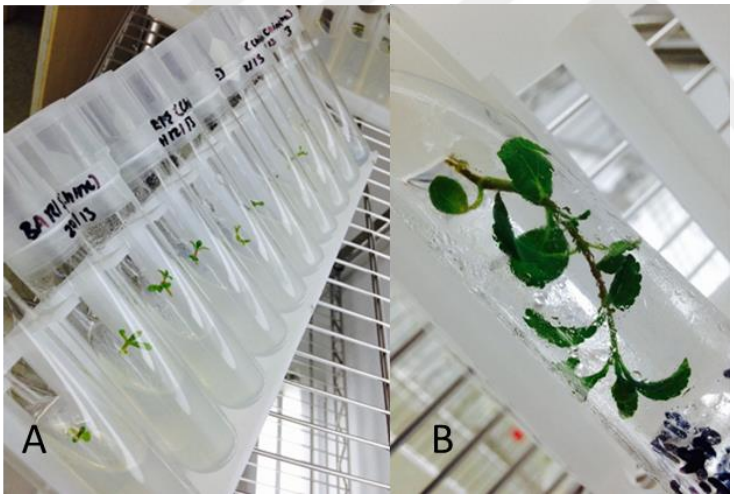


Figure 21. In vitro and ex vitro studies on stevia plants. Shoot emergence after one week (**A**), and 3 weeks cultivation of the nodal segments in culture tubes (**B**).

However, shoot length decreased from 2.7 to 1.3 cm when BAP increased from 0.1 to 2.0 mg/L. More callus formation was observed at the cut edges of the explant in contact with MS medium containing 1.0 or 2.0 mg/L BAP. It was clearly observed that there was an inverse relationship between callus formation and shoot growth. Greater amount of callus developed at the cut edges of the explant the fewer number of shoots regenerated. This pattern was observed at the increasing

concentrations of BAP, as well. There was no significant difference in term of mean shoot length among MS basal medium with or without 0.1 mg/L KIN or BAP.

Table 4. Effects of different MS media compositions supplemented with or without cytokinins at various concentrations on mean number of shoots, shoot length per nodal explant and callus response after 3 weeks culture [Mean ± SE (standard error) with the same letter within the columns are not significantly different according to Duncan's multiple range test at α=0.05; + signs represent degree of callusing]

Media Type	Concentrations (mg/L)	Mean no. of shoots/explant	Mean shoot length/explant (cm)	Nature of Callus
No PGR		2.0 ± 0.0	2.6 ± 0.1^{c}	+
KIN	0.1	2.0 ± 0.0	2.6 ± 0.2^{c}	+
	0.5	2.0 ± 0.0	2.9 ± 0.2^{ab}	+
	1.0	2.0 ± 0.0	3.1 ± 0.2^{a}	++
	2.0	2.0 ± 0.0	2.9 ± 0.1^{ab}	++
BAP	0.1	2.0 ± 0.0	2.7 ± 0.1^{bc}	+
	0.5	2.0 ± 0.0	2.0 ± 0.2^{d}	++
	1.0	2.0 ± 0.0	1.7 ± 0.1^{e}	+++
	2.0	2.0 ± 0.0	1.3 ± 0.1^{f}	+++

For root formation, different auxins (IAA, IBA or NAA) at varying concentrations (0.25 or 0.50 mg/L) were compared with MS basal medium (i.e., control group) for 3 weeks (Table 4). The highest number of roots (8.1 roots per shoot with 100% rooting frequency) developed on MS medium containing 0.25 mg/L IAA. When IAA concentration was doubled, it was observed that the mean number of roots per shoot decreased significantly (from 8.1 to 5.3 roots/shoot). Similarly, IBA was effective for rooting; however, rooting frequency was slightly lower than that induced by IAA (85.5 and 87.6% of the shoots producing a mean of 6.2 or 5.0 roots per shoot, respectively). Of the treatments tested for root formation, NAA had the weakest influence producing maximum 2.1 roots per shoot after 3 weeks of cultivation, and frequency of the rooting was the lowest (27.9 or 35.2%) as compared to IAA or IBA (Table 5). Instead, white creamy callus formation was predominant

pattern at the cut edge of the nodal segments in contact with culture medium (observed data).

Table 5. Rooting stage of the shoots derived from nodal explants on MS medium with or without auxin at different concentrations for 3 weeks [Mean ± SE (standard error) with the same letter within the columns are not significantly different according to Duncan's multiple range test at α=0.05].

Auxins	**Concentrations (mg/L)**	**Mean number of root/shoot**	**% of shoots rooting**
No PGR		3.1 ± 0.2^{d}	55.0
IAA	0.25	8.1 ± 0.8^{a}	100.0
	0.50	5.3 ± 0.4^{b}	97.3
IBA	0.25	6.2 ± 0.5^{b}	85.5
	0.50	5.0 ± 0.5^{bc}	87.6
NAA	0.25	2.1 ± 0.2^{e}	35.2
	0.50	1.6 ± 0.4^{e}	27.9

As to the regeneration protocol developed for large scale propagation for field trial studies, direct shoot regeneration from nodal explants was employed by subculturing the nodal segments in MS basal medium at 3 week intervals for 8 months (Figure 22).

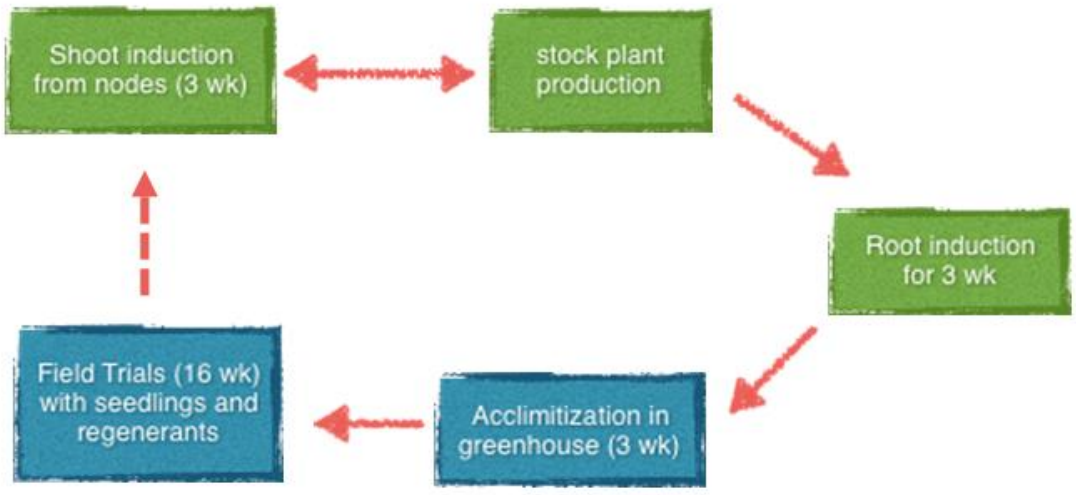


Figure 22. A strategy developed for stevia plant production at large scale associated with micropropagation techniques. Green and blue boxes represent in vitro and ex vitro studies, respectively, at different periods of cultivations, Arrows demonstrate the direction of plantations as follows: two way arrow shows multiple clone

production from node to shoot or vice-versa; broken arrow shows the best clone selection for in vitro studies.

Then, all shoots were transferred to the MS media supplemented with 0.25 mg/L IAA for root formation as described above. Different morphological parameters including mean shoot and root length, mean number of nodes and roots, and their fresh weights obtained from shoots before and after the rooting stage were given in Table 6. Based on the results, data regarding all parameters sharply increased (3 or 4 times higher) during rooting stage.

Table 6. Different morphological parameters obtained from shoots and rooted-shoots (regenerants) after 3 and 7 weeks of culture initiation, respectively (±, standard error).

	Mean length of shoots (cm)	**Mean number of nodes/plantlet**	**Mean fresh weight (gr)**	**Mean number of roots/shoot**	**Mean length of roots (cm)**
Shoots	2.6±0.1	2.1±0.1	306.1±88.7	0	0
Rooted-shoots	13.4±0.3	8.2±0.2	912.0±144.0	7.2±0.5	2.5±0.3

For example, fresh weight of shoots increased from 306 mg to 912 mg, mean number of node from 2.1 to 8.2 cm, and shoot length from 2.6 to 13.4 cm after rooting. Unless otherwise stated, all these findings obtained from a 7-week culture period (3 weeks for shooting and 4 weeks for rooting) were found to be optimal for in vitro cultivation prior to acclimatization step of *S. rebaudiana*.

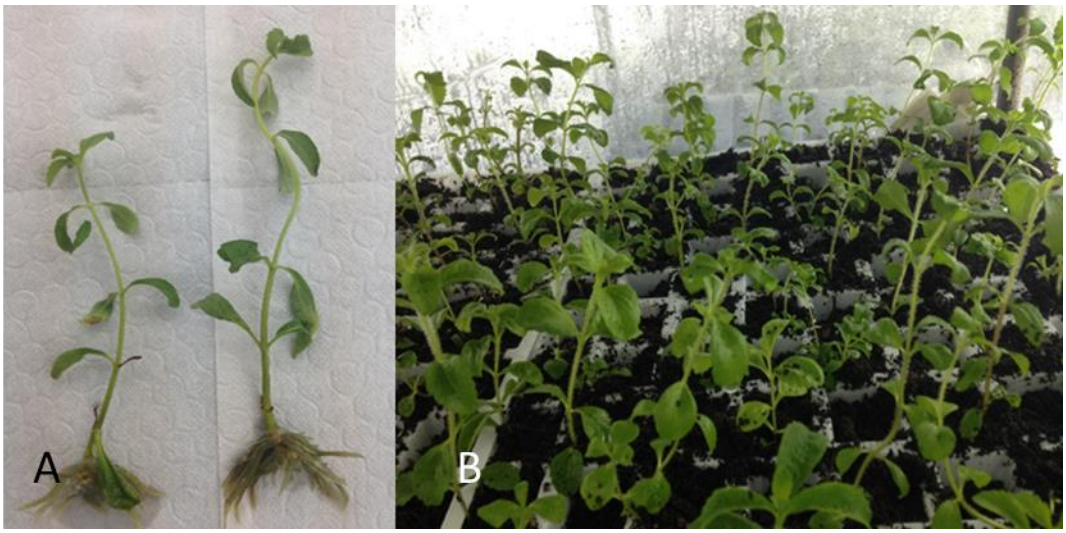


Figure 23. In vitro and ex vitro studies on stevia plants. (A) Rooting of the shoots in MS medium supplemented with 0.25 mg/L IAA for 3 weeks. (B) Hardening off the regenerants in compost mixture for 3 weeks prior to transfering to the field conditions.

For acclimatization, all regenerants (rooted-shoots) as shown in Table 3 were transferred to the compost mixture in plastic trays. High survival rate (>90%) was observed under portable greenhouse conditions (Figure 23, Figure 23.B). All the plantlets (both regenerant and seedling derived plants) in equal numbers (2500 plants for each) were then transferred to the field with a drip irrigation system, and grown well with a high survival rate (>99%) and negligible loss (<1%). At the end of 13 weeks of plantation (until flowering period), regenerants and seedlings reached a mean length of 65.5 and 68.3 cm, respectively. Additionally, no morphological differences between regenerants and seedlings (Figure 24,A,B) were observed. Harvesting was performed by hand after 16 weeks of plantation, and the total yield (dry leaf of regenerant- and seed/ling-derived plants) was estimated as 2.4 tones ha^{-1}.

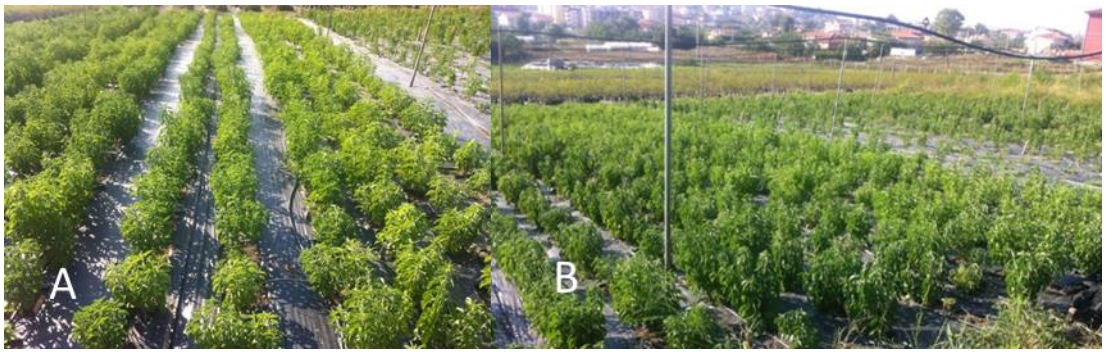


Figure 24. Seed derived mother plants (A), and regenerants after 12 weeks of plantation (B)

Extraction protocol applied for steviol glycosides (SG) was established with a high recovery rate (≥98%). Steviol glycoside analysis was employed in dried leaves sampled from the regenerants and seedlings in late vegetative stage (9 weeks of plantation) and flowering stage (13 weeks of plantation). Of the steviol glycosides analyzed, stevioside (ST) was predominantly found in dried leaves in both regenerants and seedlings. Irrespective of their vegetative periods, producing maximum 6.7% (w/w) in regenerants, and 6.9% (w/w) in seedlings (Table 7).

Table 7. Steviol glycoside contents (%) in dried leaves sampled from seedlings (S) and regenerants (R) after their late vegetative (LV) and flowering (F) stages (Reb-A: rebaudioside A; Reb-C: rebaudioside C; Reb-D: rebaudioside-D; Reb-F: rebaudioside-F; ST: stevioside; Dul-A: dulcoside A, ±: standart deviation).

	Mean Shoot length (cm)	**Reb A**	**Reb C**	**Reb D**	**Reb F**	**ST**	**Dul A**
R-LV	54.5 ±1.6	4.8± 0.2	1.0± 0.0	0.3 ± 0.0	0.2± 0.0	6.7± 0.3	0.7± 0.1
R-F	65.5 ± 1.1	5.0 ± 0.2	1.4± 0.0	0.4 ± 0.0	0.2± 0.0	6.4± 0.3	0.5± 0.0
S-LV	53.5 ±1.6	4.7 ± 0.3	0.9± 0.0	0.0 ± 0.0	0.2 ± 0.0	6.9± 0.7	0.5 ± 0.0
S-F	68.3 ± 1.1	4.8± 0.4	0.9 ± 0.0	0.0± 0.0	0.2± 0.0	6.6 ± 0.8	0.4± 0.0

For rebaudioside-A (Reb-A) content of regenerants sampled at the flowering period was slightly higher than the late vegetative period including flower budding stage of the regenerants and seedlings [compare 5.0% (w/w) and 4.7% (w/w) of Reb-A]. In Figure 27 , pie charts of SGs were demonstrated for the regenerants and seedlings at the flowering period. Therein, almost similar SG pattern was observed in leaf samples of both plant types. The ratio of ST content in overall SG was slightly

higher in regenerants (50%; w/w) than the seedlings in which a 46% (w/w) ST was detected. While Reb-A composition of both leaf sources are alike, other steviol glycoside compositions of the leaves differed slightly. The maximum difference was shown in rebaudioside-C (Reb-C) content which is also third major SG detected in leaves. More Reb-C [1.4% (w/w)] was found in regenerants flowering period. Irrespective of plant source being either regenerants or seedlings, it was clearly observed that those three predominant SGs (ST, Reb-A, and Reb-C) comprise at least 90% (w/w) of total SG in leaves (Figure 25).

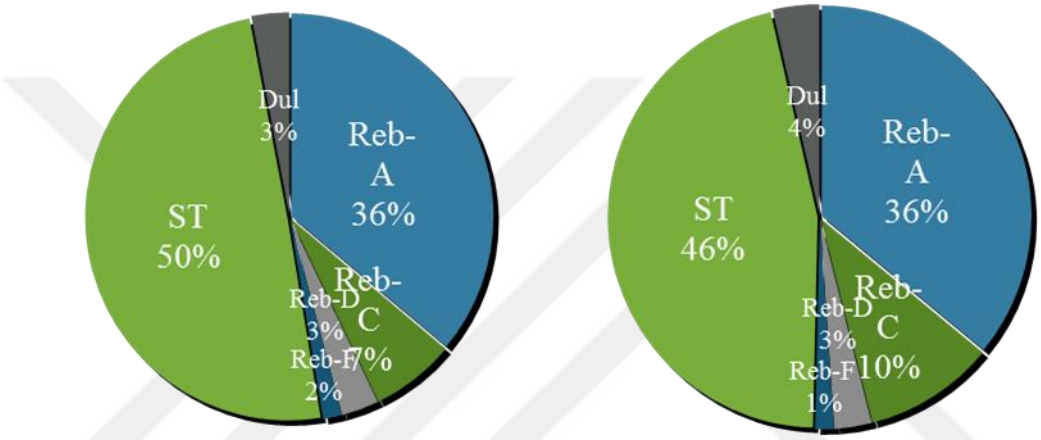


Figure 25. The pie charts of steviol glycosides (SG) contents. Each slice is proportional to the quantity (%) of SG in dried leaves of regenerants (left) or seedlings (right) sampled at their flower budding stage. Reb-A: rebaudioside A; Reb-C: rebaudioside C; Reb-D: rebaudioside-D; Reb-F: rebaudioside-F; ST: stevioside; Dul-A: dulcoside A.

4.1.2 Field Cultivation

The experiments were planted according to randomized complete block, factorial design (RBD) with four replications in 3 different locations of Turkey. Yalova, Adana and Balıkesir/İvrindi. A crop density is 90,000 plants per hectare at spacing of 40cm x 25cm. Four plot each of them has gross area 250m^2 (25x10m) were planted and observation were recorded from that area per plot. In each plot, get 4 samples and each sample contain 10 plants.

4.1.2.1 2013 Plantations

4.1.2.1.1 Yalova Spring Plantation

(Table 8) shows the average plant heights and dry leaf yields per plot in 3 different harvest times in Yalova in 2013 plantation season. The parameters average plant height and average dry leaf yield varied depending on the harvest time.

Table 8. Yalova 2014 spring plantation plants (average height and yields in 3 harvests).

	Ave.Plant Height (cm)	Ave.Dry Leaf Yield (kg/plot)
1st Harvest	58,70	71,47
2nd harvest	63,52	73,75
3rd Harvest	67,77	78,64

Based on the results; the highest plants, ave. 67,77 cm and highest yield with 78,64 kg $plot^{-1}$ were obtained from the 3rd harvest. Second average highest plant height and average dry leaf yield were observed at the 2nd (63,52 cm and 73,75 kg $plot^{-1}$) and the 3rd harvest (58,70 cm and 71,47 kg $plot^{-1}$), respectively.

Table 9. Yalova 2013 spring plantation steviol glycosides contents in 3 harvests

	1st HARVEST	**2nd HARVEST**	**3rd HARVEST**
Rubusoside	0,03	0,04	0,04
Dulcoside A	0,09	0,16	0,16
Reb B	< 0,01*	< 0,01*	< 0,01*
Stevioside	4,77	6,64	6,87
Reb C	0,47	0,77	0,79
Reb F	0,09	0,13	0,14
Reb A	2,84	4,39	4,43
Reb D	0,09	0,2	0,22
Reb E	< 0,01*	< 0,01*	< 0,01*
Steviolbioside	< 0,01*	< 0,01*	< 0,01*
TSG	**8,39**	**12,32**	**12,64**

* the mentioned value is equivalent to the detection limit

** (Method: HPLC-UV/VIS-MS/MS; IK2001)+

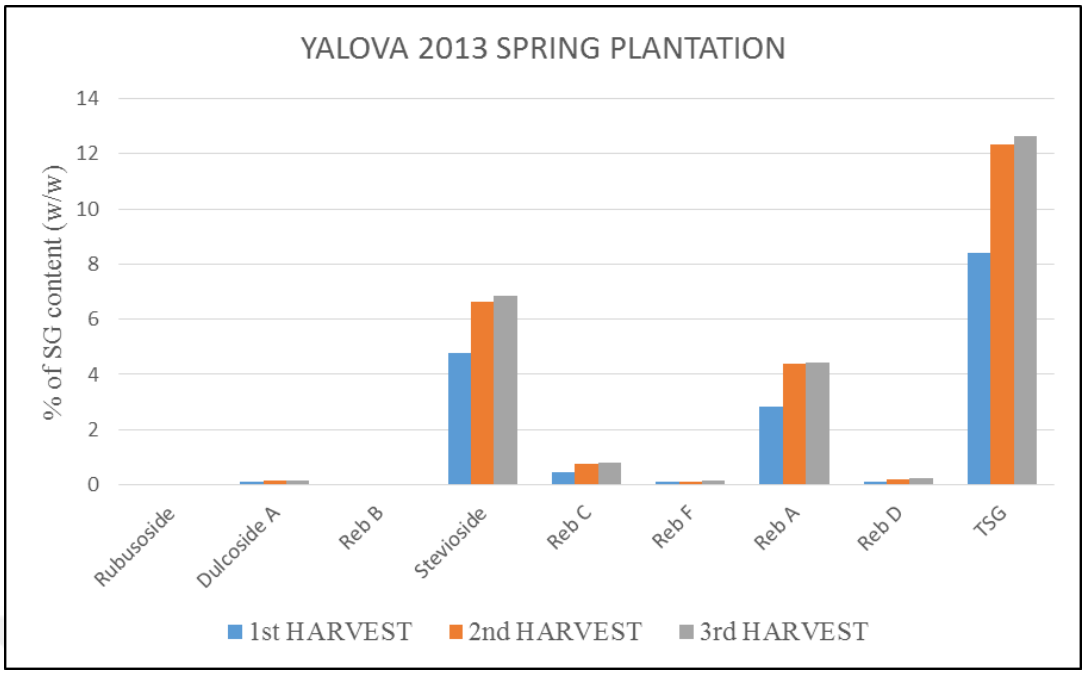


Figure 26. Yalova 2013 Spring plantation steviol glycosides contents in 3 harvests

In Yalova location, (Table 9) and (Figure 26) show steviol glycosides (SG) contents at 3 different harvest time. The highest steviol glycoside contents (TSG) and RebA were obtained from the 3rd harvest (TSG:12,64 and RebA: 4,43) followed by the 2nd (TSG:12,32 and RebA: 4,39) and 1st harvest being the least (TSG:8,39 and RebA: 2,84), respectively.

4.1.2.1.2 Adana Winter Plantation

(Table 10) shows the average plant heights and dry leaf yields per plots in 2 different harvest times in Adana in 2013 season. In our study, we have studied in 2 different planting time as winter and spring for Adana location. On the table, we observed two parameters like Yalova; average plant height and average dry leaf yield varied depending on the harvest time. Based on the results; average 99,7 cm plant height and 90,52 kg plot^{-1} average dry leaf yield were obtained from the 1st harvest. At the second harvest we obtained 86,04 cm average plant height and 82,37 kg plot^{-1} average dry leaf yield.

Table 10. Adana 2013 winter plantation plants height and yields in 2 harvests

	Ave.Plant Height (cm)	Ave.Dry Leaf Yield (kg/plot)
1st Harvest	99,7	90,52
2nd Harvest	86,04	82,37

We observed 44,5% more TSG and 87,2% more RebA content at the 2nd harvest as compared tot he 1st harvest in Adana 2013 winter plantation (Table 11 and Table 27, Figure 27.). Also, at the 2nd harvest, all steviol glycocides contents were dramatically higher than the 1st harvest.

Table 11. Adana 2013 winter plantation 2014 steviol glycosides contents in 2 harvests

	1st HARVEST	**2nd HARVEST**
Rubusoside	0,02	0,03
Dulcoside A	0,09	0,11
Stevioside	4,39	5,24
Reb C	0,39	0,72
Reb F	0,07	0,12
Reb A	2,19	4,1
Reb D	0,05	0,09
Reb E	< 0,01*	< 0,01*
Reb B	< 0,01*	< 0,01*
Steviolbioside	< 0,01*	< 0,01*
TSG	**7,2**	**10,41**

*: the mentioned value is equivalent to the detection limit

** (Method: HPLC-UV/VIS-MS/MS; IK2001)+

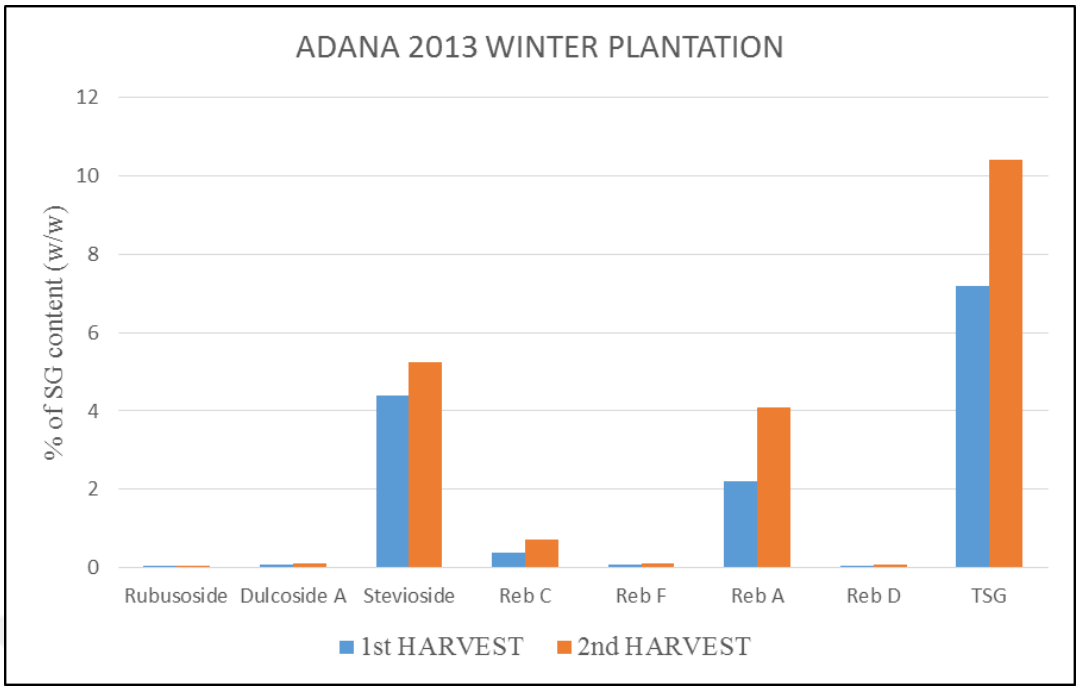


Figure 27. Steviol glycoside content oft he leaves harvested from Adana in 2013

4.1.2.2 2014 Plantations

4.1.2.2.1 Yalova Spring Plantation

In 2014 season, at Yalova spring plantation, the average plant heights and average dry leaf yields per plots in 3 different harvest time shows at the (Table 12) For two parameters, average plant height and average dry leaf yield varied depending on the harvest time. Based on the results; the highest plants were obtained from the 2nd harvest as 69,1 cm and average dry leaf yield was obtained from the 3rd harvest as 79,77 kg plot^{-1}. The highest average dry leaf yield at the 3rd harvest was 8,3%, being more than the lowest harvest at the 1st one.

Table 12. Yalova 2014 spring plantation plants height and yields in three harvests.

	Ave.Plant Height (cm)	Ave.Dry Leaf Yield (kg/plot)
1st Harvest	54,75	73,65
2nd Harvest	69,1	77,69
3rd Harvest	68,28	79,77

(Table 13) and (Figure 28. Yalova 2014 spring plantation steviol glycosides **contents** in 2 harvests) show steviol glycosides contents at 3 different harvest time in Yalova location in 2014 spring plantation. The highest steviol glycoside contents

obtained from the 2nd harvest (12,64) and RebA (4,46) and the 3rd harvest (12,49) (4,27) and 1st harvest (10,57) (3,59) respectively. And almost all highest steviol glycocides contents were observed at the 2nd harvest, except stevioside content.

Table 13. Yalova 2014 spring plantation steviol glycosides contents in 3 harvests

	1st HARVEST	**2nd HARVEST**	**3rd HARVEST**
Dulcoside A	0,14	0,17	0,17
Stevioside	6	6,98	7,07
Rubusoside	0,02	0,02	0,02
Steviolbioside	< 0,01	< 0,01	< 0,01
Reb B	< 0,01	< 0,01	< 0,01
Reb C	0,58	0,78	0,76
Reb E	< 0,01	< 0,01	< 0,01
Reb F	0,11	0,14	0,13
Reb A	3,59	4,46	4,27
Reb D	0,12	0,1	0,08
TSG	**10,57**	**12,64**	**12,49**

*: the mentioned value is equivalent to the detection limit

** (Method: HPLC-UV/VIS-MS/MS; IK2001)+

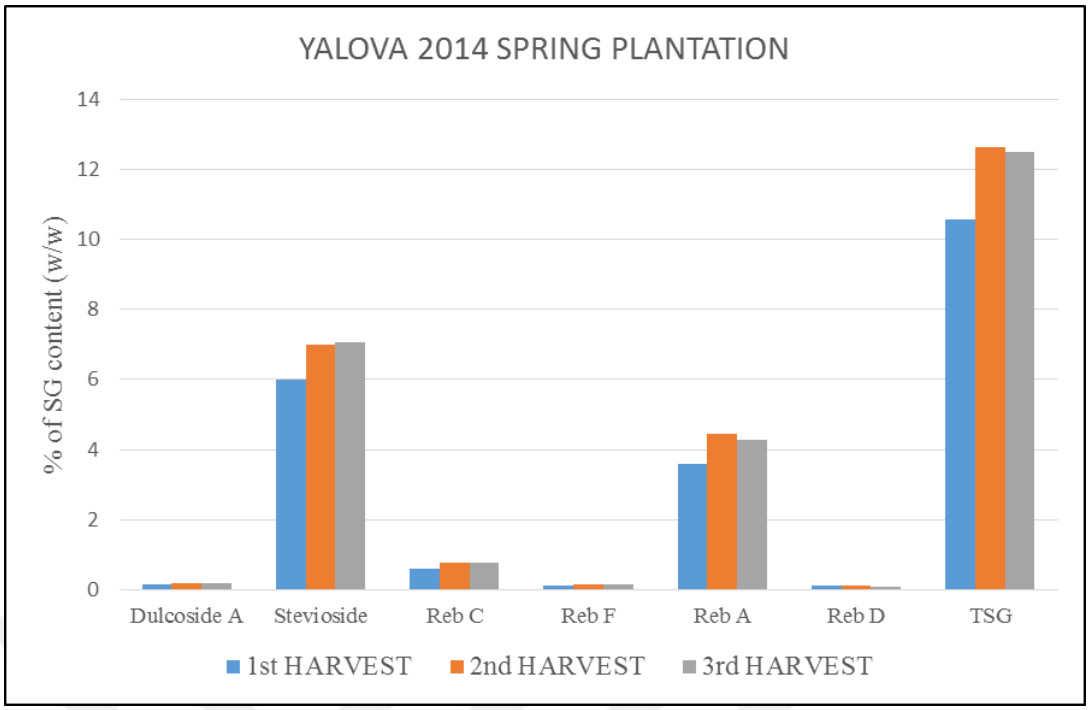


Figure 28. Yalova 2014 spring plantation steviol glycosides contents in 2 harvests

4.1.2.2.2 Adana Spring Plantation

In Adana region 2014 spring plantation average plants height was 112.23cm which is very high and average dry leaf yield was 92,84kg plot^{-1} in a single harvest.

(Table 14) and (Figure 29. Adana 2014 spring plantation steviol glycosides contents in single harvest. show Steviol Glycosides contents at single harvest time in Adana in 2014 Spring plantation. The total Steviol Glycoside contents (TSG) obtained as 7,2 and RebA 2,19.

Table 14. Adana 2014 spring plantation steviol glycosides contents in single harvest

	SINGLE HARVEST
Rubusoside	0,02
Steviolbioside*	0,01
Dulcoside A	0,09
Reb B *	0,01
Stevioside	4,39
Reb C	0,39
Reb F	0,07
Reb A	2,19
Reb E *	0,01
Reb D	0,05
TSG	**7,2**

*: the mentioned value is equivalent to the detection limit

** (Method: HPLC-UV/VIS-MS/MS; IK2001)+

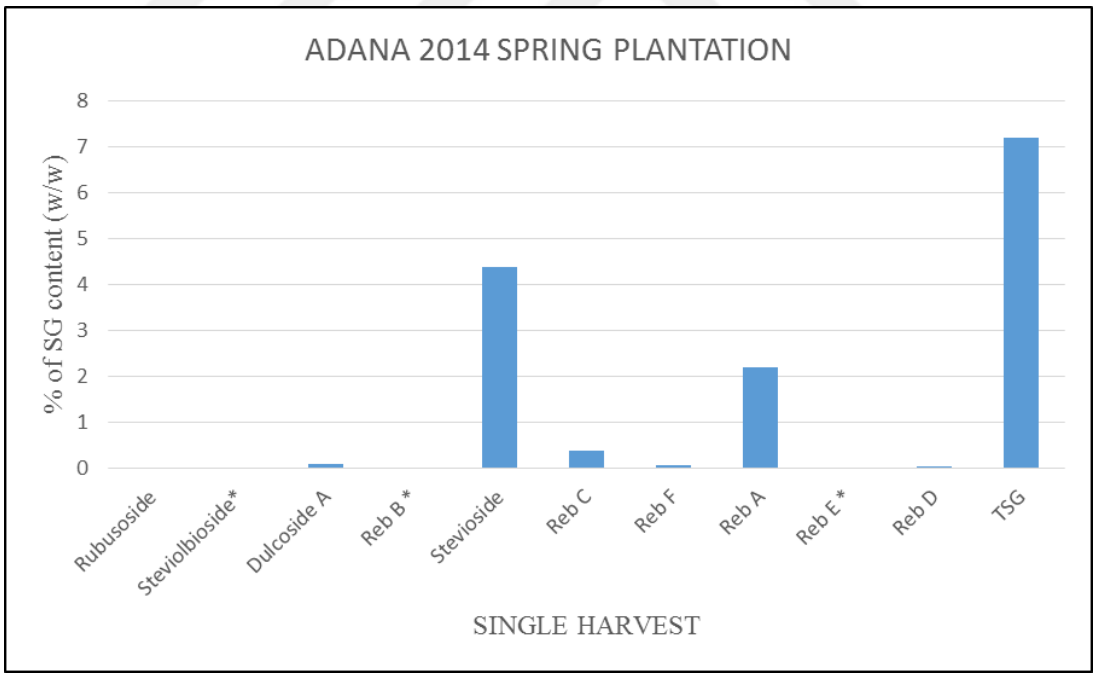


Figure 29. Adana 2014 spring plantation steviol glycosides contents in single harvest.

4.1.2.2.3 Balıkesir Spring Plantation

(Table 15) shows the average plant heights and average dry leaf yields per plots in 2 different harvest time in Balıkesir in 2014 spring plantation. We observed two parameters; average plant height and average dry leaf yield varied depending on the harvest time. The 2nd harvest average plant heights was 59.30 cm and average dry leaf yield was 77.69 kg plot^{-1} and higher than the 1st harvest 56.63 and 76.09 respectively.

Table 15. Balıkesir 2014 spring plantation plants height and yields in two harvests

	Ave. Plant Height (cm)	Ave.Dry Leaf Yield (kg/plot)
1st Harvest	56,63	76,09
2nd Harvest	59,30	77,69

(Table 16) and (Figure 30) shows Balıkesir 2014 Spring Plantation Steviol Glycosides Contents in 2 Harvests. Base on results; the second harvest TSG (Total steviol glycocides) were 15,1% higher than the 1st harvest and RebA contents 35,1%.

Table 16. Balıkesir 2014 spring steviol glycosides contents in 2 harvests

	1st HARVEST	**2nd HARVEST**
Rubusoside	0,03	0,03
Dulcoside A	0,08	0,08
Stevioside	4,09	4,18
Reb C	0,44	0,59
Reb F	0,08	0,1
Reb A	2,42	3,27
Reb B	< 0,01*	< 0,01*
Reb D	0,05	0,09
Reb E	< 0,01*	< 0,01*
Steviolbioside	< 0,01*	< 0,01*
TSG	**7,2**	**8,33**

* the mentioned value is equivalent to the detection limit

** (Method: HPLC-UV/VIS-MS/MS; IK2001)+

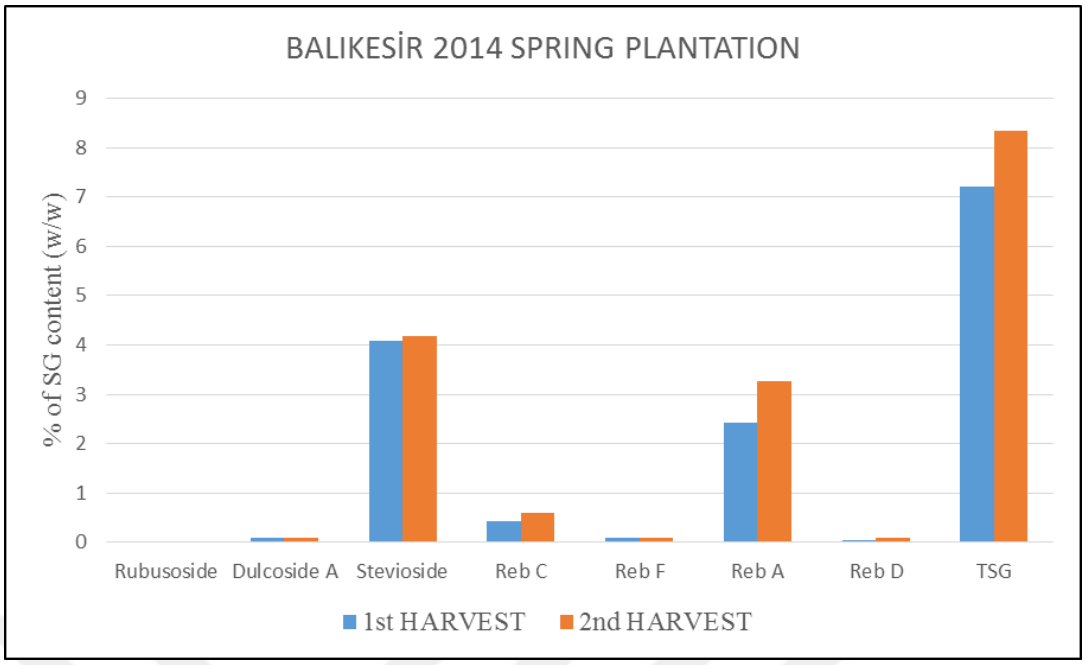


Figure 30. Balıkesir 2014 spring plantation steviol glycosides contents in 2 harvests.

4.1.2.2.4 Balıkesir Winter Plantation

Lost all crops due to frost & very low temperature. (Figure 31)

Figure 31. Low temperature damage on stevia

4.1.2.3 Data Analysis

4.1.2.3.1 Plant Height

Analysis of variance showed that there was a significant difference among locations for plant height ($p \leq 0.0001$). The highest plant height was observed in Adana location (100.8 cm). On the other hand, the lowest plant height was found in Balıkesir location (56.62 cm) (Table 17). These results clearly indicated that location has significant effect on plant height of stevia plant.

Table 17. Means of plant height from 3 locations.

Locations	**Means**
Adana	100.8 A
Yalova	64.09 B
Balıkesir	56.62 C

*The same letter within the same column is not significantly.

Harvesting time had also significant effect on plant height ($p \leq 0.0001$). The highest plant height was observed in first harvesting time (76.39 cm). On the other hand, the lowest plant height was found in third harvesting time (68.65 cm) (Table 18).

Table 18. Means of plant height from three harvesting time

Harvesting Time	**Means**
1	76.39 A
2	70.24 B
3	68.65 C

*The same letter within the same column is not significantly.

Analysis of variance showed that there was a significant difference years ($p \leq 0.0001$). The highest plant height was observed in year 2013 (76.46 cm). On the other hand, the lowest plant height was found in year 2014 (69.65 cm) (Table 19).

Table 19. Means and standard deviation of plant height from 2 years

Year	Mean	Std. Error
2013	76.465 [A]	.367
2014	69.654 [B]	.335

*The same letter within the same column is not significantly.

4.1.2.3.2 Yield

Analysis of variance showed that there was no significant difference between years regarding to yield ($p \leq 0.907$). On the other hand, harvesting time had significant effet on yield. The lowest yield was observed in the second harvesting time (74.71). (Table 20).

Table 20. Means of yield from three harvesting time

Harvesting Time	**Means**
1	81.21 A
3	80.01 A
2	74.71 B

*The same letters within the same column is not significantly.

Analysis of variance showed that there was a significant difference among locations for yield ($p \leq 0.0001$). The highest yield was observed in Adana Location (87.03). On the other hand, the lowest yield was found in Balıkesir Location (71.03) (Table 21). These results clearly indicated that location has significant effect on yield of stevia plant.

Table 21. Means of plant height from three locations

Locations	**Means**
Adana	87.03 A
Yalova	76.94 B
Balıkesir	71.03 C

*The same letters within the same column are not significantly.

Analysis of variance showed that there was a spring season (80.63). On the other hand, the lowest yield was found winter season (73.98).

4.2 DISCUSSION

4.2.1 In Vitro Propagation

Nowadays, the demand for zero-calorie sweeteners has prompted researchers to investigate alternative high intensive sweeteners substituted for the use of sugar. The emergence of plant-derived sources in global sweetener market has been of great value since consumers seek out alternative ingredients which are more "natural". As *S. rebaudiana* pioneers sweetener market among other natural sources including monk fruit and monatin, agricultural facilities of stevia plants are very important for stable and cost effective plant production. Since seed propagation of *S. rebaudiana* results in a great variation in the levels of steviol glycosides (Tamura et al.1984), this phenomenon has been attributed to the fact that *S. rebaudiana* is a self-incompatible plant, with steviol glycoside variation due to gene segregation.

Similarly, low germination pattern is also another serious problem for the seeds when industrial-scale cultivation is taken into consideration. In order to eliminate these drawbacks, several attempts have been made to propagate homogeneous populations of *S. rebaudiana* through plant tissue culture techniques in the last decade. Based on our literature survey ascertained, there are a large number of studies for stevia propagation from shoot tips (Tamura et al. 1984; Das et al. 2011), nodal segments (Mitra and Pal 2007; Singh et al. 2014), leaf explants (Sreedhar et al. 2008), hypocotyl explants (Ramirez-Mosqueda and Iglesias-Andreu 2015), flowers (Ahmad et al. 2011), callus cultures (Swanson et al. 1992; Preethi et al. 2011; Manthur and Shekhawat 2013) and stem-cuttings under ex vitro conditions (Khalil et al. 2014).

Nevertheless, tissue cultures studies incorporated with field experiments are yet a major challenge in large-scale production underlying steviol glycoside spectra of the samples. Similarly, vegetative propagation through stem-cutting seems effective to produce consistent plant production for certain chemotypes (for example high rebaudioside-A containing varieties), however; it is limited to the number of

individual plant, and is time-dependent for the successful adaptation to the soil. Since *S. rebaudiana* is photoperiod sensitive, both in vitro and ex vitro studies are to fulfill optimizations for the leaf mass and steviol glycoside content (Ramesh et al. 2006; Tavarini et al. 2013). For the large-scale plant production, micropropagation provides a number of advantages with true-to-type plant production in relatively less time and limited space.

In the present study, commercial scale *S. rebaudiana* production associated with micropropagation from nodal segments was demonstrated. In this view, field grown seed/ling-derived plants having high value-added steviol glycosides can be selected for the next year plantation, and those of the plants dispatched from the fields can be propagated from nodal segments continuously under in vitro conditions throughout the year. This way of propagation using shoot-tip culture incorporated with ex vitro studies in field conditions was also noticed by Tamura et al. (1982). However, in our study, for the first time, a detailed SG analysis was shown in both *S. rebaudiana* plants derived from micropropagation and seedling-derived plants.

For the micropropagation, nodal segments are highly capable of shoot induction in vitro conditions (Sivaram and Mukundam 2003; Thiyagarajan et al. 2012; Singh et al. 2014; Yücesan et al. 2015). It is also noteworthy to mention that MS medium without any growth regulator was considered highly practical for the direct clonal propagation in this study. Increasing concentration of BAP from 0.5 to 2.0 mg/L influence the length of shoots negatively (reducing the mean number of shoot from 2.0 to 1.3 per explant).

This finding is also consistent with earlier reports in which BAP ceases the growth and development of *S. rebaudiana* cultivars. For example, Das et al. (2011) reported that KIN was more efficient cytokinin than BAP, since it promoted more shooting response without any callus symptom. Similarly, Thiyagarajan and Venkatachalam (2012) reported that increasing concentrations of BAP (from 0.5 to 3.0 mg/L) decreased the percentage of shoot induction (from 85 to 50%) and number of shoot per explant (from 2.0 to 1.25 shoots). Contrarily, Sivaram and Mukundan (2003) reported progressively elevating concentrations of BAP resulted in more multiple-shoot formation in all the explants of *S. rebaudiana*. For the cost effective

production, Ibrahim et al. (2008) reported that the use of MS basal medium only gave results comparable to those containing growth regulators.

To sum up these earlier contributions, it is obvious that optimal composition and concentration of growth regulators may depend on the genotype, origin of explant, and cultivation time. Moreover, several tissue culture protocols of *S. rebaudiana*, in terms of mean shoot number per explant that is ranging from 12 to 126 shoots (Aman et al. 2013; Khalil et al. 2014; Rathore et al. 2014). It is difficult to claim that these reports are applicable for industrial scale propagation, as they requires more labor and time with subsequent sub-cultivations of the micro-shoots derived from intervening callus tissues.

For example, Sivaram and Mukundan (2003) reported that following the three cycles on the shoot-multiplication media (40 days for each) and 30 days on rooting media, about 36,000 can be propagated from single nodal explant after 6 months. However, in our study, it is possible to produce 560,000 plants in the same length of period from a single nodal explant multiplying at every three-week intervals. As seen in Table 3 and Fig. 2, each nodal explants give rise to 2 shoots with three axillary meristematic tissues (two nodes and one shoot tip) that lead to further shoot multiplication by multiples of 6 (i.e. 2, 12, 72, 432, 2592 shoots and so on) during subsequent cultivations on MS medium without any PGRs.

For effective acclimatization step, rooting stage was of very pivotal role in *S. rebaudiana*, as well-developed root system has positive effect on acclimatization success in non-axenic natural conditions. For example, less than 5 roots with minimum 2.5 cm length were not adapted to the soil (Table 6), unless they were dipped into the solution prepared by high auxin concentration for a while (unpublished data). Swanson et al. (1992) reported that total steviol glycoside content was regulated by such enzymatic reactions which probably existed in a well-developed root system. High survival rate of regenerants (>90%) in plastic trays might be due the success of rooting stage followed by 3 weeks of intermediate stage in portable greenhouse conditions for effective stomatal functioning and water balancing in leaf tissues of regenerants prior to the field experiments.

Seedlings produced from seed materials and regenerants derived from nodal segments were successfully transferred to the soil with a negligible loss (less than

1%). It was clearly shown that micropropagation pioneers the homogenous plant production with high phenotypical (shoot length) and chemotypical (SG content) similarities to the mother plant derived from seeds samples. In terms of Reb-A and stevioside contents (%), there was no significant differences in seed/ling- and regenerant-derived plants. This finding can be assessed as the best outcome of the results since homogeneity is the main goal of the research as compared to previous studies in which homogenous *S. rebaudiana* production is restricted by genetic marker tests only (see Das et al. 2011) without employing field experiments and steviol glycoside analysis, thereof.

Additionally, different vegetative periods in our plantation (late vegetation and flowering) did not affect the steviol glycoside contents in the leaves. Despite the fact that stevia leaves need to be harvested prior to flowering stage (Ramesh et al. 2006), our findings concerning steviol glycoside spectra reveal that the leaf harvesting can be established during flowering period without changing the SG contents. However, once the plants harvested at their flowering stage, the risks of seed loss and quality are the most likely encountered problems in field.

Due to these shortcomings, micropropagation techniques paved the way for the stable plant production especially in scarcity of the stock plants or seed materials. Tamura et al. (1984) reported that the micropropagation studies in *S. rebaudiana* could be applied not only to the genetic conservation of a selected superior stock, but also for mass propagation of the uniform and better characteristics in the plant. In spite of some attempts on steviol glycoside production through several tissue culture techniques with or without elicitation (Striedner et al.1991; Swanson et al. 1992; Bondarev et al. 2003; Ladygin et al. 2008; Sreedhar et al. 2008), none of the reports has provided a continuous and reasonable amount of value-added SG under in vitro conditions.

Instead, these reports contribute new insights for steviol glycoside biosynthesis. Cell suspension cultures seem not the way of cost-effective steviol glycoside production unless fermentation technologies used in this manner (see rebaudioside M production in Prakash et al. 2014). Ceunen and Geuns (2013) reported that long-day photoperiod (≥14 h) prolonged the vegetative growth in *S. rebaudiana* resulting in a greater amount of leaf mass and SG accumulation. Tavarini

et al. (2012) also reported steviol glycoside accumulation in different growing seasons, steviol glycoside contents having a tendency to vary which is influenced by sun light and temperature the plants were exposed to.

In conclusion, depending on the genotype it is clear that total steviol glycosides increases progressively during the growth stages in the field conditions. Therefore, as described in this study, homogenous plant production through micropropagation does not only play a key role for a continuous plantlet production, but also provides a powerful tool for the selective breeding studies in future studies on *S. rebaudiana.*

4.2.2 Field Cultivation Studies

The aim of this chapter was to describe the possibilities of cultivation of stevia in Turkey in 3 different regions and importance of the plant and define to its production requirements in Turkey, but major emphasis is given to the agronomic and management aspects of the plant to be grown as a crop in Turkey as an alternative source of natural, safe and healthy sweetener.

The experiments were planted according to randomized complete block, factorial design (RBD) with four replications. Four plots, each having a gross area of about 250m^2, were planted and observations were recorded. In Yalova and Balıkesir locations both plantation times, the second and the third harvest plant heights were higher than first harvest due to length of harvest period (Table 8, 12 and Table 15). The highest plants and highest yield obtained from the 3rd harvest due to vegetation term. That result is consistent with Maheshwar (2005), related with prolonge of vegetation period, *Stevia rebaudiana* plant height increased. Varbanov et al. (2008) observed that after a 60–80 days vegetation period stevia plant height reached 60–70 cm.

However, probably due to climate, length of day time, temperature and humidty during the vegetation term in Adana, we obtained two harvests in 2013 winter plantation and single in 2014 plantation. Most of researchers agree on the temperature has been observed to influence on the availability of soil nutrients, germination, the growth of the plant and shoots, winter survival, photosynthesis, and respiration. It is rather difficult to say a particular process that is most affected by

temperature. But it is generally accepted from the early work of Sumida (1980) (and as cited by Sakaguchi and Kan, 1982) that the optimal temperature range for the growth of stevia is 15–30ºC, though the plant can tolerate critical temperature of 0–2ºC. Mizukami et al. (1983) had postulated that day night temperature variation is another determinant for stevioside production. They obtained best growth and stevioside yield under 25/20ºC day/night regime. Temperature influences yield (Parsons, 2003) either directly or through the diurnal variation as it plays a vital role in stevia production as discussed by Barathi (2003).

In this study, we observed that the different harvest time and vegetation length has seriously affected the yield of steviol glycosides and content of them. In 2013 plantations both spring and winter showed us there was a serious difference on TSG contents between harvests. It was 46,8% between 2nd vs 1st and 50.6% between 3rd vs 1st in Yalova spring plantations and 44,6% more 2nd vs 1st at Adana winter plantations. Similarly, Tavarini and Angelini (2012) observed that harvest time, indepedently of site and year, was certainly one of the major factors determining the most important qualitative traits of *S. rebaudiana* in both the first and second years of growth.

In 2014 plantations, we obtained similar results as 2013. But slightly different in Yalova that the 2nd harvest spring plantation had 1,2% higher SG value than the 3rd harvest and 19,6% higher than the first one. In this respect, soil texture and nutrition uptake of the plants may play a critical role. Supporting these results, Utumi et al. (1999) reported that; the role in growth and development, deficiencies of K, Ca, and S decreased the concentration of stevioside in the plant on dry weight basis while all deficiencies, except that of P, decreased the stevioside content in the plant. On the contrary Mizukami et al. (1983) had postulated that day night temperature variation is another determinant for stevioside production.

In Adana, we had single harvest and the results was exactly same as previous year 1st harvest that 7,2%. In Balıkesir, the results were same as Yalova spring plantation (19,6 % more TSG in 2nd harvest than first harvest) and 2nd harvest contained 15,7% more TSG than the 1st.

For TSG contents, variation between 2013 and 2014 spring plantations in Yalova were average 11,12% and 11,9% respectively, which was a match with the results of Tavarini at al.(2012) that the ratio between rebaudioside A and stevioside gradually increased in the stevia leaves from the first to the third harvest in both years of cultivation. In Adana, it is interesting that 2014 spring plantation results obtained exactly the same as 2013 winter plantation first harvest as 7,2%. That is also do not match with Tavarini at al (2012) reported as "the stevioside decreased significantly in the second year of cultivation at both Jesi and PieveCesato regions, while rebaudioside A did not change at Jesi but increased significantly at Pieve Cesato region". Therefore, results can be caused by probably genotype and environmental conditions.

As dry leaf yield per plot, in all plantations and in 2013 and 2014 were obtained average 71 kg/plot to 92 kg/plot related with regions, harvest and plantation time. That yield results quite close the results of Jordan Molero (1984) in terms of economic biomass productivity, the dry leaf yield in the natural habitat, Paraguay, was between 1500 and 2500 kg/ha under dry land conditions and around 4300 kg ha^{-1} with irrigation per year. And Brandle and Rosa (1992), leaf yields of 3000 kg ha^{-1} with a stevioside concentration of 105 mg g^{-1} equivalent to 66.2 ton ha^{-1} of sugar was obtained in Canada. Additionally, our average dry leaf yield per plot in between different regions and harvest time; first harvest yields were less than second and third harvest except Adana region. That result also match with Kumar et al (2013).

Additionally, in locations; differentiation of dry leaf yields ranged from 2,1% (Balıkesir, spring plantations, 2014) to 10,03% (Yalova, spring plantation, 2013, 3^{rd} harvest vs 1^{st}) . These results support the findings of Sunk (1975) as dry leaf yield of 1^{st} harvest was 3000 kg ha^{-1} and 2^{nd} harvest 3500 kg ha^{-1} in the first year, while 4000–4500 kg ha^{-1} in the second and 4000–6000 kg ha^{-1} in third third year, respectively.

5. CONCLUSIONS

The results obtained in this study show that for the in vitro propagation of stevia, only two parameters, shoot length and callus appearance, varied depending on the medium composition. As to the regeneration protocol developed for a large scale propagation for field trial studies, direct shoot regeneration from nodal explants was employed by subculturing the nodal segments in MS basal medium at 3 week intervals during a period of 8 months. Harvesting was performed by hand after 16 weeks of plantation, and the total yield (dry leaf of regenerant- and seedling-derived plants) was estimated as 2.4 tones per ha. Our findings highlight the ratio of ST content in overall SG was slightly higher in regenerants (50%; w/w) than the seedlings in which a 46% (w/w) ST was detected. While Reb-A composition of both leaf sources were alike, other steviol glycoside compositions of the leaves differed slightly. The maximum difference was shown in rebaudioside-C (Reb-C) content which is also third major SG detected in leaves.

It can also be outlined that the harvest time certainly plays an important role in determining the quality of stevia leaf extracts and dry leaf yield. This factor is also important in affecting the rebaudioside A/stevioside ratio, which is an accepted measure of sweetness quality. This study highlights that the best-quality products and higher dry leaf yields could be obtainedd with the late harvest, since the rebaudioside A/stevioside ratio increased in the stevia leaves from July to September in both years of cultivation. This result is in line with market trends, which require final products that are characterised by a high content of rebA and consequently a low or absent bittertaste due to the presence of stevioside and high dry leaf yield for profitable industrial production process.

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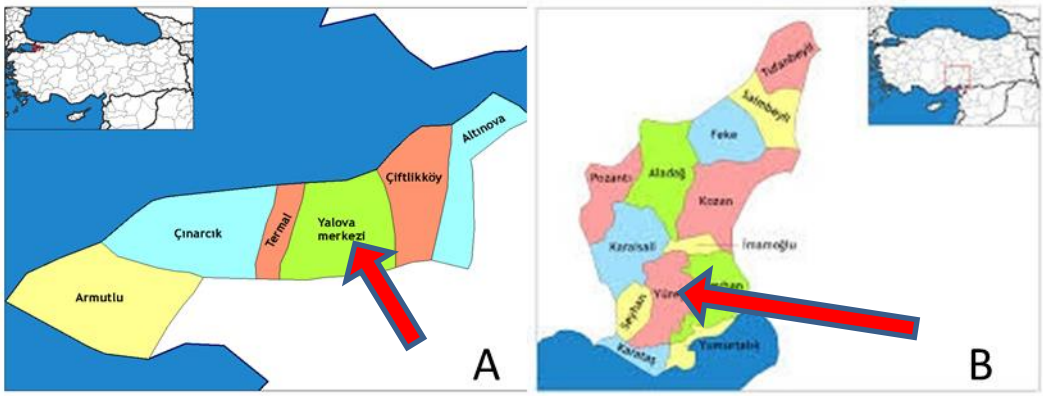
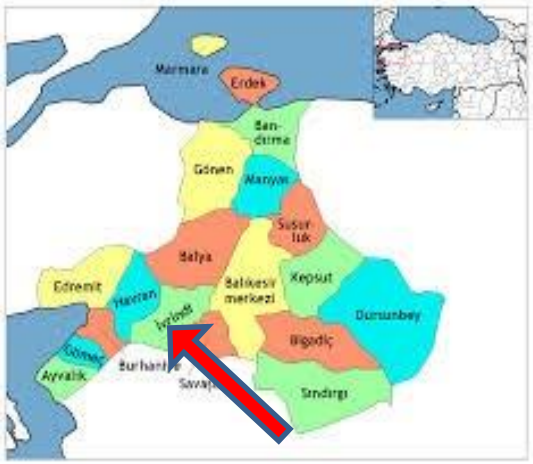
APPENDICES

APPENDIX



Map 1. A.Yalova City, B. Adana City



Map 2. Balıkesir City

Table 22. Yalova city climate statistics (1954–2014)

YALOVA	January	February	March	April	May	June	July	August	September	October	November	December
Ave.Temp (°C)	6,5	6,7	8,3	12,4	17	21,4	23,6	23,5	19,9	15,7	11,7	8,7
Ave.Max. Temp (°C)	10,1	10,5	12,5	17	21,4	26,1	28,5	28,6	25,1	20,5	16,1	12,2
Ave.Min Temp (°C)	3,2	3,3	4,5	8,1	12,1	15,9	18,1	18,2	15	11,8	8,1	5,4
Ave. Sunshine Hours(h)	1,6	3	4,2	5,4	7,3	9,1	9,3	9	7,3	5,6	2,4	1,3
Ave.Number of Rainy days	15,7	13,6	13	11,1	7,7	5,7	3,9	3,8	6,2	10,3	11,8	14,9
Ave Monthly Rainfall (kg/m^2)	89,8	71,5	73,3	52,4	34,2	37,4	23,5	28,1	52,8	81,3	79,5	115,3
Max Temp (°C) (1954-2013)	25	27,2	31,4	36,5	37	42,1	45,4	40,2	37,5	36,6	29,7	27,4
Min Temp(°C) (1954-2013)	-9	-11	-7,4	-1,6	1,2	7,8	10,8	10,3	6,2	1,3	-3,2	-5,6

1

Table 23. Adana city climate statistics (1954–2014)

ADANA	January	February	March	April	May	June	July	August	September	October	November	December
Ave.Temp (°C)	9,6	10,5	13,5	17,5	21,7	25,6	28,1	28,4	25,9	21,3	15,4	11,2
Ave.Max. Temp (°C)	15	16,2	19,5	23,8	28,2	31,7	33,8	34,6	33,1	29	22,4	16,8
Ave.Min Temp (°C)	5,5	6,1	8,5	12,2	15,9	20	23,2	23,5	20,3	15,9	10,7	7,1
Ave. Sunshine Hours(h)	4,4	5,2	6	7,1	9,1	10,4	10,5	10,3	9	7,2	5,6	4,3
Ave.Number of Rainy days	10,4	10,3	9,9	9,1	6,7	3	0,9	0,7	2,6	5,5	7,2	10,5
Ave Monthly Rainfall (kg/m^2)	109,9	84,2	66,8	55,2	47,3	20	7,1	5,2	15,8	40,7	73,7	128,7
Max Temp (°C) (1954-2013)	26,5	26,7	32	37,5	40,6	41,3	44	45,6	43,2	39,4	33,3	30,8
Min Temp(°C) (1954-2013)	-8,1	-6,4	-3,6	-1,3	5,6	11,2	11,5	14,8	9,3	4,8	-4,3	-4,4

[1] Turkish State Meteorological Service

Table 24. Balıkesir city climate statistics (1954–2014)

BALIKESİR	January	February	March	April	May	June	July	August	September	October	November	December
Ave.Temp (°C)	4,8	5,9	8,3	13	17,8	22,5	24,8	24,6	20,7	15,7	10,4	6,7
Ave.Max. Temp (°C)	9	10,6	13,9	19,1	24,5	29,3	31,2	31,2	27,6	22,1	15,9	10,7
Ave.Min Temp (°C)	1,2	1,7	3,3	6,9	10,9	14,9	17,6	17,8	13,9	10,1	5,7	3,1
Ave. Sunshine Hours(h)	2,9	3,6	4,7	6,2	8,7	10,8	11,6	10,9	8,7	6,3	4,2	2,5
Ave.Number of Rainy days	14,1	11,8	11,4	9,6	7,6	4,3	1,6	1,5	3,5	6,6	9,6	14
Ave Monthly Rainfall (kg/m^2)	79,2	66,9	58,8	51,6	39,8	21,8	8,1	5,7	22,3	42,6	78	97,3
Max Temp (°C) (1954-2013)	23,5	24,8	29,8	32,5	38,2	42,5	43,2	43,7	40,3	36,4	29	26,1
Min Temp(°C) (1954-2013)	-13,8	-18,8	-8	-4	0,6	4	9,1	8,7	4	-2,3	-7,9	-12,9

* Source : Devlet Meteoroloji İşleri Genel Müdürlüğü (Turkish State Meteorological Service)

Table 25. Report of soil analysis Yalova

Report of Soil Analysis								
Name	**POLİSAN FIDANLIK**					**Date of Report**		**21.04.2013**
Province	**YALOVA**							
Village	**-**					**Report No**		**286**
Crop	**STEVIA**							
RESULTS OF THE ANALYSIS								
Lab.No:	**Deep**	**Saturation**	**EC_{25} (1:2.5) (mmhos/cm**	**pH** (1:2.5)**	**CaCa3 (%)**	**Organic Matter (%)**	**Available Phosphorus (ppm)**	**Mutable Potassium (ppm)**
9871	0-30	58	0.19	7.9	39.78	3.68	16	160
		Clay loam	Saltless	Alkaline	Very High	Good	Medium	Low
9872	30-60	60	0.15	7.8	44.56	2.09	40	103
		Clay loam	Saltless	Alkaline	Very High	Medium	High	Low

Table 26. Report of soil analysis Adana

Report of Soil								
Adı-Soyadı	**POLİSAN**					**Rapor Tarihi**		**04.08.2013**
İli-İlçesi	**ADANA**							
Köyü	**-**					**Rapor No**		**825**
Bitki	**STEVIA**							
ANALİZ SONUÇLARI								
Lab.No:	**Deep**	**Saturation**	**EC25 (1:2.5) (mmhos/cm**	**pH (1:2.5)**	**CaCa3 (%)**	**Organic Matter (%)**	**Available Phosphorus (ppm)**	**Mutable Potassium (ppm)**
1617	0-30	53	0,2	7,3	1,61	4,57	3	470
		Clay loam	Saltless	Notr	Az	High	Low	Very High
1618	30-60	51	0,17	7,5	19,7	3,59	4	290
		Clay loam	Saltless	Slightly Alkaline	High	Good	Low	High

Table 27. Report of soil analysis Balıkesir/İvrindi

Report of Soil Analysis								
Name	**POLİSAN**					**Date of Report**		**16.05.2013**
Province	**BALIKESİR- İVRİNDİ**							
Village	**-**					**Report No**		**825**
Crop	**STEVIA**							
RESULTS OF THE ANALYSIS								
Lab.No:	**Deep**	**Saturation**	**EC_{25} (1:2.5) (mmhos/cm**	**pH (1:2.5)**	**CaCa3 (%)**	**Organic Matter (%)**	**Available Phosphorus (ppm)**	**Mutable Potassium (ppm)**
384	0-30	58	0,08	7,7	4,3	2	37	39
		Clay loam	Saltless	Slightly Alkaline	Low	Low	Low	Low
385	30-60	57	0,07	7,8	3,9	2,2	43	41
		Clay loam	Saltless	Slightly Alkaline	Low	Low	Low	Low

* T.C. Ministry of Food, Agriculture and Livestock, Atatürk Horticulture Central Research Institute, Soil and Water Analysis Labs

UNIVARIATE ANALYSIS OF VARIANCE

Table 28 . Yield

Between-Subjects Factors

		N
Harvest	1	20
	2	16
	3	8
Season	Spring	36
	Winter	8
Year	2013	20
	2014	24
Location	Adana	12
	Balıkesir	8
	YALOVA	24

Table 29. Tests of Between-Subjects Effects

Dependent Variable: Yield

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1801.995[a]	6	300.333	22.148	.000
Intercept	83632.493	1	83632.493	6167.370	.000
Harvest	216.603	2	108.302	7.987	.001
Season	77.261	1	77.261	5.698	.022
Year	.189	1	.189	.014	.907
Location	999.703	2	499.851	36.861	.000
Error	501.738	37	13.560		
Total	274387.124	44			
Corrected Total	2303.733	43			

a. R Squared = .782 (Adjusted R Squared = .747)

Estimated Marginal Means

Table 30. Harvest

1. Harvest

Dependent Variable: Yield

Harvest	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1	77.573	1.200	75.142	80.004
2	73.979	1.076	71.799	76.159
3	80.385	1.689	76.963	83.807

Table 31. Season

2. Season

Dependent Variable: Yield

Season	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Spring	80.639	1.025	78.561	82.717
Winter	73.986	2.184	69.560	78.412

Table 32. Year

3. Year

Dependent Variable: Yield

Year	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
2013	77.224	1.076	75.044	79.404
2014	77.401	1.382	74.600	80.202

Table 33. Location

4. Location

Dependent Variable: Yield

Location	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Adana	89.159	1.288	86.549	91.769
Balıkesir	69.158	1.832	65.447	72.870
YALOVA	73.620	1.583	70.411	76.828

Post Hoc Tests

Harvest

Table 34. Multiple Comparisons

Multiple Comparisons

Dependent Variable: Yield

	(I) Harvest	(J) Harvest	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1	2	6.4981*	1.23513	.000	3.4826	9.5137
		3	1.1987	1.54048	.719	-2.5623	4.9598
	2	1	-6.4981*	1.23513	.000	-9.5137	-3.4826
		3	-5.2994*	1.59455	.006	-9.1924	-1.4063
	3	1	-1.1987	1.54048	.719	-4.9598	2.5623
		2	5.2994*	1.59455	.006	1.4063	9.1924
LSD	1	2	6.4981*	1.23513	.000	3.9955	9.0007
		3	1.1987	1.54048	.441	-1.9226	4.3201
	2	1	-6.4981*	1.23513	.000	-9.0007	-3.9955
		3	-5.2994*	1.59455	.002	-8.5302	-2.0685
	3	1	-1.1987	1.54048	.441	-4.3201	1.9226
		2	5.2994*	1.59455	.002	2.0685	8.5302

Based on observed means.

The error term is Mean Square(Error) = 13.560.

*. The mean difference is significant at the

Homogeneous Subsets

Table 35. Yield

Yield

	Harvest	N	Subset 1	Subset 2
Tukey HSD[a,b,c]	2	16	74.7194	
	3	8		80.0188
	1	20		81.2175
	Sig.		1.000	.694

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 13.560.

a. Uses Harmonic Mean Sample Size = 12.632.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha =

Location

Table 36. Location

Multiple Comparisons

Dependent Variable: Yield

	(I) Location	(J) Location	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Adana	Balıkesir	16.0458*	1.68080	.000	11.9422	20.1495
		YALOVA	10.1371*	1.30194	.000	6.9584	13.3158
	Balıkesir	Adana	-16.0458*	1.68080	.000	-20.1495	-11.9422
		YALOVA	-5.9088*	1.50336	.001	-9.5792	-2.2383
	YALOVA	Adana	-10.1371*	1.30194	.000	-13.3158	-6.9584
		Balıkesir	5.9088*	1.50336	.001	2.2383	9.5792
LSD	Adana	Balıkesir	16.0458*	1.68080	.000	12.6402	19.4515
		YALOVA	10.1371*	1.30194	.000	7.4991	12.7751
	Balıkesir	Adana	-16.0458*	1.68080	.000	-19.4515	-12.6402
		YALOVA	-5.9088*	1.50336	.000	-8.9548	-2.8627
	YALOVA	Adana	-10.1371*	1.30194	.000	-12.7751	-7.4991
		Balıkesir	5.9088*	1.50336	.000	2.8627	8.9548

Based on observed means.

The error term is Mean Square(Error) = 13.560.

*. The mean difference is significant at the

Homogeneous Subsets

Table 37. Yield

Yield

	Location	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b,c]	Balıkesir	8	71.0375		
	YALOVA	24		76.9463	
	Adana	12			87.0833
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 13.560.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha =

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List of Publications **:**

1- Yücesan B, Büyükgöçmen R, Mohammed A, Sameeullah M, Altuğ C, Gürel S, Gürel E (2016) "An efficient regeneration system and steviol glycoside analysis of *Stevia rebaudiana* Bertoni, a source of natural high intensive sweetener" In vitro Cell Dev Biol–Plant (accepted)

2- Yücesan B, Büyükgöçmen R, Mohammed A, Mohammad, A., Kavas, A., Altuğ C, Mohammad, S., Gürel S, Gürel, E, (2016) "Comparison of Conventional and Biotechnological Approaches for Stevia Production with High Rebaudioside A Content"

Awards **:**