BOLU ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

INVESTIGATION OF PHYSIOLOGICAL ROLES OF STRIGOLACTONES IN SUGAR BEET (*BETA VULGARIS* L.) UNDER *IN VITRO* TISSUE CULTURE AND *EX VITRO* ABIOTIC STRESS CONDITIONS

DOCTOR OF PHILOSOPHY

FATEMEH AFLAKI

BOLU, AUGUST 2019

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APPROVAL OF THE THESIS

Investigation of Physiological Roles of Strigolactones in Sugar Beet (*Beta vulgaris* L.) under *In Vitro* Tissue Culture and *Ex Vitro* Abiotic Stress Conditions submitted by **Fatemeh AFLAKI** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Department of Biology, The Graduate School of Natural and Applied Sciences of BOLU ABANT IZZET BAYSAL UNIVERSITY in 21/08/2019** by

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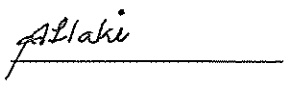
I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Fatemeh AFLAKI



ABSTRACT

INVESTIGATION OF PHYSIOLOGICAL ROLES OF STRIGOLACTONES IN SUGAR BEET (*BETA VULGARIS* L.) UNDER *IN VITRO* TISSUE CULTURE AND *EX VITRO* ABIOTIC STRESS CONDITIONS
PHD THESIS
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BOLU, AUGUST 2019

Strigolactones (SLs), as carotenoid-derived compounds and recently introduced plant hormones, have wide-ranging biological roles ranged from both shoot and root architecture, plant communication in the rhizosphere, stimulation of germination in root parasitic plants, such as *Striga*, *Orobanche* and *Phelipanche* species, seed germination, responses to environmental stresses, modulators of root and shoot development in response to nutrient-deficient conditions, regulation of plant defense, and stimulation of secondary growth. Since this class of plant hormones has various biological roles in the growth and development, the recognition of responsible genes, which have not been recognized yet in sugar beet (*Beta vulgaris*), will provide a valuable knowledge in order to select better and productive genotypes, or to be manipulated *in vitro*, then to breed this highly important and industrial crop species. On the other hand, environmental stresses like salinity and drought lead to a reduction in the productivity of plants due to their adverse effects on plant growth. Different phytohormones are involved in stress responses; however, the role of strigolactones (SL) in this important respect has not been elucidated yet. In addition to the above-mentioned importance, deciphering SLs roles in *in vitro* culture of sugar beet may contribute to a greater success in its tissue culture methods and optimization of the certain stages. To know more, it is encouraged to read the Introduction section of the thesis (Chapter I). Therefore, this thesis has been defined to evaluate the roles of *MAX1* gene in biosynthesis or signaling of SLs and hopefully to suggest/introduce the responsible gene for the first time in sugar beet (Chapter II). It will include estimates how SLs could contribute to alleviating the adversely affected plant conditions under abiotic stresses, which are one of the undeniable problems for plant production throughout the world (Chapter III). In addition, one part of the thesis research is to understand the SLs effects on tissue culture of sugar beet from germination to root growth (Chapter IV and V). The next purpose is investigating the relationship between SLs and auxin as a hormonal interaction (Chapter VI). In addition, it is hoped that such findings will contribute to agricultural research activities aiming at the development of plant varieties with high abiotic stress tolerance.

Chapter II describes an experiment for the evaluation of a putative ortholog of MAX1 gene in sugar beet. The effects of strigolactone hormones (*rac*-GR24, (±)-strigol and (±)-5-deoxystrigol) and one SL inhibitor (TIS108) on the expression level

of *Beta vulgaris* subsp. *vulgaris* the gene encoding Cytochrome P450 711A1 were studied. A few sets of different primer pairs for this gene and a reference gene (β-actin) were designed to estimate the expression level of the gene of interest quantitatively. Before starting the main experiments, a few sets of pre-experiments were devised to check the feasibility and reliability of the main experiment. The pre-experiments were done for checking the spraying conditions, and examining the quality and quantity of the molecular results. Since the results of the pre-experiments were highly promising, the main experiments were done. To evaluate the effects of exogenously applied SLs on the expression of the gene of interest, sugar beet cv. Serenada seeds were sown in pots. They were sprayed with four levels (0, 2.5, 5, and 7.5 μM) of the chemicals (*rac*-GR24, strigol, 5-deoxystrigol, and TIS108), once every two days for seven times, each time with 5 or 10 ml of an aqueous solution of the chemicals. After applying the treatments, leaf samples of the plants were collected, their whole RNA was extracted, cDNAs were synthesized, and the changes in the expression levels of the gene were investigated based on a quantitative PCR (qPCR) method using BioRad CFX connect Real-Time PCR instrument. The results obtained from the qPCR analysis indicated that exogenous application of the SL hormones decreased the expression of the gene. On the other hand, application of the SL inhibitor increased the expression of the gene of interest. Generally, the decrease and increase in the expression of the gene were respectively inversely and directly proportional to the concentrations of the applied chemicals.

Chapter III describes an experiment designed to evaluate the effects of exogenous application of SLs on salinity- and drought-stress exposed sugar beet plants growing in pots. After analyzing the properties of soil, a pre-experiment was carried out to assess the response of the sugar beet plants to different levels of salinity and drought stresses. The salinity pre-experiment was performed by irrigating the potted plants with 50 ml of an aqueous solution of NaCl containing 150 mM, 200 mM, or 250 mM over ten days. The drought pre-experiment was performed by withholding irrigation for four, six, or eight days. The observed results from the pre-experiments suggested that 250 mM NaCl and withholding irrigation for six days were the proper conditions for conducting the main experiments. For the main experiments, ten-day-old seedlings were sprayed with 10 μM of the hormones (*rac*-GR24, St, and dSt) once or twice per day over two weeks. After collecting shoot samples, the treatment effects on morphology, catalase (CAT) enzyme activity, chlorophyll (Chl) content, and malondialdehyde (MDA) content were compared with water-sprayed control plants. Observation of the plants after running the main experiments indicated that all the hormonal treatments increased the plants' tolerance to salinity and drought stresses. In a general point of view, the hormonal treatment effects on increasing the amounts of Chl a and the Chl total (Chl T) were significant, whereas their effects on Chl b were not significant. However, the hormonal treatment effects on CAT enzyme activity in the stress-subjected plants were not statistically significant. On the other hand, the hormonal treatments generally decreased the amount of MDA in the plants.

Chapter IV describes a method proposed to examine the effects of the SL hormones on the germination of sugar beet seeds. The applied hormonal treatments were *rac*-GR24, St, dSt, and TIS108, mixed in a half-strength MS medium, and supplemented with 10 g L^{-1} sucrose. The concentrations of the hormonal treatments were 0 as control, 2.5, 5 and 7.5 μM. The experiment was done in *in vitro* conditions. Fourteen days after sowing the seeds in the hormone-containing media, the percentage

of the germinated seeds were calculated. The results indicated that the effects of the hormonal treatments on seed germination of sugar beet were not statistically significant.

Chapter V describes a method to study SLs effects on *in vitro* tissue culture of sugar beet. In this experiment, to germinate the seeds, a half-strength MS was used for the common medium preparation. After germinating seeds in *in vitro* medium, the explants were subcultured on a common medium containing full-strength MS medium supplemented with 30 g L^{-1} sucrose, plus 0 as control, 2.5, 5 and 7.5 µM *rac*-GR24. One month after culture initiation, shooting pattern and root growth were observed. Treating sugar beet explants with *rac*-GR24 significantly decreased the number of leaves, the total length of leaves, and the total area of leaves. Similarly, *rac*-GR24 addition to the *in vitro* medium decreased the length of root in sugar beet explants. The decrease in the measured parameters was statistically significant, and it was inversely proportional to the concentration of *rac*-GR24 in the medium.

Chapter VI describes a protocol to study the interactions of SL with auxin in *in vitro* conditions. After germinating seeds in the medium, the explants were subcultured on a common medium containing a full-strength MS medium supplemented with 30 g L^{-1} sucrose, but with varied amounts of SL and auxin hormones (*rac*-GR24 and IAA), and SL and auxin inhibitors (TIS108 and TIBA). Totally seven different combinations of the chemicals were prepared. Four weeks after culture initiation, the effects of the interaction of different plant growth regulators on the number of leaves, the length of leaves, the area of leaves, the length and the number of roots in sugar beet explants were observed. The effects of the applied treatments were statistically significant. The highest number of leaves and the longest leaves were observed for control, TIS108, and TIS108+IAA treatments, whereas the lowest numbers were recorded for the treatments containing TIBA+GR24, TIBA+GR24+IAA, and TIBA+GR24+IAA+TIS108. The largest leaf area was recorded for control and TIS108 treatments, while the smallest areas were produced in the media containing TIBA+GR24, TIBA+GR24+IAA, and TIBA+GR24+IAA+TIS108. The sugar beet explants growing in control conditions produced the longest roots, whereas the shortest ones were recorded for the explants treated in TIBA+GR24, TIBA+GR24+IAA, and TIBA+GR24+IAA+TIS108 supplemented media. Control, TIS108, and TIS108+IAA treatments produced the highest number of roots, but the other treatments resulted in the lowest number of roots.

Chapter VII includes the conclusions driven from the experiments described in this research thesis. It seems that the studied gene (encoding Cytochrome P450 711A1) in sugar beet (*B. vulgaris*) is most likely an ortholog of *MAX1* gene in *Arabidopsis*. The gene is likely involved in the biosynthesis pathway of SLs in sugar beet. In addition, SLs are effective in modifying sugar beet shoot and root architectures in *in vitro* conditions. SLs seem to have an inhibitory effect on the growth of the *in vitro* sugar beet explants. It seems that SLs can alleviate the deleterious effects of salinity and drought conditions for sugar beet plants. Moreover, it suggests that SLs can act directly and independent of auxin.

KEYWORDS: Abiotic stress, *Beta vulgaris*, Strigolactones, *MAX1* gene, Tissue culture, Gene expression

ÖZET

STRİGOLAKTONLARIN ŞEKER PANCARINDA (*BETA VULGARIS* L.) FİZYOLOJİK ETKİLERİNİN *İN VİTRO* DOKU KÜLTÜRÜ VE *EX VİTRO* ABİYOTİK STRES KOŞULLARI ALTINDA İNCELENMESİ
DOKTORA TEZİ
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BOLU, AĞUSTOS - 2019

Karotenoidlerden kökenlenen ve yeni nesil hormonlar olarak bilinen strigolaktonlar (SL), kök ve gövde oluşumunu düzenleyici, *Striga*, *Orobanche* ve *Phelipanche* gibi kök paraziti bitkilerin çimlenmesini uyarıcı, besin stresine karşı kök ve gövde oluşumunu düzenleyici, metabolik ve çevresel uyarıcılara tepki ve savunma mekanizmalarında etkili, ve sekonder büyümeyi teşvik eden bitki büyüme düzenleyicileridir. Bu bitki hormonları sınıfının, büyüme ve gelişmede çeşitli biyolojik rolleri olduğu için, şeker pancarında (*Beta vulgaris*) henüz tanımlanmamış sorumlu genlerin belirlenmesi, daha iyi ve üretken genotiplerin seçilmesi, veya in vitro olarak manipüle edildikten sonra, bu son derece önemli ve endüstriyel bitkinin üretimi için değerli bir bilgi sağlayacaktır. Öte yandan, tuzluluk ve kuraklık gibi çevresel stresler, bitki büyümesi üzerindeki olumsuz etkilerinden dolayı bitki verimliliğinde düşüşe yol açmaktadır. Farklı fitohormonlar stres tepkilerine katılır; ancak, bu önemli açıdan strigolaktonların (SL) rolü henüz açıklanamamıştır. Yukarıda belirtilen öneme ek olarak, SL'lerin in vitro şeker pancarı kültüründeki rollerinin açıklanması, doku kültürü yöntemlerinde ve belirli aşamaların optimizasyonunda daha büyük bir başarıya katkıda bulunabilir. Daha fazla bilgi için, tezin Giriş bölümünün okunması önerilir (Bölüm I). Bu nedenle, bu tez, MAX1 geninin SL'lerin biyosentezindeki veya sinyallemesindeki rollerini değerlendirmek ve şeker pancarında ilk kez sorumlu geni önermek/tanımlamak için hazırlanmıştır (Bölüm II). Bu, SL'lerin, dünyadaki bitki üretimi için inkar edilemez sorunlardan biri olan abiyotik stresler altında olumsuz etkilenen bitki koşullarının hafifletilmesine nasıl katkıda bulunabileceğini tahmin etmeyi içerecektir (Bölüm III). Ek olarak, tez çalışmasının bir bölümü, şeker pancarının doku kültürü üzerindeki çimlenmeden kök büyümesine kadar SL'lerin etkilerini araştırmaktır (Bölüm IV ve V). Bir sonraki amaç, SL'ler ile oksin arasındaki ilişkiyi hormonal bir etkileşim olarak incelemektir (Bölüm VI). Ek olarak, bu bulguların abiyotik stres toleransı yüksek bitki çeşitlerinin geliştirilmesine yönelik tarımsal araştırma faaliyetlerine katkıda bulunacağı da ümit edilmektedir.

II. Bölümde, şeker pancarındaki MAX1 geninin olası ortolojisinin değerlendirilmesi için bir deney tanımlamıştır. Strigolakton hormonlarının (rac-GR24, (±) -strigol ve (±) -5-deoksistrigol) ve bir SL inhibitörünün (TIS108), *Beta vulgaris* subsp. *vulgaris* sitokrom P450 711A1 ekspresyon düzeyi üzerine etkisi çalışılmıştır. Bu gen ve referans gen (β-aktin) için birkaç farklı primer çifti kümesi, ilgilenilen genin

ekspresyon seviyesini kantitatif olarak tahmin etmek için tasarlanmıştır. Ana deneylere başlamadan önce, ana deneyin fizibilitesini ve güvenilirliğini kontrol etmek için bir takım ön deneyler yapıldı. Püskürtme koşullarını kontrol etmek ve moleküler sonuçların kalitesini ve miktarını incelemek için ön deneyler yapıldı. Ön deneylerin sonuçları oldukça ümit verici olmuş, sonrasında ana deneyler yapılmıştır. Eksojen olarak uygulanan SL'lerin ilgilenilen genin ekspresyonu üzerindeki etkilerini değerlendirmek için, şeker pancarı cv. Serenada tohumları saksılara ekildi. Dört seviye (0, 2.5, 5 ve 7.5 μM) kimyasal (rac-GR24, strigol, 5-deoksistrigol ve TIS108), her iki günde bir kez yedi kez, her seferinde 5 veya 10 ml ile püskürtülmüş, işlemler uyguladıktan sonra, bitkilerin yaprak örnekleri toplanmış, toplam RNA'ları çıkarılmış, cDNA'lar sentezlenmiş ve genin ekspresyon seviyelerindeki değişiklikler BioRad CFX connect Real-Time PCR cihazı kullanılarak kantitatif bir PCR (qPCR) yöntemine göre araştırılmıştır. qPCR analizinden elde edilen sonuçlar SL hormonlarının eksojen uygulamasının gen ekspresyonunu azalttığını göstermiştir. Öte yandan, SL inhibitörünün uygulanması ilgilenilen genin ifadesini arttırmıştır. Genel olarak, genin ekspresyonundaki azalma ve artış, uygulanan kimyasalların konsantrasyonları ile sırasıyla ters ve doğrudan orantılı olmuştur.

Bölüm III, SL'lerin eksojen uygulamasının, saksıda yetişen tuzluluk ve kuraklığa strese maruz kalan şeker pancarı bitkileri üzerindeki etkilerini değerlendirmek için tasarlanmış bir deneyi anlatmaktadır. Toprağın özelliklerini analiz ettikten sonra, şeker pancarı bitkilerinin farklı tuzluluk seviyelerine ve kuraklık streslerine karşı tepkilerini değerlendirmek için bir ön deney yapılmıştır. Tuzluluk öncesi deneyi, saksı bitkilerinin, 50 gün boyunca 150 ml, 200 mM veya 250 mM içeren sulu bir NaCl çözeltisi ile sulanmasıyla gerçekleştirildi. Kuraklık öncesi deney, dört, altı veya sekiz gün boyunca sulamayı keserek gerçekleştirildi. Ön deneylerden gözlemlenen sonuçlar, 250 mM NaCl'nin ve altı gün boyunca sulamanın durdurulduğu koşulların asıl deneylerin yapılması için uygun olduğunu göstermiştir. Asıl deneyler için, on günlük fidelere iki hafta boyunca günde bir veya iki kez 10 μM hormon (rac-GR24, St ve dSt) püskürtülmüştür. Sürgün numunelerinin toplanmasından sonra, morfoloji, katalaz (CAT) enzim aktivitesi, klorofil (Chl) içeriği ve malondialdehit (MDA) içeriği üzerindeki etkileri su püskürtülmüş kontrol bitkileriyle karşılaştırılmıştır. Asıl deneyler yapıldıktan sonra bitkilerin gözlemlenmesi, tüm hormonal işlemlerin bitkilerin tuzluluk ve kuraklık streslerine karşı toleransını arttırdığını göstermiştir. Genel bir bakış açısına göre, Chl a ve toplam Chl (Chl T) miktarının arttırılması üzerindeki hormonal uygulma etkileri önemliyken, Chl b üzerindeki etkileri anlamlı değildi. Bununla birlikte, strese maruz kalan bitkilerde CAT enzim aktivitesi üzerindeki hormonal uygulama etkileri istatistiksel olarak anlamlı değildi. Öte yandan, hormonal uygulamalar genellikle bitkilerde MDA miktarını azaltı.

Bölüm IV, SL hormonlarının şeker pancarı tohumlarının çimlenmesi üzerindeki etkilerini incelemek için önerilen bir yöntemi açıklamaktadır. Uygulanan hormonal uygulamalar, yarı kuvvetli bir MS ortamına eklenmiş ve 10 g L^{-1} sukroz ile takviye edilmiş, rac-GR24, St, dSt ve TIS108 olmuştur. Hormonal uygulamaların konsantrasyonları 0 (kontrol), 2.5, 5 ve 7.5 μM idi. Deney, in vitro koşullarda yapıldı. Hormon içeren ortamda tohumların ekiminden on dört gün sonra, çimlenmiş

tohumların yüzdesi hesaplandı. Sonuçlar, hormonal uygulamaların şeker pancarı tohumunun çimlenmesi üzerindeki etkilerinin istatistiksel olarak anlamlı olmadığını göstermiştir.

Bölüm V, SL'lerin şeker pancarının in vitro doku kültürü üzerindeki etkilerini inceleyen bir yöntemi tanımlamaktadır. Bu deneyde, tohumları çimlendirmek için, hazır ortam olarak yarı-kuvvetli bir MS kullanılmıştır. Tohumların in vitro ortamda çimlenmesinden sonra, eksplantlar, 30 g L^{-1} sukroz, ve 0 (kontrol), 2.5, 5 ve 7.5 µM rac-GR24 içeren tam güçte MS ortamı içeren bir ortam üzerinde alt kültüre alındılar. Kültür başlangıcından bir ay sonra, sürgün oluşumu ve kök büyümesi gözlendi. Şeker pancarı eksplantlarına rac-GR24 uygulanması, yaprak sayısını, toplam yaprak uzunluğunu ve toplam yaprak alanını önemli ölçüde azalttı. Benzer şekilde, in vitro ortama rac-GR24 ilavesi, şeker pancarı eksplantlarındaki kök uzunluğunu azaltmıştır. Ölçülen parametrelerdeki düşüş istatistiksel olarak anlamlıydı ve ortamdaki rac-GR24 konsantrasyonu ile ters orantılıydı.

Bölüm VI, SL'nin oksin ile etkileşimlerini in vitro koşullarda incelemek için bir protokol tanımlamaktadır. Ortamdaki tohumların çimlenmesinden sonra, eksplantlar, 30 g L^{-1} sukroz ile takviye edilmiş, ancak çeşitli miktarlarda SL ve oksin hormonları (rac-GR24 ve IAA) ve SL ve oksin inhibitörleri (TIS108 ve TIBA) içeren tam kuvvetli bir MS ortamı içeren bir ortamda alt kültüre alındılar. Kimyasalların toplam yedi farklı kombinasyonu hazırlandı. Kültürün başlamasından dört hafta sonra, farklı bitki büyüme düzenleyicilerinin etkileşiminin yaprak sayısı, yaprakların uzunluğu, yaprakların alanı, şeker pancarı eksplantlarındaki köklerin uzunluğu üzerindeki etkileri gözlenmiştir. Uygulamaların etkileri istatistiksel olarak anlamlıydı. En yüksek yaprak sayısı ve en uzun yaprak, kontrol, TIS108 ve TIS108 + IAA uygulamalarında gözlenirken, en düşük veriler TIBA + GR24, TIBA + GR24 + IAA ve TIBA + GR24 + IAA + TIS108 içeren uygulamalarda kaydedilmiştir. . En büyük yaprak alanı kontrol ve TIS108 uygulamaları için kaydedildi. En küçük alanlar TIBA + GR24, TIBA + GR24 + IAA ve TIBA + GR24 + IAA + TIS108 içeren ortamlarda elde edildi. Kontrol koşullarında yetişen şeker pancarı eksplantları en uzun kökleri oluştururken, en kısa olanları TIBA + GR24, TIBA + GR24 + IAA ve TIBA + GR24 + IAA + TIS108 ile desteklenmiş ortamlarda kültüre alınan eksplantlar için kaydedilmiştir. Kontrol, TIS108 ve TIS108 + IAA uygulamaları en fazla kök üretti, ancak diğer uygulamalar en az kök sayısına neden oldu.

Bölüm VII, bu araştırma tezinde açıklanan deneylerden elde edilen sonuçları içermektedir. Şeker pancarındaki (*B. vulgaris*) incelenen genin (cytochrome P450 711A1) Arabidopsis'te MAX1 geninin bir ortologu olduğu görülüyor. Gen, muhtemelen şeker pancarı içindeki SL'lerin biyosentez yolunda rol oynamaktadır. Ek olarak, SL'ler, şeker pancarı sürgün ve köklerinin mimarilerinin in vitro koşullarda değiştirilmesinde etkilidir. SL'lerin, in vitro şeker pancarı eksplantlarının büyümesi üzerinde inhibe edici bir etkisi olduğu görülmektedir. SL'ler şeker pancarı bitkileri için tuzluluk ve kuraklık koşullarının zararlı etkilerini hafifletebilir gibi görünüyor. Ayrıca, SL'lerin doğrudan ve oksinden bağımsız olarak hareket edebildiğini göstermektedir.

ANAHTAR KELİMELER: Abiyotik stres, *Beta vulgaris*, Strigolaktonlar, MAX1 geni, Doku kültürü, Gen ekspresyonu

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LIST OF ABBREVIATIONS AND SYMBOLS

ABA	**:** Abscisic acid
ABCB19	**:** ATP-BINDING CASSETTE B19
AM	**:** Arbuscular mycorrhizal fungi
atD14	**:** *Arabidopsis* DWARF14
B. vulgaris	**:** *Beta vulgaris*
B1	**:** Thiamin
B6	**:** Pyridoxine
BRC1	**:** BRANCHED1
BRs	**:** Brassinosteroids
CCD7	**:** Carotenoid cleavage dioxygenase 7
CCD8	**:** Carotenoid cleavage dioxygenase 8
CKs	**:** Cytokinins
CL	**:** Carlactone
CLA	**:** Carlactonoic acid
CYP450)/P450	**:** Gene encoding Cytochrome P450
D27	**:** DWARF 27
dSt	**:** 5-Deoxystrigol
ETH	**:** Ethylene
GAs	**:** Gibberellins
GR24	**:** *rac*-GR24
IAA	**:** Indole-3-acetic acid
JAs	**:** Jasmonates
MAX1	**:** MORE AXILLARY GROWTH1
MAX2	**:** MORE AXILLARY GROWTH2
MAX3	**:** MORE AXILLARY GROWTH3
MAX4	**:** MORE AXILLARY GROWTH4
MeCLA	**:** Methyl carlactonoate
osD14	**:** *Oryza sativa* D14
PATs	**:** Polar auxin transport stream
PGR	**:** Plant growth regulator
PIN1	**:** Auxin efflux carrier family protein
POD	: Peroxidase

SAs	**:** Salicylates
SCF complex	**:** Skp, Cullin, F-box containing complex
SKP1	**:** S-Phase kinase associated protein 1
SLs	**:** Strigolactones
SMXL6/7/8	**:** SUPRESSOR OF MAX2-LIKE 6 to 8
SOD	: Superoxide dismutase
St	**:** Strigol
TFs	**:** Transcription factors
TIBA	**:** 2,3,5-Triiodobenzoic acid
TIS	**:** TIS108

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CHAPTER I

1 Introduction

1.1 Plant Hormones

1.1.1 Overview of plant hormones

"Plant hormones" or "phytohormones" are expressions referring to naturally occurring chemicals that can be found in small amounts in plants (Davies, 2010). They have various critical roles which are fundamental to plant growth and development, plant interactions with the environment and other organisms, alongside plant tolerance or resistance to environmental stresses (abiotic/biotic stresses) (Le Xu et al., W., 2018, Davies, 2010). In other words, plant physiological processes can be strongly influenced by a very low concentration of phytohormones (Davies, 2004). Phytohormones are mobile substances that move from the site of synthesis to a target tissue to control different physiological functions e.g. growth (Davies, 2010).

The first identified phytohormone, auxin, dates back to the early 90s (Went, 1937) after the earlier observations by Sachs (1880) and Darwin (1880). On the other hand, the last discovered one thus far is a new class of phytohormones calling strigolactones (SLs) (Gomez-Roldan et al., 2008). After introducing the world to SLs, the number of phytohormones categories has been changed to nine including auxins, gibberellins (GAs), abscisic acid (ABA), ethylene (ETH), cytokinins (CKs), brassinosteroids (BRs), jasmonates (JAs), salicylates (SAs), besides strigolactones (SLs) (Su et al., 2017).

Phytohormones have vitally and extremely important functions in various processes in plants from germination to reproduction/yield. The processes can be

summarized in plant growth, development, and senescence (Davies, 2010). Plant hormones are deeply involved in cellular, molecular, and tissue levels (Takatsuka and Umeda, 2014). Plant hormones are also critical in both gene expression, regulation and transduction. They effectively function from the earliest stage in plant life until the latest one. Therefore, identifying new plant hormones, understanding their functions, their biosynthesis, and transduction pathways can elucidate different aspects of plant growth and developmental processes.

1.1.2 Discovery of strigolactones

SLs' initial identification dates back to 1966 when its compound was isolated from cotton (*Gossypium hirsutum*) root exudate as a stimulator of parasitic weed germination, *Striga lutea* (witchweed). At that time, it was the only defined role for SLs (Zwanenburg and Pospíšil, 2013). The name "strigolactone" was selected based on *Striga lutea* and lactone rings in their structures (Cook et al., 1966).

On the other hand, some efforts were put in discovering the compounds dealing with AM (Arbuscular mycorrhiza) growth like promoting hyphal branching. In the late 20th century, several mutants of pea (*Pisum sativum*), *Arabidopsis thaliana*, and petunia (*Petunia hybrida*) showed extremely branchy shoots that their phenotypes were different from auxin or cytokinin mutants. It was revealed (by grafting studies) that a substance originated from root have a prominent role in controlling shoot branching. In the first 21st century, these findings came to an important realization by which it was indicated that strigol is a substance to which both AM fungi and parasitic plants respond and is a critical determinant in AM hyphal branching, hence SLs contribute to symbiotic interactions of plants in the rhizosphere (Lopez-Obando et al., 2015).

In 2008, the research ends up to the discovery of a new dimension of SLs as a plant hormone discovered by Gomez-Roldan et al. (2008). Later, it was demonstrated that SLs are among the plant hormones seem to be involved in eliciting responses to biotic and abiotic plant stresses like salinity, drought and cold stresses (Xiong et al., 2002). It seems that SLs in conjunction with different phytohormones regulate plant architecture (Shinohara et al., 2013). Despite the investigations that have recently been

conducted, there is a lot to be discovered in synthesis, transduction, and function of SLs.

1.1.3 Structure and biosynthesis and signal transduction of SLs

1.1.3.1 Structure

In 1972, Cook and coworkers reported the structure of strigol as a new potent stimulant of parasitic witchweed (*Striga lutea* Lour.) (Figure 1.1). They isolated strigol from the root exudates of cotton (*Gossypium hirsutum* L.). They then observed that germination percentage of parasite witchweed seeds was zero whereas this amount reached 50% after application of strigol (Cook et al., 1972).

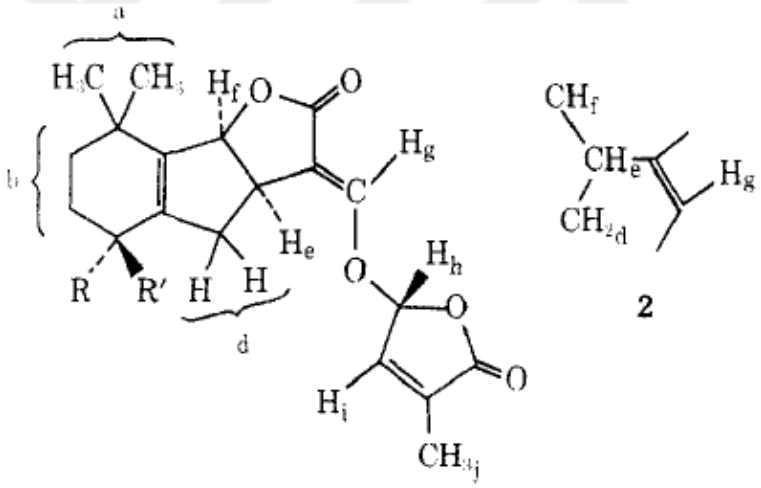


Figure 1.1. The structure of strigol (Cook et al., 1972).

Later, the absolute stereochemistry of strigol was obtained through X-ray crystallography (Brooks et al., 1985). Discovery of SL continued by Sorgolactone (Hauck et al., 1992), alectrol (Müller et al., 1992), and Orobanchol (Yokata et al., 1998).

The structure of natural SLs is divided into two main categories, strigol- or orobanchol-like SLs (Figure 1.2 and Figure 1.3).

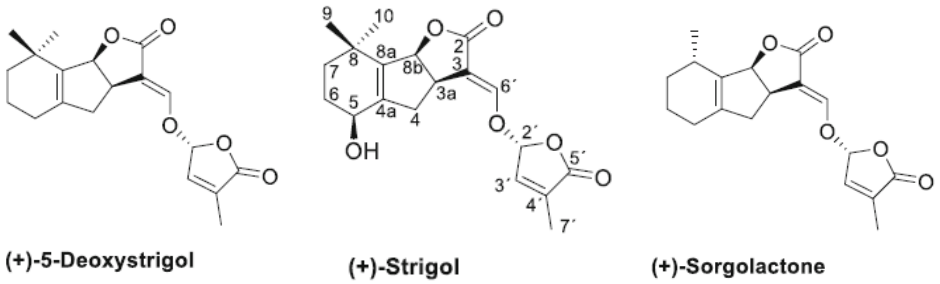


Figure 1.2. SLs with stereochemistry of strigol, or strigol-like SLs (Zwanenburg et al., 2016).

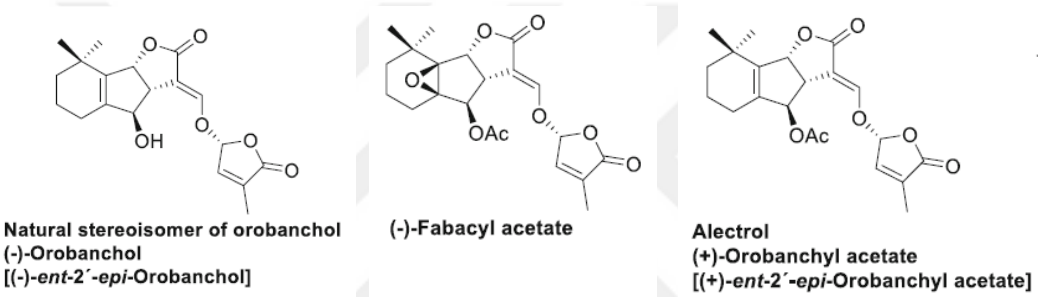


Figure 1.3. SLs with stereochemistry of orobanchol, or orobanchol-like SLs (Zwanenburg et al., 2016).

The difference between strigol-like SLs and orobanchol-like ones is related to the stereochemistry of the junction of B–C ring whereby the former one contains β-oriented C ring, whereas, orobanchol-like one has α-oriented C ring (Scaffidi et al. 2014). The simple structures of a natural and a synthetic SL are provided in Figure 1.4.

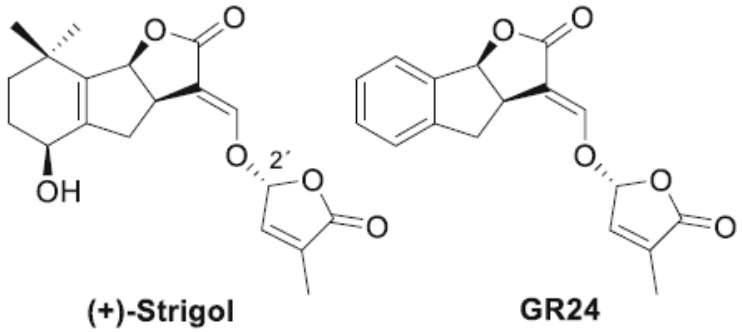


Figure 1.4. Simple structures of strigol and *rac*-GR24 (Zwanenburg et al., 2016).

In general, the structure of SLs has two moieties: a) Tricyclic lactone, and b) butenolide.

Tricyclic lactone consisting of three rings (A, B, and C) is the core structure of SLs with possible slight differences in A size and substitution patterns on AB rings. Tricyclic lactone as the one moiety is bonded to another one called D ring (butenolide) through enol ether bridge (Lopez-Obando et al., 2015; Zwanenburg et al., 2016) (Figure 1.5).

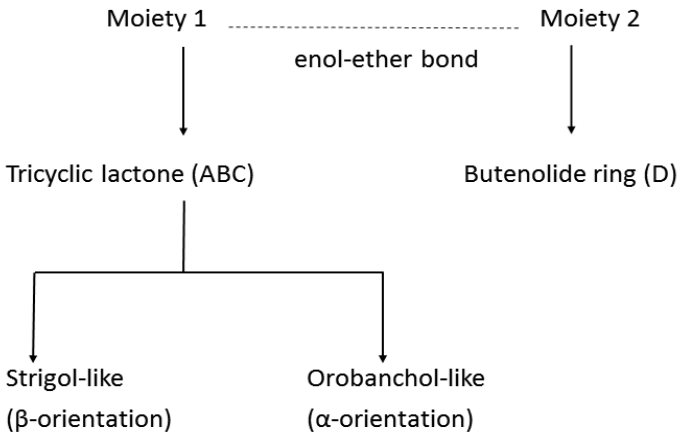


Figure 1.5. Moieties of the structure of SLs.

1.1.3.2 Biosynthesis

In 2005 based on a study using fluridone (an inhibitor of carotenoid biosynthesis) and mutants of carotenoid metabolism, it was indicated that SLs might be carotenoid derivatives (Matusova et al., 2005). The result of some research reported in 2008 supported the above-mentioned hypothesis. In both pieces of research, it was proved that the mutants of *d17* and *d10* in rice which were defective in carotenoid cleavage dioxygenase 7 (CCD7) and carotenoid cleavage dioxygenase 8 (CCD8) and mutant of *ccd8* in pea were SL-deficient mutants (Umehara er al., 2008; Gomez-Roldan et al., 2008). Therefore, it was demonstrated that SLs are synthesized from carotenoids (Seto and Yamaguchi, 2014). Carotenoids are isoprenoid compounds from which SLs emanate in plastids. It happens through a pathway involving carotenoid cleaving deoxygenase 7 (CCD7), carotenoid cleaving deoxygenase 8 (CCD8), DWARF27 (D27) and cytochrome CYP450 (P450). D27, an iron-containing protein

localized in chloroplasts, is a b-carotene isomerase which is responsible for conversion of all-trans-b-carotene into 9-cis-b-carotene (Lin et al., 2009).

CCD7 and CCD8, carotenoid cleavage dioxygenases 7 and 8, are non-heme iron enzymes which can oxidatively cleave C-C double bonds. They are plastid-localized proteins (anonymous, 2011). The product of *D27* is cleaved by CCD7 and its resultant product is rearranged by CCD8 (Alder et al., 2012).

The product of three mentioned biosynthetic enzymes (D27, CCD7, and CCD8) is "carlactone" (CL). CL, a carbon skeleton similar to SL, is a mobile compound (Seto and Yamaguchi, 2014), which can move from plastid to cytoplasm. Based on mutant studies, CL was considered as the precursor of SL (Alder et al,. 2012; Seto and Yamaguchi, 2014). In a step further, *MAX1*-encoded CYP450 (a class III Cytochrome P450) (Booker et al., 2005) can convert CL into carlactonic acid (CLA) or 5-deoxystrigol (Pulido et al. 2012; Alder et al., 2012; Kramna et al., 2019). In the process explained up till now, the corresponding genes to enzymes and CYP450 are as follows:

{D27: *D27*}, {CCD7: *MAX3*}, {CCD8: *MAX4*} and {CYP450: *MAX1*}

1.1.3.3 Signal perception and transduction

SLs signal transduction at a single glance includes SL-mediated interaction of the receptor with F-box protein, degradation of repressor proteins, activation of transcription factors (Marzec, 2016).

MAX2 through encoding F-box protein acts in the perception of SL (Stirnberg et al., 2002). F-box proteins can be considered as a shared feature in transduction pathways of hormones (Vierstra, 2003). *D3* in rice and *RMS4* in pea are orthologous to *MAX2* in *Arabidopsis* (anonymous, 2011).

The proteins, atD14 and F-box are of basic necessities in the signal transduction pathway of SLs. atD14 and F-box proteins are encoded by *atD14* and *MAX2* genes in *Arabidopsis*, respectively. *OsD14* in rice is equivalent to *atD14* in *Arabidopsis*. atD14 is a member of α/β hydrolase superfamily, which acts as a receptor for SLs (Waters et

al., 2012). F-box protein is a component in SKP1-CULLIN-F-BOX complex (SCF). atD14 and F-box are responsible for reception and SL-mediated binding of target molecules to Skp1, Cullin and ubiquitin ligase for ubiquitination and subsequently proteasomal degradation. The target molecules consist of repressors proteins, e.g., SUPPRESSOR OF MAX2-LIKE6 to 8 (SMXL6/7/8) in *Arabidopsis* and D53 in rice. Degradation of repressors of TCP transcription factor family allows TFs expression (Marzec, 2016) (Figure 1.6).

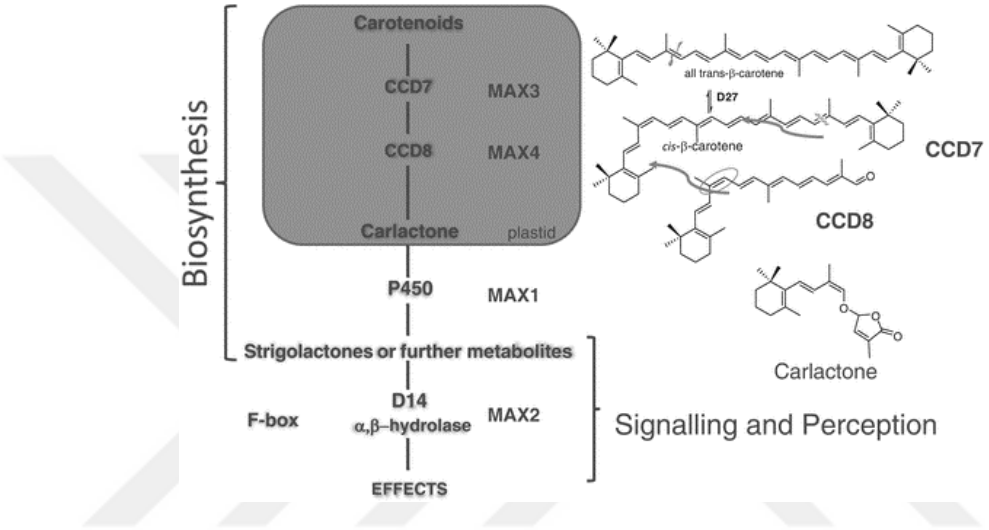


Figure 1.6. Biosynthesis, perception and signaling of SL (Koltai and Prandi, 2014).

1.1.4 The importance of *MAX1*

As earlier mentioned the main gene in converting CL into CLA in cytoplasm is *MAX1* in *Arabidopsis*. In a mutant-based experiment, it has been shown that *MAX1* is crucial for SL biosynthesis pathway. In *Arabidopsis*, *MAX1* is classified as CYP711A1 (a single-member family) (Booker et al., 2005).

max1 Arabidopsis mutants created a change in their structures. The different morphological structures of the mutants with the increased number of lateral inflorescences lie in SL deficiency because without *MAX1* gene and its encoded protein (CYP450), CL or its methyl ester, methyl carlactonoate (MeCLA), are not converted into SL to suppress lateral branches. *MAX1*-encoded CYP450 is a

monooxygenase which is responsible for the oxidation of CL (Abe et al., 2014). *MAX1*, a functional gene involved in SLs biosynthesis pathway, has been identified in model plants thus far like *Arabidopsis*, rice, and pea. However, research groups are attempting to identify the orthologous genes to *MAX1* encoding CYP450 in different plants (anonymous, 2011).

By recognizing the probable homologous gene in sugar beet (*Beta vulgaris*) as a non-model plant, beneficial knowledge can be acquired which can be exploited for selection, breeding, and manipulation of sugar beet genotypes. Therefore, it was aimed to blast the genome of sugar beet to see whether there is putative *MAX1* homologous gene or not and next to check if the given gene is involved in SL biosynthesis or not.

1.2 Sugar Beet

1.2.1 Sugar beet overview

Beta vulgaris, a herbaceous and an allogamous dicotyledon (Smigocki et al., 2008), is one of the four major groups of Amaranthaceae family which are leaf beet, garden beet, fodder beet, and sugar beet plants. Sugar beet is classified as *Beta vulgaris* L. ssp. *vulgaris* (Lange et al., 1999) (Table 1.1). Sugar beet is a biennial plant that is a rosette of leaves in the first year while its root expands through sucrose accumulation in the second year. In other words, it has two phases: 1) a vegetative phase and 2) a reproductive phase. In the reproductive phase, an aerial stem is produced with flowering branches (Smigocki et al., 2008). Vernalisation is unavoidable for flowering in the course of the second year in the reproductive phase (Milford, 2006; Winner et al., 1993). The flowers consist of a tricarpellate pistil surrounded by five stamens and five narrow sepals (Smigocki et al., 2008). Sugar beet can be cultured in many different areas; however, between 30° and 60° North latitude is the best for its commercial production. In addition, it can be grown in different soil types even in soils with high salinity due to being a halophyte (Draycott and Christenson, 2003).

Table 1.1. The genus Beta taxonomy (Ford-Lloyd, 2005; Letschert, 1993).

	Genus *beta*		
	Section *Beta* (syn. *Vulgares* Ulbrich)		
2*x*, 3*x*, 4*x*[a]	*Beta vulgaris* L.	ssp. *vulgaris*	Leaf beet group[b]
		ssp. *maritima* (L.) Arcang.	Garden beet group[c]
		ssp. *adanensis* (Pamuk.) Ford-Ll. et Will.	Fodder beet group[d]
			Sugar beet group
2*x*, 4*x*	*Beta macrocarpa* Guss.		
2*x*	*Beta patula* Ait		
	Section *Corollinae* Ulbrich		
2*x*, 4*x*	*Beta lomatogona* Fisc. et May.		
2*x*	*Beta macrorhiza* Stev.		
4*x*	*Beta corolliflora* Zos. ex Buttler		
4*x*	*Beta intermedia* Bunge		
4*x*, 6*x*	*Beta trigyna* Waldst. et Kit.		
	Section *Nanae* Ulbrich		
2*x*	*Beta nana* Boiss. et Heldr.		
	Section *Procumbentes* Ulbrich (syn. *Patellares* Tranzschel)		
2*x*	*Beta procumbens* Sm.		
2*x*	*Beta webbiana* Moq.		
4*x*	*Beta patellaris* Moq.		

[a]Number of chromosomes ($2x = 18$; $3x = 27$; $4x = 36$; $6x = 72$).
[b]Also named Mangold, Spinach beet, Chard, Swiss chard etc.
[c]Also named red beet.
[d]Also named forage beet.

Up to 1740s, the only source for the production of sugar was sugar cane (Alamzan et al., 1998). During that particular time, a German chemist by extracting the sugar from *B. vulgaris* and understanding that it is the exact material existed in sugar cane; sucrose provided a valuable window into sugar production era (Cooke and Scott, 1993). Large scale production of sugar beet became possible about 50 years later. Plant breeders tried to select better varieties thereafter. Sugar beet production has seen the continuation of selection and breeding programes for sugar content, disease and pest resistance, yield, and nutrition (Stevanato and Panella, 2013).

Sugar beet is one of two important sucrose-producing plants. Along with sucrose, pulp and molasses are sugar beet by-products that are extensively used as livestock feed. Except for sugar and animal food source, many co-products can be

provided by sugar beet, e.g., pharmaceuticals, biofuels, plastics (Finkenstadt, 2013). Therefore, the quality of sugar beet is a matter of importance to both farmers and industrialists. Because sugar beet is clearly of the utmost importance, it was selected as plant material in this project.

For a better understanding of central and crucial importance of sugar beet, its products and co-products are summarized in a schematic chart (Figure 1.7).

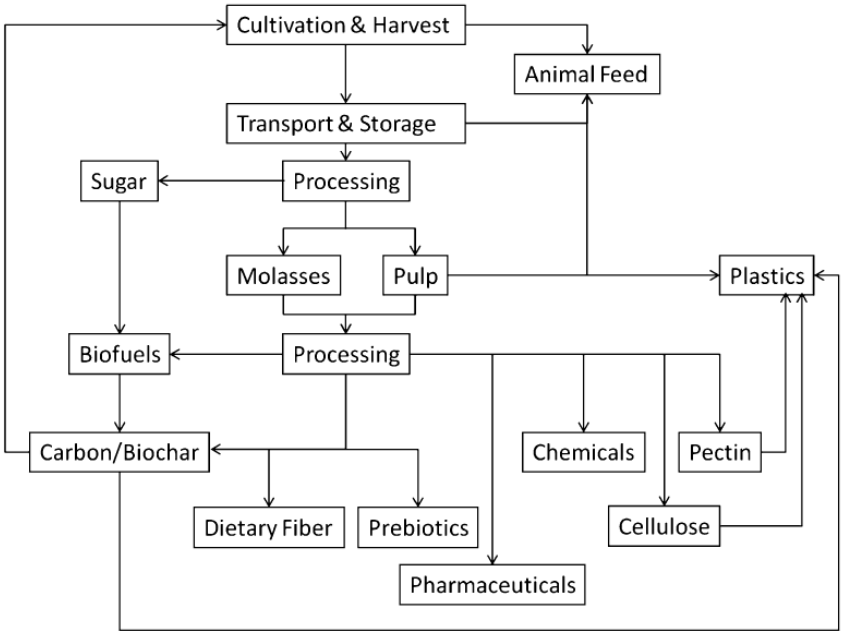


Figure 1.7. Schematic of complete utilization of Sugarbeet (Finkenstadt, 2013).

The figure below shows the increasing economic value of sugar which could imply the growing prominence of sugar beet (Figure 1.8).

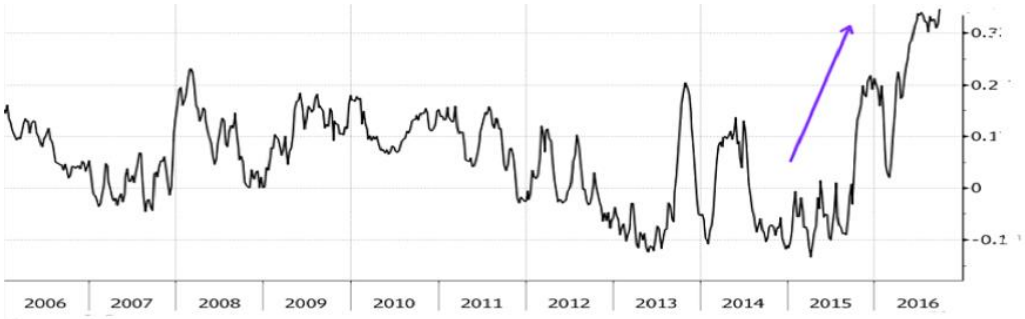


Figure 1.8. Sugar price development (in US cent/lb). Source: CFTC (Commodity Futures Trading Commission), Bloomberg, 2017 (Maitah and Smutka, 2018).

1.2.2 Sugar beet in Turkey

Sugar beet is a very important crop for Turkey's industry and economy. Sugar beet production is the second most agricultural produce after wheat in Turkey (FAOSTAT, 2016a). Turkey, after France, Germany, Poland, the United States, and Russia is the fifth largest producer of sugar beet in the world. Sugar is one of the most important commercial products in Turkey with a significant contribution to the Turkish economy (Akcay and Uzunoz, 2006).

This crop mostly is produced in the central Anatolian region, where has the lowest amount of precipitation as well, which subjects agricultural plants to drought stress and subsequenly salinity stress (Sensoy et al., 2008).

Based on the significance of sugar beet (production, yield, and resistance) and the expected tolerance to increasing stress conditions in Turkey, it was selected as a non-model plant of the thesis in the hope that the obtained results could provide valuable information.

1.3 Abiotic Stress

The general perception about stress was shaped by Selye (1936), in which unfavorable agents could place stress on plants and provoke plant responses. Later, “environmentally unfavorable conditions for plants” was defined as stress (Levitt, 1980). Today, it is a widely-held view that any conditions that adversely influences plant growth, development, productivity, and survival is considered as stress (Rhodes and Nadolska-Orczyk, 2001).

By taking an overall look at the term stress, it can be classified into three categories as follows:

1. Biotic stress e.g. pathogens, insects, fungi, and vira.
2. Natural abiotic stress e.g. drought, low temperature, salinity, and heat stresses.

3. Unnatural abiotic stress (anthropogenic stress) e.g. climate change, air pollution, and acid rain.

The mentioned stresses are environmental constraints. They as external signals set off a series of actions which include signal perception and signal transduction. Signal transduction can be in two ways: first, gene expression and second metabolic responses.

One of the metabolic responses is synthesis, and transport of stress hormones (either internal or external ones). They can mitigate the stress' detrimental effects on plants (Lichtenthaler, 1998). It has been revealed that the main phytohormones e.g. gibberellins (GAs), cytokinins (CKs), abscisic acid (ABA), auxin, salicylic acid (SA), etc. serve a role in regulating stress responses of plants (Kohli et al. 2013). For instance, ABA has a significant role in mediating plant responses to abiotic stress e.g., salinity, heat, cold, and drought stress (Zhang et al., 2006). Another example is a recently-demonstrated role of indole-3-acetic acid (IAA) in ameliorating abiotic stresses (Zörb et al. 2013), and recently-reported function of CKs in inducing defense responses to both biotic and abiotic stresses (Choi et al., 2010; O'Brien and Benkova, 2013). Additionally, it has been shown other well-known plant hormones like abscisic acid, ethylene, and jasmonate can have regulartory function in plant responses to environmental conditions, e.g. abiotic stresses (Pandey, 2017).

Thus far, it has extensively been tried to study and understand the effects of the main phytohormones on inducing proper responses in plants to different environmental conditions such as abiotic stresses (Kaya et al., 2009). However, SL roles in stress-mediated signaling have much more to discover. Therefore, it was decided to assess the effects of SL as a newly-discovered hormone on sugar beet responses to abiotic stresses i.e. salinity and drought and to reveal that this group of hormones can help plants to cope with stress-related strains or not. Among the identified and synthetic SLs, three of them were selected. Firstly, it was considered that using only one SL hormone may not fully and certainly clarify the above-explained assessment. On the other hands to assuredly elucidate the effects of SLs on drought and salinity-exposed plants, one synthetic analog of SLs, *rac*-GR24, and two naturally occurring SLs, strigol and 5-deoxystrigol were chosen.

Among all abiotic stresses, drought and salinity were selected because they are geographically widespread. Both stresses adversely affect plant growth, development, and reproduction. Concerning the broad spread of affected regions and damaging impacts of salinity and drought stresses, they are classified as major environmental stresses, which is the reason behind choosing them for this thesis study. It is expected that estimating SLs effects on plants under salinity and drought stresses could result in providing basic information for applied sciences.

1.4 Tissue Culture

Plant tissue culture is one of the fundamental techniques in plant sciences that facilitates plant production, as well as propagation, and preservation. It contributes towards fundamental and applied sciences along with commercial applications. In brief, tissue culture is generally the production of new plants from tissues, organs, or cells of an intact plant in a proper growth medium. A growth medium generally contains inorganic nutrients (both macro- and micro-nutrients), an energy source like carbon, a reduced nitrogen source, some vitamins e.g. thiamin (B_1) and (pyridoxine) B_6, and phytohormones. Exogenous phytohormones can directly/indirectly affect growth and development of explants in a cultured medium. Cytokinins and auxin can be pointed up in *in vitro* explant morphogenesis. Their ratio is mainly considered paramount in the growth and development of tissues/organs cultured in a medium (Gaspar et al., 1996). For example, when the ratio of cytokinin to auxin is high, it leads to shoot induction and vice versa (low ratio results in root induction). The other phytohormones like gibberellic acid and abscisic acid can play roles in the modulation of plant responses (Bhojwani, 1990). In other words, the exogenous application of other classes of hormones besides cytokinins and auxin and hormone-like compounds provides a culture medium with regulatory roles. They make a significant impact via interaction with cytokinins and auxin on development, differentiation, and growth of explants particularly in recalcitrant ones such as sugar beet. However, their functions vary according to genotype, explant, and medium composition. Later, new natural growth substances e.g. brassinosteroids, polyamines, jasmonates, and salicylic acid were identified. Although the effects of many of the mentioned hormones have been examined and principally identified, however, their convoluted interaction need to be

elucidated (Gaspar et al., 1996). In recent years, many searching questions have been addressed by introducing a new class of phytohormones, SLs. One series of them is related to their effects on explant morphology in *in vitro* condition. This part of the study was designed because of a question mark over the impacts of SLs on *in vitro* shooting and rooting of sugar beet which had two distinct differences with plant materials of other research works; first, it was a non-model plant and second, it was a recalcitrant species.

1.5 Seed Germination

Seeds as enclosed packages carry energy and information for angiosperms and gymnosperms. They contain an inactive embryo and essential materials. All the mentioned contents of seeds are required for germination, morphogenesis, growth, and emergence of a new plant. Seed germination is a truly remarkable process in which consecutive chemical, physical, and morphological alternations transform an embryo into a new-emerged seedling. The germination process can be summarized as follows:

1. Imbibition; the process of physical absorption of water,
2. Hydration, and subsequently activation,
3. Cell division, and extension,
4. Radicle protrusion,
5. Completion of morphogenesis and formation of the primary plant body (Berlyn, 1972).

Therefore, germination as the initial step of plant establishment is of prime importance that cannot be disregarded. Germination is controlled by some factors. One of them is “environmental conditions” in which seeds can germinate directly or after breaking of dormancy. The other influential factor is “plant hormones” at both endogenous and exogenous (produced by the plant itself or bacteria of soil) levels. They variously affect processes involved in germination e.g. division, growth, and differentiation of seed cells, seed dormancy, and seed development (Miransari and Smith, 2014). Some, such as gibberellins, can act as a germination enhancer and some, like ABA, as a germination inhibitor. It was indicated that 1–10 μM ABA in plants like *Arabidopsis thaliana* can restrain seeds from germination (Kucera et al., 2005).

Gibberellins can act as seed germination stimulator through some processes e.g. gene induction required for the production of amylases (such as proteases and α-amylase), endosperm weakening, and embryo expansion (Yamaguchi, 2008).

Plant hormones interactively act in which production of one hormone may be regulated by the other hormone. Besides alternation in both proteins (converting by enzymes) and plant hormones, hormonal balance (e.g. between gibberellins and ABA) is a requisite for germination. There are, in addition to gibberellins (Seo et al., 2009), the other plant hormones e.g. cytokinins, ethylene, and brassinosteroids, that can act antagonist to ABA and stimulate seed germination (Hermann et al., 2007). The role of major phytohormones in germination and dormancy processes has been determined. Even though the early role of SLs refers to the stimulation of parasitic seeds, however, there are not any specific studies to examine the effects of SLs on germination of non-parasitic seeds like sugar beet seeds to clarify whether there are any effects or not. If so, are they adverse or positive effects? Thus, in this study it was designed to assess the effects of SLs on the germination of sugar beet seeds.

1.6 The Relationship Between SLs and Auxin

It has been showed that hormone-induced responses are not solely related to individual contributions of each plant hormone. Thus, one of the crucial points in understanding plant hormone roles in mediating environmentally induced responses is their crosstalk. The crosstalk between plant hormones (either positive or negative) is fundamental in plant responses (Verma et al., 2016).

Shoot branching has two key steps; 1) axillary meristem formation 2) axillary meristem development comprising initiation and outgrowth. One of the factors owning a critical role in the control of bud outgrowth is hormonal signals.

SLs and auxin, two systemic hormones, can play a role in shoot branching by inhibition of lateral buds (Domagalska and Leyser, 2011). In addition, it is indicated that SL and auxin distribution and level for axillary bud inhibition can be regulated by their feedback loop (Hayward et al., 2009).

Auxin after being produced in stem apex inhibits bud outgrowth through polar auxin transport stream (PATS) (Wisniewska et al 2006). It has been assumed that the other hormones as second messengers of auxin are involved in directly regulating bud activity because it seems a very small amount of auxin that enters the buds is inadequate for direct inhibition (Booker et al., 2003). SLs can be of possible messengers of auxin contributing towards bud inhibition. The hypothesis is that SLs may have direct or indirect inhibitory effects on bud outgrowth and subsequently shoot branching. The direct effect can be through up-regulation of bud specific gene BRANCHED1 (BRC1), and down-regulation of ABCB19 and PIN1 as auxin transport genes. The indirect effects can be exerted by a reduction in auxin canalization from axillary buds and reduction in auxin transport in the main stem.

The relationship between auxin and SLs are of probing questions, thus, it was decided to outline a plan for assessing their interplay. Different combination of hormones (*rac*-GR24 and IAA) and hormone inhibitors (TIS108 and TIBA) was selected to study their interaction.

1.7 Aim and Scope of the Study

This thesis research was defined to achieve the following main objectives;

(i) to evaluate the role of an ortholog of MORE AXILLARY GROWTH1 gene (*MAX1*) in biosynthesis/signaling of SLs in sugar beet and to identify/introduce the corresponding responsible gene for the first time in sugar beet,

(ii) To understand SLs' morphological effects on tissue culture of sugar beet from shooting to rooting stages,

(iii) To understand SLs' effects on germination of sugar beet seeds as the seeds of a non-parasitic plant,

(iv) To investigate the interplay of SLs and auxin as a hormonal interaction,

(v) To estimate how SLs could contribute to the alleviation of the adverse effects of abiotic stress conditions on sugar beet plants.

Therefore, it is hoped that such findings will contribute to agricultural research and breeding activities aiming at the development of high-yield and -quality sugar beet varieties, preferably with tolerance to abiotic stress factors.

CHAPTER II

2 Evaluation of a Putative *MAX1* Gene in Sugar Beet

2.1 Introduction

Strigolactones are a new class of plant hormones. The phytohormones recently have been the center of attention for many research topics. Preliminary studies on the effects of strigolactones on model plants have suggested that it has an important role in plant shooting, root growth, and stress tolerance. Therefore, this study was conducted to evaluate the effects of the phytohormones on sugar beet. This part includes investigating the effects of three types of strigolactones on the expression of a proposed homolog gene of *MAX1* in sugar beet for the first time to clarify/prove its assumed function in SL biosynthesis pathway. *MAX1* has a central role in the biosynthesis of SLs. Therefore, finding its homologous gene in different plants beside model plants can provide valuable information for genetic, breeding, and physiological studies.

This chapter of the thesis, reports the results of a method evaluating the effects of strigolactone hormones (*rac*-GR24, (±)-strigol and (±)-5-deoxystrigol) and one SL inhibitor (TIS108) on the expression level of a putative *MAX1* gene in sugar beet:

1. Spraying plantlets in the pots with 2.5, 5 and 7.5 µM *rac*-GR24, (±)-strigol, (±)-5-deoxystrigol and TIS108 as treatments in comparison with control
2. RNA isolation
3. cDNA synthesis
4. qPCR

This part of the study aims to evaluate whether a putative gene in sugar beet that is homolog to *MAX1* gene in *Arabidopsis* is one of the responsible genes for

biosynthesis pathway of SLs or not. It was tried to check the transcription level of the putative gene after applying different SL hormones and one SL inhibitor.

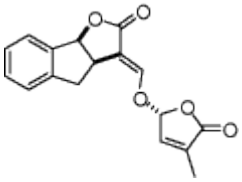
2.2 Materials and Methods

2.2.1 Plant material

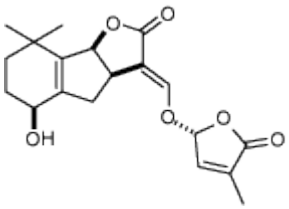
A commercial variety of sugar beet, cv. Serenada, from KWS Company (Germany) was used in this study.

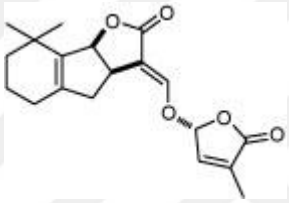
2.2.2 Hormone information

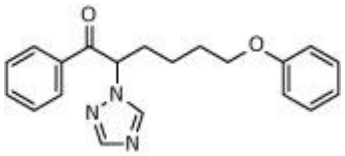
The applied chemicals are:

Hormone Name	*rac*-GR24
Chemical Name	(3aR*,8bS*,E)-3-(((R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one
Structure	
Molecular Formula	$C_{17}H_{14}O_5$
Molecular Weight	298.29
Purity	>98%

Hormone Name	(±)-strigol
Chemical Name	(3aR*,5S*,8bS*,2'R*)-3-[(E)-2',5'-dihydro-4'-methyl-5'-oxo-2'-furanyloxymethylene]-5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-b]furan-2-one

Structure	
Molecular Formula	$C_{19}H_{22}O_6$
Molecular Weight	346.37
Purity	>98%

Hormone Name	(±)-5-deoxystrigol
Chemical Name	(3aR*,8bS*,E)-8,8-dimethyl-3-((((R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)-3,3a,4,5,6,7,8,8b-octahydro-2H-indeno[1,2-b]furan-2-one
Structure	
Molecular Formula	$C_{19}H_{22}O_5$
Molecular Weight	330.37
Purity	>97%

Inhibitor Name	TIS108
Chemical Name	6-phenoxy-1-phenyl-2-(1H-1,2,4-triazol-1-yl)hexan-1-one
Structure	
Molecular Formula	$C_{20}H_{21}N_3O_2$
Molecular Weight	335.40
Purity	>98%

2.2.3 The gene of interest

It was aimed to study a putative gene in sugar beet that was postulated to be involved in SLs biosynthesis pathway and has effects on the phenotype of the plant. This speculation is based on *MAX1* (in cDNA and protein levels) in *Arabidopsis*. For achieving this purpose, *MAX1* gene, cDNA (AT2G26170.1), and protein sequence of *Arabidopsis* were found in www.arabidopsis.org. Then, the cDNA and protein of *MAX1* were blasted on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find similar cDNA and protein in sugar beet. The role of selected putative cDNA and protein from sugar beet genome is classified as "a proposed gene" and has not been still confirmed whether they have the same roles or not.

Arabidopsis thaliana MAX1 gene information from TAIR database (https://www.arabidopsis.org/index.jsp):

GenBank Accession: NM_128175

Arabidopsis thaliana cytochrome P450, family 711, subfamily A, polypeptide 1 (CYP711A1), mRNA

NCBI Reference Sequence: NM_128175.3

The results from BLAST of *MAX1* gene with sugar beet transcriptome:

The blasted mRNA identity in sugar beet: "PREDICTED: *Beta vulgaris* subsp. vulgaris cytochrome P450 711A1 (LOC104886613), mRNA".

Gene ID: 104886613

cDNA Reference Sequence: XM_010671076.1

Protein RefSeq: XP_010669378.1

Identities: 80%

Gaps: 2%

Number of Matches: 1

2.2.4 Primer designing

To find the best primer sets, some different primer pairs were designed to clone the desired gene's (*MAX1* homolog in sugar beet) complementary DNA (cDNA). In addition, some sets of primer pairs based on a reference gene in sugar beet were designed as an internal control to quantify the expression of *MAX1* homolog gene by quantitative real-time polymerase chain reaction (qPCR), and detecting fluorescence of ethidium bromide intercalating PCR product amplicons in an agarose electrophoresis gel.

By using NCBI primer designing tool, it was tried to design and select the best available primer pairs. Three types of primers were designed for the gene of interest in transcription level (Table 2.1). The primer pairs' clone a sequence on a single exon, two exons with a middle intron, or two exons with while spanning an exon and partially another exon as an "exon-exon junction" (Figure 2.1).

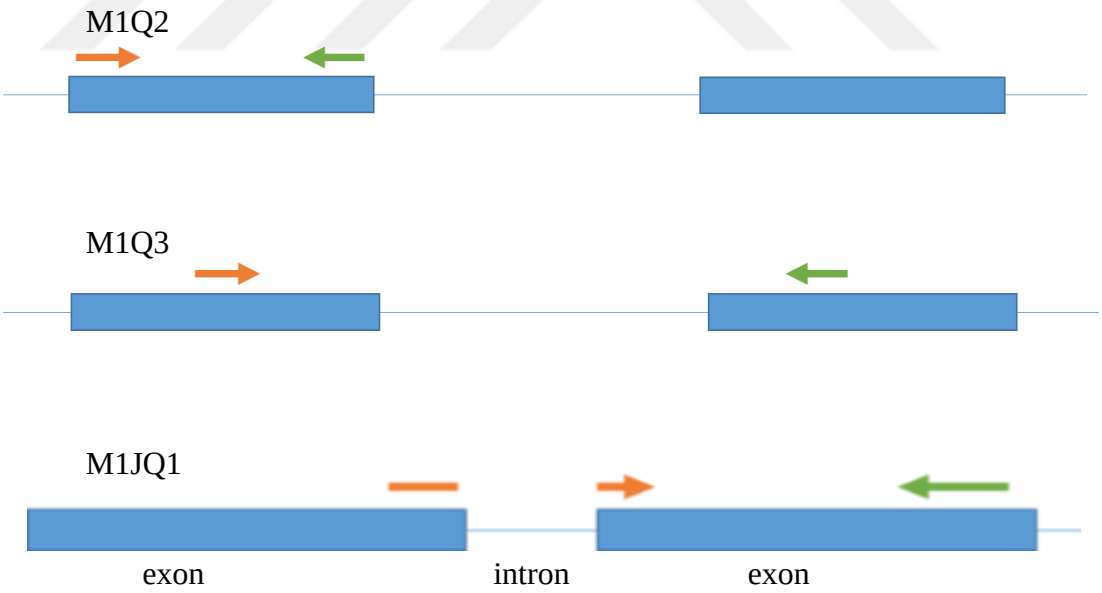


Figure 2.1. The first designed primer pairs are complementary to a single exon sequence (M1Q2) and only clone it. The second designed primer (M1Q3) with "intron inclusion" and the third one (M1JQ1) with "exon-exon junction" are efficient and specific. Such a design is useful for limiting the amplification specificity to mRNA.

Table 2.1. The sequence and the length of three types of primer pairs designed to clone a part of XM_010671076.1 transcript in sugar beet.

M1JQ1	Sequence (5'->3')	Length (bp)
Forward	CTTCACCAGGGATTCAAGGT	20
Reverse	TGGTGGGTAGTAAACTGGCT	20
Product		89

M1Q2	Sequence (5'->3')	Length (bp)
Forward	GATCCAGAGCTATGCAGAGAAG	22
Reverse	TGGTGTAGGTGTGAAGCATAAA	22
Product		95

M1Q3	Sequence (5'->3')	Length (bp)
Forward	CAGCAGTTGAGATAGGAGGTTAC	23
Reverse	TCGGGTTCTGGAAAGTTCTTC	21
Product		100

As a reference gene to compare with the desired gene and normalize the expression level, primers were designed to clone actin gene of sugar beet (Table 2.2).

NCBI database: *Beta vulgaris* β-actin (ACT1) mRNA, GenBank: DQ866829.1.

Table 2.2. The sequence and the length of two sets of primer pairs designed to clone a part of *Beta vulgaris* β-actin (ACT1) transcript in sugar beet.

AC1	Sequence (5'->3')	Length (bp)
Forward	CCCACTGAATCCCAAGGC	18
Reverse	TTTCCCGTTCGGCTGATG	18
Product		299

AC2	Sequence (5'->3')	Length (bp)
Forward	TCAATGTGCCTGCTATGTATGT	22
Reverse	GTGACTAACACCATCACCAGAG	22
Product		104

2.2.5 Preparation of required solutions and stocks for molecular study

2.2.5.1 Stock preparation of SL hormones and SL inhibitor (*rac*-GR24, St, dSt and TIS)

The stocks of SL hormones and SL inhibitor (10 µM) were prepared. Considering the molecular weight, the proper amount of pure acetone was added to their powders. The powders immediately dissolved in the solvent. The stocks were kept at −20 °C until use.

2.2.5.2 Preparation of Tween-20 Solution Stock

One droplet of tween was added to 1 ml distilled water, vortexed and kept in room temperature for use. One hundred µl of tween 20 (diluted from the stock in the ratio of 1:100) was added per 10 ml of the solution.

2.2.5.3 Polyethylene glycol (PEG) 4000 (1%, w/w)

1 g PEG 4000 was dissolved in 100 ml water.

2.2.5.4 Preparation of 0.5 M EDTA

To dissolve EDTA, sodium hydroxide (NaOH) stock was made by dissolving 20 g NaOH in 50 ml distilled water.

14.61 g EDTA was added into 100 ml milli-Q water. Then it was dissolved at pH = 8 while it was titrating by NaOH.

2.2.5.5 Ethidium bromide stock

For ethidium bromide stock, 5 mg ethidium bromide powder was weighted and then 1000 µl ultra-pure water (Thermo Scientific™ Barnstead™ Smart2Pure™ Water Purification System) was added to dissolve. The tube containing the stock was covered by aluminum foil to prevent light. After vortexing, it was kept in 4 °C.

2.2.5.6 10× TBE buffer

- 54 g Tris-base
- 27.5 g boric acid
- 20 ml (0.5 M) EDTA (pH = 8)
- 500 ml milli-Q water

After adding EDTA to the weighed Tris-base plus boric acid, about 100 ml milli-Q water was poured on them. pH of the solution was checked. In this step, the pH should be 8.3. After checking the pH, volume of the solution was reached to 500 ml with milli-Q water to have a 10× buffer.

2.2.5.7 Preparation of 1 M Tris-HCl

Concerning the molecular weight for preparation of 1 M Tris-HCI, 3.94 g of its powder was dissolved in 25 ml milli-Q water.

2.2.5.8 Preparation of 1× TE buffer

1× TE buffer	Amount (100 ml)	Final conc.
1 M Tris-HCl, pH: 7.5	1 ml	10 mM
0.5 M EDTA, pH: 8	200 µl	1 mM
Milli-Q water	98.8 ml	

2.2.5.9 Ladder dilution (50 bp, ready-to-load ladder, NEB #B7025)

Appropriate amount of TE buffer was added to the ladder. For 10 use, 5 µl TE buffer was added to 5 µl 50 bp ladder.

2.2.5.10 Dilution of primers

100 µM Stock tubes:

- Centrifuged for 1 min at 15000 g
- Appropriate amount of DNase/RNase-Free water was added to each tube
- Vortexed for about 1 min
- Short centrifuged at 1000 g

Then 10 µM of the stock was prepared:

- 5 µl of 100 µM stock plus 45 µl of DNase/RNase-Free water

2.2.6 Pre-experiment for evaluation of different combinations of foliar application

For foliar application of the hormonal treatments, as a preliminary experiment, six combinations of solutions were sprayed on candidate plants to examine the solutions effects' on shoots. The effects of Tween 20 and PEG 4000, as additives to the solutions, on plant shoots were examined. The additives were used to increase penetration and cohesion of the hormonal treatments. Therefore, the additives were sprayed without the hormones to observe their probable advantages and disadvantages over sugar beet leaves. The applied solutions were:

1) 10 ml of distilled-water (DW) without any additive: W
2) 10 ml of DW with 100 µl of Tween 20 (diluted 1:10): T
3) 10 ml of DW with polyethylene glycol (PEG) 4000 (1%, w/w): P
4) 10 ml of DW with 100 µl of Tween-20 (diluted 1:10) + PEG 4000 (1%): PT

5) 10 ml of DW with 100 µl of Tween 20 (diluted 1:100): Td
6) 10 ml of DW with 100 µl of Tween 20 (diluted 1:100) + PEG 4000 (1%): PTd

2.2.7 Evaluation of gene expression of *MAX1* homolog by designed primers in untreated sugar beet as a pre-experiment

In this part, the aim was to examine whether our putative gene (which is in sugar beet genome) can be expressed or not. As a preliminary experiment, RNA isolation using TRIzol (NucleoZOL-Macherey Nagel) was conducted as follows:

2.2.7.1 RNA isolation with TRIzol

- Untreated leaf tissue of sugar beet was ground with liquid nitrogen using a mortar and pestle
- 50 mg powder was transferred to a DNase/RNase-Free 2 ml tube
- 500 µl NucleoZOL (TRIzol) was added to the tube
- Vortexed vigorously for 5 min
- 200 µl DNase/RNase-Free water was added (for 500 µl TRIzol)
- Shacked vigorously for 1 min
- Incubated at room temperature for 15 min
- Centrifuged for 15 min at 12000 ×g – room temperature
- 500 µl supernatant was transferred to a new DNase/RNase-Free tube
- 500 µl isopropanol (100%) was added to the tube
- Tubes were mixed by inverting 10 times
- Incubated for 10 min at room temperature
- Centrifuged for 10 min at 12000 g
- Supernatant was discarded and white pellet was kept
- 500 µl of 75% ethanol was added
- Centrifuged for 3 min at 8000 g
- Ethanol was removed using pipette
- 500 µl of 75% ethanol was added

- Again centrifuged for 3 min at 8000 g
- Ethanol was removed by pipette
- White pellet was dissolved in 60 µl DNase/RNase-Free water to get 1 µg/µl

2.2.7.2 RNA concentration

To determine RNA concentration, 1 µl of the dissolved pellet was diluted with 99 µl DNase/RNase-Free water.

OD260 was recorded (Hitachi U-1900, UV-VIS Spectrophotometer, Tokyo, Japan). Then the below formula was used to estimate total RNA concentration:

Conc. = OD260 × 40 × Dilution = RNA ng/µl

2.2.7.3 cDNA synthesis

For the cDNA synthesis, all components of the kit (NEB first-strand synthesis kit) and PCR tubes were put on ice (Table 2.3). The process was as follows:

Table 2.3. cDNA synthesis components and amounts.

Components	Amounts
Total RNA (X)*	3 µl
dT23VN (50 µM)	2 µl
Nuclease-free H2O (X)	3 µl
Total Volume	8 µl**

*The amount of RNA is chosen based on its concentration.
**Total volume is recommended to be 8 µl.

After adding the components as written above:

- RNA was denatured for 5 min at 70 °C
- Spun briefly
- Put immediately on ice
- 10 µl M-MulV reaction mix and 2 µl enzyme mix were added to the tube
- Spun briefly

Then the tube containing 20 µl reaction was placed in a PCR Thermocycler (XP Cycler, Bioer Technology, Hangzhou, China) according to Table 2.4:

Table 2.4. PCR cycling conditions.

Temperature	Duration
42 °C	60 min
80 °C	5 min

After cDNA synthesis, 30 µl DNase/RNase-Free water was added to the tube to have 50 µl reaction mixture for PCR. It was kept at −20 °C.

2.2.7.4 PCR performing

After dilution of cDNA for PCR, the PCR method was done in the same thermocycler (XP Cycler, Bioer Technology, China) as follows (Table 2.5 and 2.6).

Table 2.5. Components, volume, and final concentration of PCR for cDNA synthesis.

Components	Volume	Final concentration
Taq reaction buffer	5 µl	1X
dNTP	1 µl	10 mM
F-primer*	1 µl	10 µM
R-primer*	1 µl	10 µM
DNA template	1 µl	1,000 ng
Taq polymerase	0.25 µl	1.25 units/50 µl PCR
DNase/RNase-Free water	40.75 µl	--------

*PCR was performed with designed primers separately (M1Q2, M1Q3 and, AC1 plus M1JQ1).

Table 2.6. Components, temperature, and duration of PCR for cDNA synthesis.

Components		Temperature	Duration
Initial denaturation		95 °C	2 min
C1	Denaturation	95 °C	30 Sec
C2	Annealing (C)	X °C**	30 Sec
C3	Extension	68 °C	1 min
Final extension		72 °C	5 min
Hold		4 °C	

* 35 cycles.
** Changeable according to the primer.

2.2.7.5 Gel electrophoresis

- Agarose gel 3%
- Buffer: TBE
- Voltage: 80
- Time: 145 min
- For each sample, 6 µl was loaded in each well including 5 µl sample and 1 µl dye

2.2.7.6 Gel visualization

After staining with ethidium bromide (30 min), gel was visualized by UV Trans Illuminator (MiniLumi, DNR Bio Imaging System, Neve Yamin, Israel).

2.2.8 SL hormones and SL inhibitor application

2.2.8.1 Seed culturing for spraying SL hormones and SL inhibitor

A commercial variety of sugar beet cv. Serenada, from KWS Company (Germany) was used in this study. Water-soaked seeds for one day were cultured in

pots filled with loamy sand soil (around 400 g soil). For each pot, 6 seeds were placed in the center of soil (2 cm beneath the top of soil). After culturing the seeds, about 100 ml water was added to each pot. Then, all the pots were covered with plastic bags to keep humidity for germination and early seedling growth. Five days after germination, when at least one seed germinated in all the pots, the plastic bags were removed and only one seedling was kept in each pot (the seedlings kept in pots were rather similar in size to guarantee their homogeneity). Therefore, we let the chosen seedlings grow and later, sprayed them with the treatments. Three days after removing the plastic bags, watering was started. Every day 50 ml water was poured in the pot saucers.

2.2.8.2 Spraying SL hormones and SL inhibitor

Ten days after germination, each plantlet was sprayed with 5 ml of the treatments solution as follows (Table 2.7):

Table 2.7. Hormonal treatment concentration, Tween-20 amount, with total volume of 5 ml.

Concentration (µM)	Tween-20 amount (µl) diluted 1:100	Total volume*
0	5	5
2.5	5	5
5	5	5
7.5	5	5

* 5 ml of the aqueous solution of each treatment for each plant.

The plants were sprayed once every two days for seven times like the plan below:

1 - - 2 - - 3 - - 4 - - 5 - - 6 - - 7 - -

For the first, second, and third times of spraying, the amount of 5 ml was enough for each plantlet. However, the volume of the solution for spraying was increased to 10 ml from the fourth time onwards for each plant considering the larger size of the treated plants (Table 2.8 and Figure 2.2).

Table 2.8. Hormonal treatment concentration, Tween-20 amount, with total volume of 10 ml.

Concentration (µM)	Tween-20 amount (µl) diluted 1:100	Total volume*
0	10	10
2.5	10	10
5	10	10
7.5	10	10

* 10 ml of the aqueous solution of each treatment for each plant.

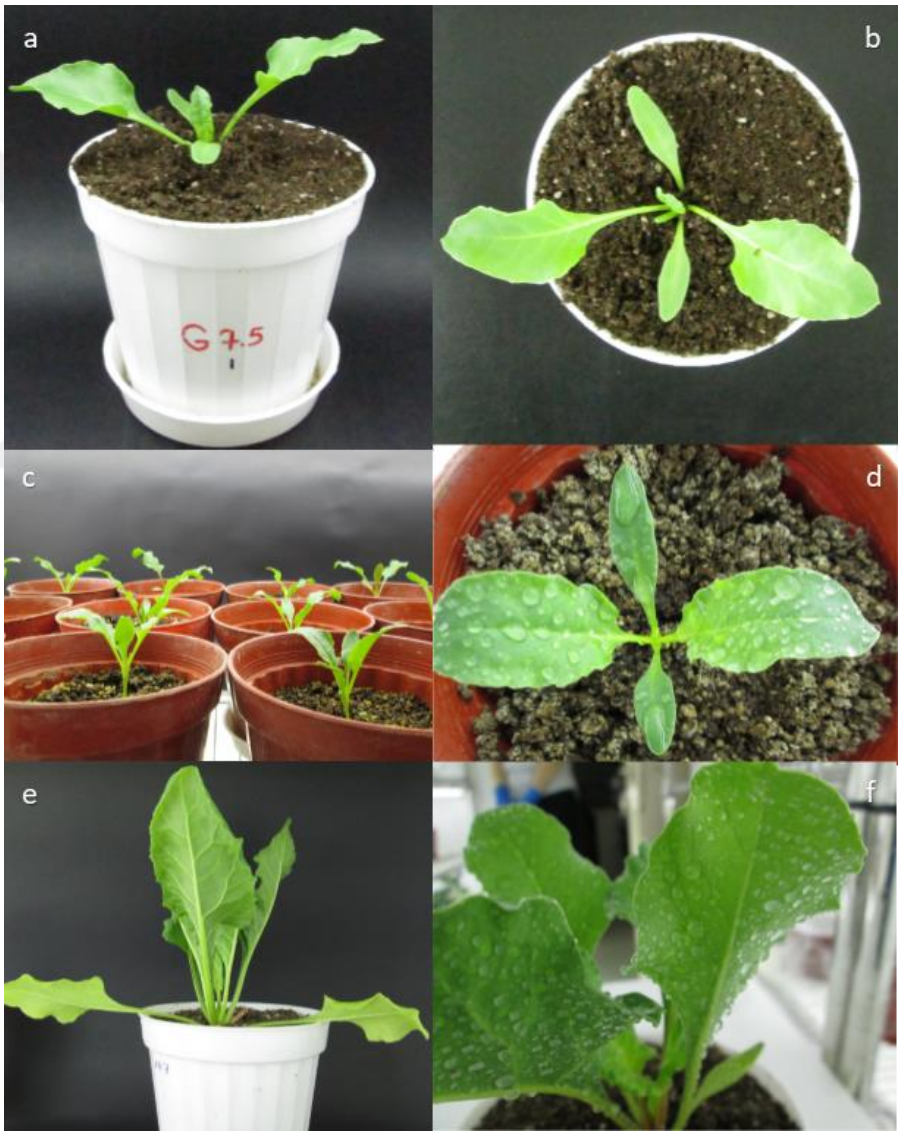


Figure 2.2. Ten days after germination (a, and b), homogeneously grown seedlings (c) were selected to spray (d). The spraying was continued for three weeks, when the plants become larger (e and f).

2.2.8.3 Sampling of SL-treated and TIS-treated plants

At the end of the treatment with SL hormones and SL inhibitor, the leaves were collected, and after snap freezing in liquid nitrogen, they were kept in −80 °C for upcoming experiments.

2.2.9 Molecular studies of a few *rac*-GR24-treated sugar beet plants (gel-based checking as a preliminary experiment before qPCR)

To see and to examine whether there is any difference between different concentrations of *rac*-GR24, it was decided to evaluate the gene expression by PCR and agarose gel electrophoresis in a few *rac*-GR24-treated samples before performing qPCR.

The total RNA was extracted by the method already explained and the sequences using the primer pairs (M1JQ1-AC1, M1JQ1-AC2, and M1Q3-AC2) were cloned. The PCR cloning products were separated after running in a gel electrophoresis, and then visualized.

2.2.9.1 Total RNA integrity

To assess the integrity of total RNA, an aliquot of the RNA samples was run (7.5 μM of four hormones) on a denaturing agarose gel stained with ethidium bromide (EtBr).

2.2.10 Quantitative PCR (qPCR)

For qPCR, BioRad CFX connect Real-Time PCR was used.

2.2.10.1 RNA isolation, cDNA synthesis, q-PCR components, conditions, and expression analysis

The methods for RNA isolation and cDNA synthesis for all the treatments (*rac*-GR24-, dSt-, St-, TIS-treated leaves) and all concentrations (0, 2.5, 5, or 7.5 µM for each chemical) were explained previously. For qPCR, primers M1Q3 (primer for putative gene) and AC2 (reference gene: β-actin) were used (Table 2.9).

Table 2.9. qPCR components and volume

Components	Volume	Concentration
Primer F	1 µl	10 µM
Primer R	1 µl	10 µM
cDNA	4 µl	100 ng
SYBR Green	10 µl	1×
ddH2O	4 µl	—
Total volume	20 µl	—

qPCR was run according to the above mentioned methods and conditions. The schematic running method and condition of the main experiment has been shown in Figure 2.3.

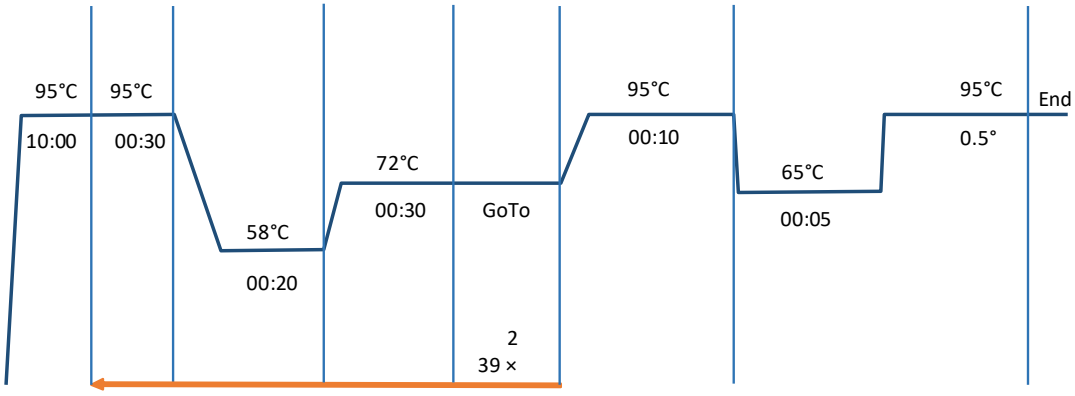


Figure 2.3. The schematic running method and condition of the qPCR experiments.

Quantifying gene expression levels was done according to the method defined by Livak and Schmittgen (2001) using the results obtained from real-time quantitative PCR and the equations for the $2^{-\Delta\Delta C_T}$.

2.2.11 Statistical analysis

The data were analyzed using appropriate methods of analysis. Levene's test of homogeneity of variances and Shapiro–Wilk (S-W) and Lilliefors corrected Kolmogorov–Smirnov (K-S) tests of normality assumptions were used for the data. Wherever appropriate, the data were subjected to one-way analysis of variance (ANOVA), and means were compared using Tukey's test, or Welch's adjusted *F* ratio for one-way ANOVA and Games–Howell post hoc tests were run to examine the differences between groups. Alternatively, the data were analyzed by the Kruskal–Wallis (K-W) test followed by the Bonferroni corrected Dunn's post hoc test or a stepwise step-down multiple comparison post-hoc test, adopted from Campbell and Skillings' method (1985). Comparisons between two independent samples were done with a two-sided Student's *t* test. The level of significance for the analyses was considered $p < 0.05$. A Windows™ based SPSS® program (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and graph drawing.

2.3 Results

2.3.1 Results of pre-experiment for evaluation of different combinations of foliar applicatıon

The leaves under W and Td treatments were healthy. However, T treatment resulted in sparse discoloration spots and malgrowth on the leaves.

The leaves under TP, PTd, and P treatments were unhealthy with large discoloration spots and necrotic margins (Figure 2.4).

Therefore, among applied treatments with healthy results, Td [tween 20 (diluted 1:100)] was used to increase cohesion of the hormones.

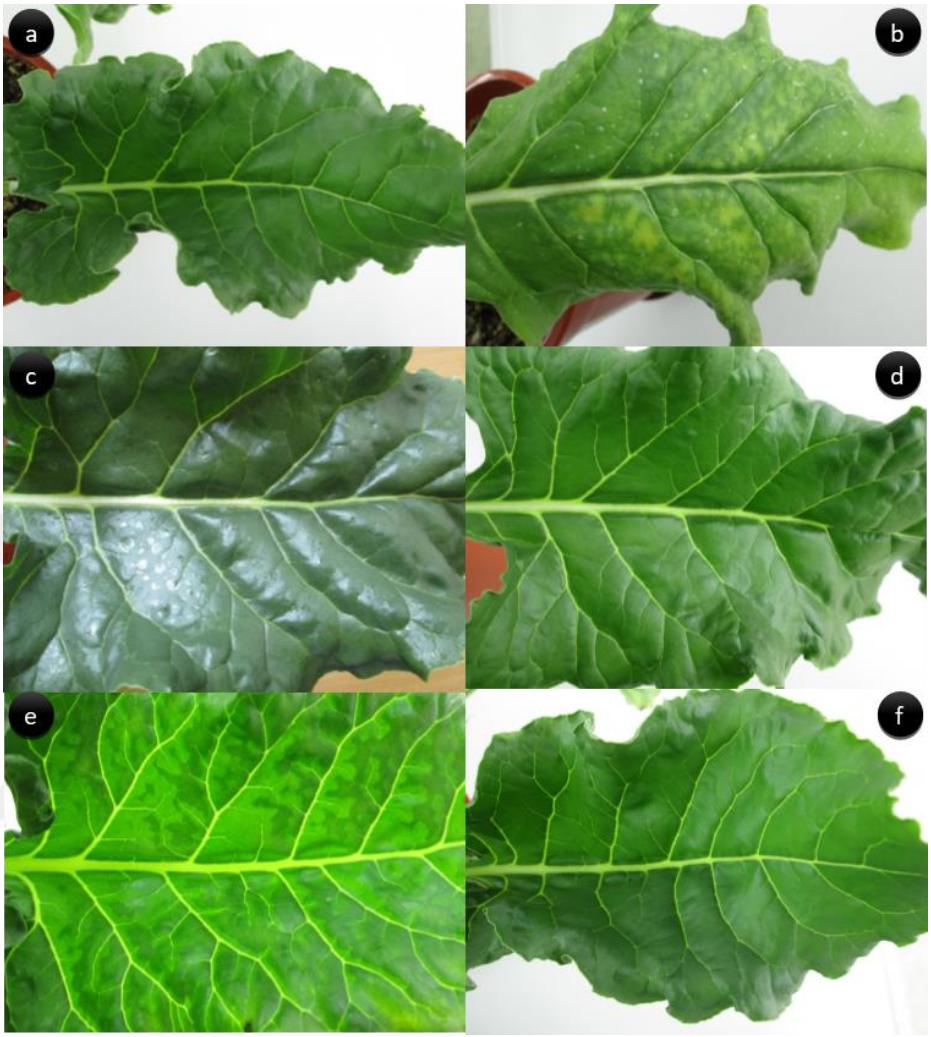


Figure 2.4. In a preliminary experiment, 5 treatments of Ttween-20 and PEG4000 were sprayed on plant leaves once and the effect of the solutions on the leaves was observed after one week. Control (w) (a), PEG4000 (P) (b), Tween-20 and PEG4000 (after 1 day) (PT) (c), Tween-20 (T) (d), diluted Tween-20 and PEG4000 (PTd) (e), and diluted Tween-20 (Td) (f). PEG4000 alone wilted the sprayed leaves and resulted in chlorosis (b). Since Tween-20 and PEG4000 (c) after just one spray covered the leaf surface and clogged stomata, therefore this treatment was not followed up more than one day. Spraying Tween-20 (d) resulted in leaf mal growth, so it could not be recommended. The treatment with diluted Tween-20 and PEG4000 (e) infiltrated into veins and damaged them, which showed that this treatment cannot not be appropriate. Spraying the leaves with diluted Tween-20 did not stimulate any negative reactions (f), thus this treatment was added to the hormonal solutions to facilitate penetrance of the hormones.

2.3.2 Results of evaluation of gene expression of *MAX1* homolog by designed primers in untreated sugar beet as a pre-experiment

The PCR products of the primer pairs indicated that our putative gene is expressed (Figure 2.5).

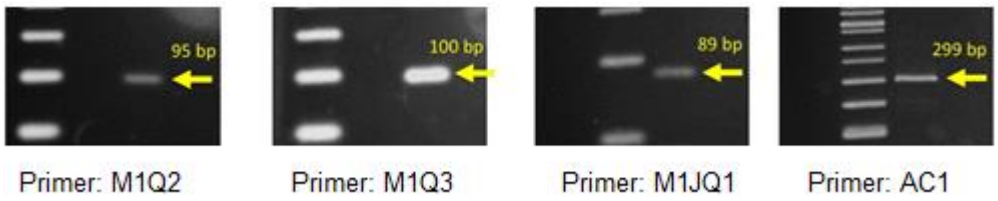


Figure 2.5. The PCR products of the primer pairs.

It was repeatedly proved that our putative gene exists in sugar beet genome, and above all, it can express.

2.3.3 Results of molecular studies of a few strigol-treated sugar beet plants (Gel-based checking as a preliminary experiment before qPCR)

Our searching question was fully answered in all the gels; it was demonstrated that there are differences among the gene bands of sprayed seedlings with different concentrations of strigol as PCR products with above-mentioned primer pairs (Figure 2.6).

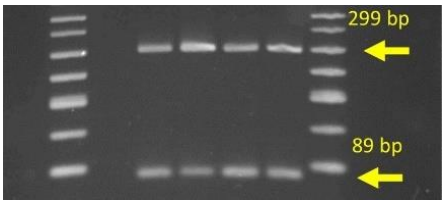


Figure 2.6. Strigol sprayed seedlings gene bands based on M1JQ1 PCR clone product, the band at 89 bp position for *MAX1* homologous gene. The band at 299 bp position came from AC1 primer pairs' amplification (Actin). The wells from left to right: Ladder, empty, control, 2.5 µM, 5 µM, 7.5 µM of hormonal solution treated plant samples, and again the same ladder.

2.3.4 Result of total RNA integrity

Based on sharp and clear 28S and 18S rRNA bands and the ratio of 28S:18S, which is approximately twice in the visualized gel, it was indicated that total RNA was completely intact (Figure 2.7).

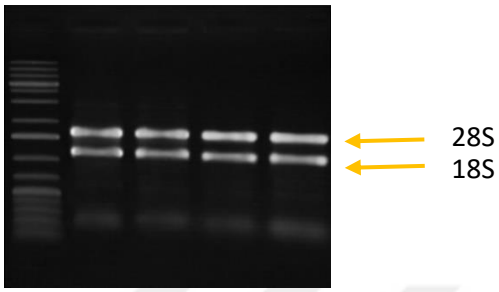


Figure 2.7. Assessment of total RNA integrity by denaturing agarose gel electrophoresis.

2.3.5 Result of Quantitative PCR (qPCR)

2.3.5.1 Result of qPCR performing for *rac*-GR24-treated samples

Rac-GR24, a synthetic SL, was sprayed on sugar beet seedlings. The effect of three different concentrations of *rac*-GR24 was compared with control and each other. *Rac*-GR24 at all the concentrations significantly decreased the expression level of *MAX1* homologous gene in sugar beet. *Rac*-GR24 effect at 2.5 and 5 µM on the expression level was not statistically different. However, the results showed that 7.5 µM of the hormone was statistically significantly different from any other treatments. To be exact, the highest concentration of the hormone suppressed the expression level of the gene at transcription level (Figure 2.8).

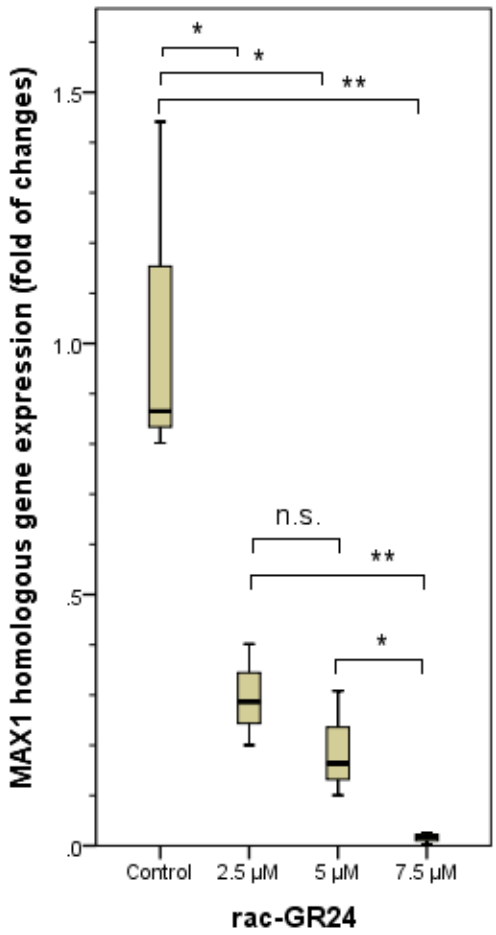


Figure 2.8. The effect of three different concentrations of *rac*-GR24 as compared with control and each other. n.s., *, and **: non-significant, significance at 5 and 0.1% levels, respectively.

2.3.5.2 Result of qPCR performing for St-treated samples

Strigol, a natural-based SL, was sprayed on sugar beet seedlings. The effect of three different concentrations of strigol was compared with control and each other. Spraying strigol at 2.5 µM was not statistically significantly different from control. The effects of 5 and 7.5 µM of strigol on transcription level of the gene (*MAX1* homologous gene) were also statistically similar. However, the effects of control and 2.5 µM of strigol were statistically significantly different from 5 and 7.5 µM of strigol (Figure 2.9).

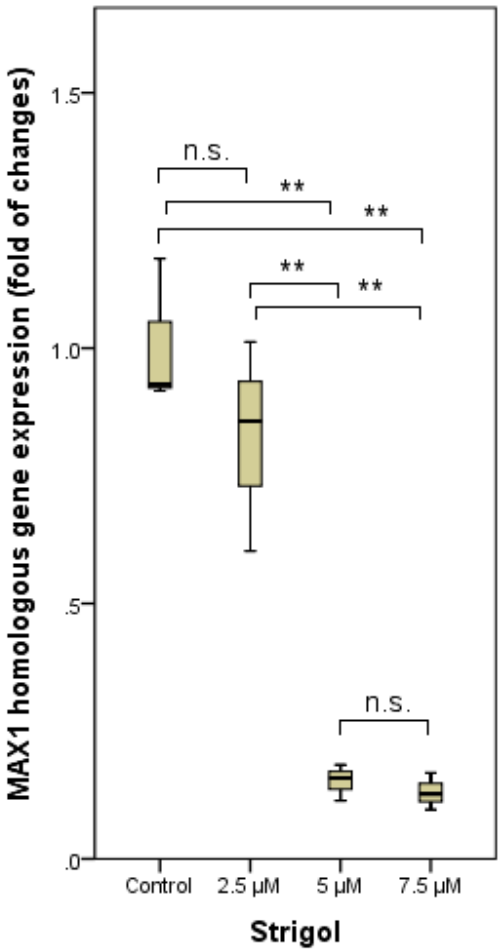


Figure 2.9. The effect of three different concentrations of Strigol as compared with control and each other. n.s., and **: non-significant, and significance at 5%, respectively.

2.3.5.3 Result of qPCR performing for 5-deoxy-strigol -treated samples

5-Deoxy-strigol, a natural-based SL, was sprayed on sugar beet seedlings. The effects of three different concentrations of 5-deoxy-strigol was compared with control and each other. The hormone effects at 2.5 µM on transcription level of the gene (*MAX1* homologous gene) was not significantly lower than control. However, the higher concentrations of the hormone (5 and 7.5 µM) significantly decreased the expression level of the gene in comparison with control. The hormone application at 5 µM statistically lowered the gene expression level in comparison with 2.5 µM of the hormone. However, the highest level of the applied hormone did not change the level of expression in comparison with the lower amounts of the hormone. Spraying 5-

deoxy-strigol to the seedlings confirmed that the hormone statistically and significantly decreased the level of expression of *MAX1* homologous gene in sugar beet (Figure 2.10).

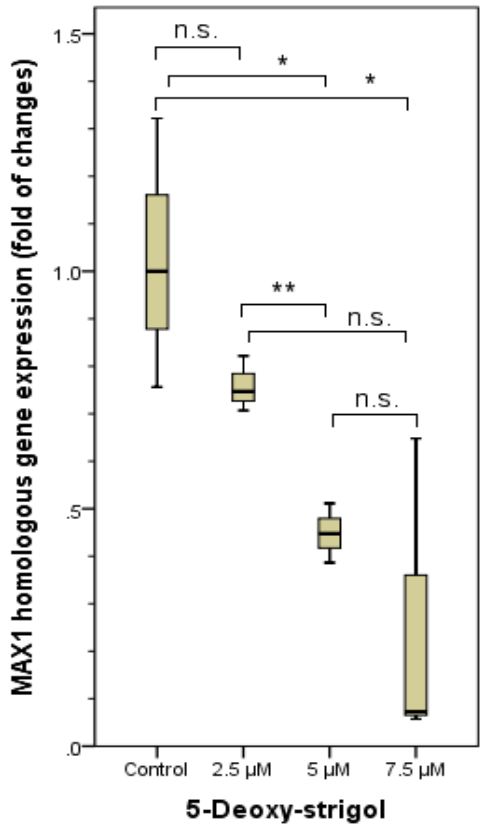


Figure 2.10. The effect of three different concentrations of 5-Deoxy-strigol as compared with control and each other. n.s., *, and **: non-significant, significance at 5 and 0.1% levels, respectively.

2.3.5.4 Result of qPCR performing for TIS-treated samples

TIS108, a triazole-type strigolactone (SL)-biosynthesis inhibitor, was sprayed on sugar beet seedlings. The effect of three different concentrations of TIS108 was compared with control and each other. In comparison with control, the inhibitor at 2.5 µM increased the level of expression of the gene, but this increase was not statistically significant. At higher concentrations, TIS108 significantly increased the expression level up to 8 and 14 times more than control. The inhibitor effects at 2.5 and 5 µM were statistically similar. Similarly, the inhibitor effects at 5 and 7.5 µM were statistically similar. However, the expression level after spraying 7.5 µM of TIS108 was statistically significantly higher than 2.5 µM of it (Figure 2.11).

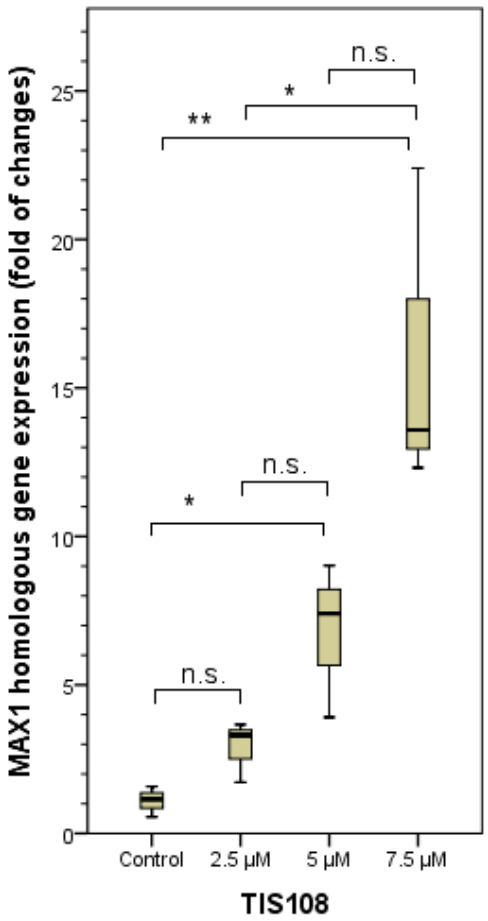


Figure 2.11. The effects of three different concentrations of TIS108 as compared with control and each other. n.s., *, and **: non-significant, significance at 5 and 0.1% levels, respectively.

2.3.5.5 The effects of spraying the three SL hormones on expression level of *MAX1* homologous gene in sugar beet

The effects of spraying the three SL hormones on expression level of *MAX1* homologous gene were analyzed. The analysis indicated that *rac*-GR24, a synthetic SL, suppressed the expression level more than the two other hormones, although statistics suggests that *rac*-GR24 and strigol effects were similar. 5-Deoxy-strigol showed the lowest effect on suppressing the expression level. However, it is worthy to note that strigol and 5-deoxy-strigol effects were statistically similar (Figure 2.12).

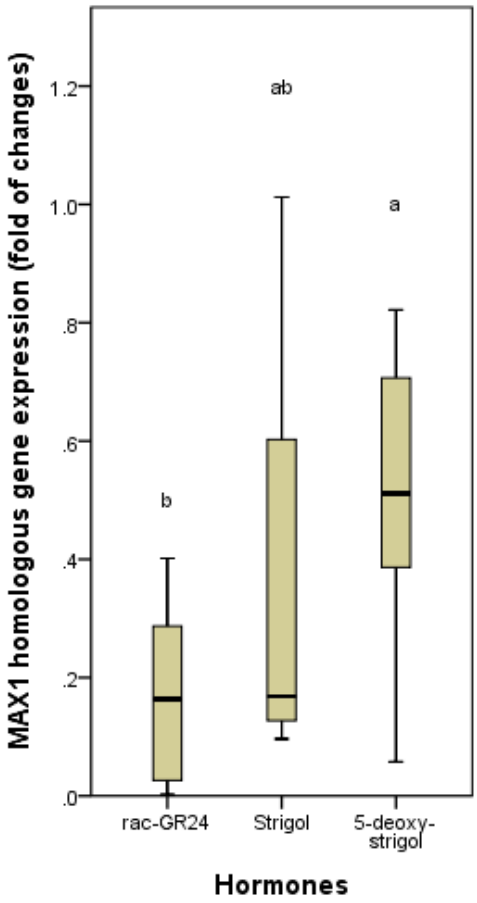


Figure 2.12. The effects of spraying the three SL hormones on expression level of *MAX1* homologous gene. The letters (a, b, c, and d) indicate significant differences between the treatments. The box-and-whiskers with similar letters are not statistically significantly different.

2.3.5.6 The effects of applied SL hormones at different concentrations on the expression level of *MAX1* homologous gene in sugar beet

The effects of the applied SL hormones at different concentrations on the expression level of *MAX1* homologous gene in sugar beet were also analyzed. All the levels of sprayed hormones were statistically significantly different from each other and control. The hormones at 7.5 µM decreased the expression level lower that 10% of the expression level of the gene in control. At 2.5 and 5 µM, the expression level was respectively 30% and 80% lower the control (100%). The results suggest that three hormones' effects on the expression level were dose-dependent (Figure 2.13).

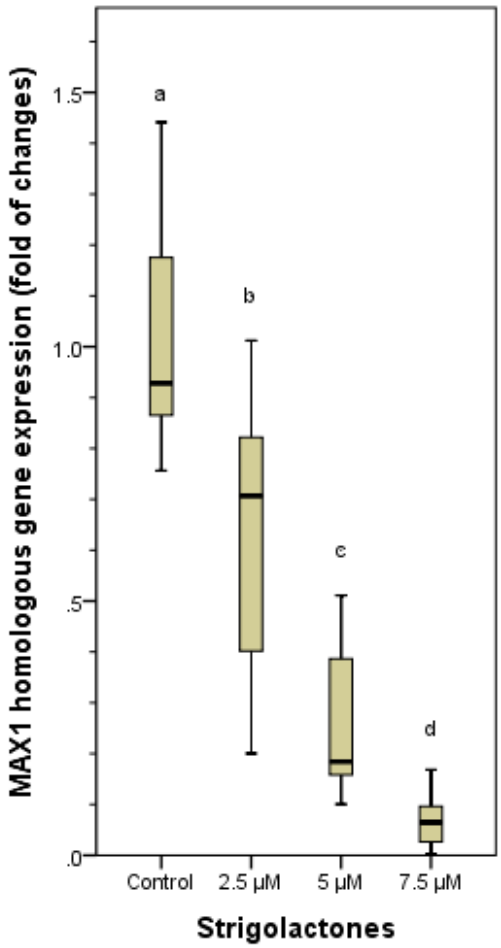


Figure 2.13. The effects of the applied SL hormones at different concentrations on the expression level of *MAX1* homologous gene in sugar beet. The letters (a, b, c, and d) indicate the significant differences between treatments.

2.4 Discussion

In all the hormonal treatments including *rac*-GR24, strigol, and 5-deoxy-strigol it can be observed that by increasing their concentration, the expression level of the gene of interest decreased. In all of them, the higher concentration (7.5 µM) always was statistically different from control (without any hormonal treatments). This feedback regulation suggests that the putative gene is an ortholog of Arabidopsis *MAX1* gene in the biosynthesis pathway. Assessment of the regulation of transcriptional changes in biosynthesis and/or transduction pathway of plant hormones is one of the widely used methods to locate the genes involved (Merchante et al., 2013). Cho et al. (2016) applied the same method for OsWOX3A as a transcription factor in

the biosynthetic pathway of gibberellic acids in Rice. Our results are consistent with Mashiguchi et al. (2009) in the feedback regulation of SL synthesis pathway.

It seems that the synthetic SL (*rac*-GR24) has the highest effect on suppressing the expression level of the gene (Figure 2.14). The same effect was reported previously for other types of hormones. For example, in auxin family hormones, alpha-naphthaleneacetic acid (NAA), Picloram, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,3,5-triiodobenzoic acid Free acid (TIBA), and 2,4-dichlorophenoxyacetic acid (2,4-D) have more and stable effects on plant growth and development than the natural auxins (indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA)). Similarly, in comparison with kinetin, a type of cytokinin, synthetic hormones such as 6-benzylaminopurine (BA), have more intense effects on plants. In addition, since there are many types of SL hormones, the effects of various compounds of SLs on a species of plants can differ.

In brief, *rac*-GR24 at 7.5 µM suppressed the gene expression level more than any other hormones at all the concentrations. After that, strigol at 7.5 µM decreased the expression level of the gene more than other treatments. Among the hormone-sprayed explants, 5-deoxy-strigol and strigol at 2.5 µM showed the weakest effects on lowering the expression level of the gene (Figure 2.15).

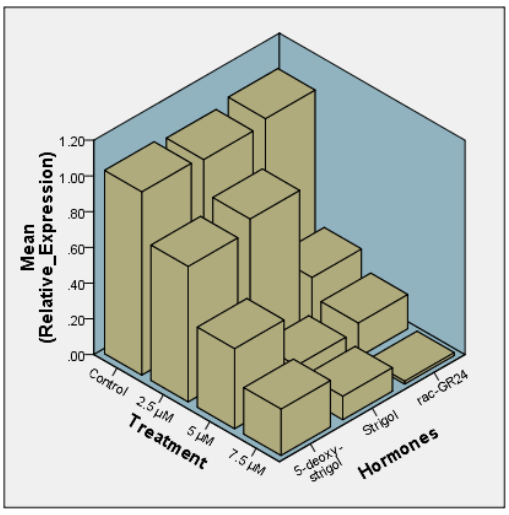


Figure 2.14. The effects of the three types of SLs in four different concentrations on the expression level of *MAX1* homologous gene in sugar beet.

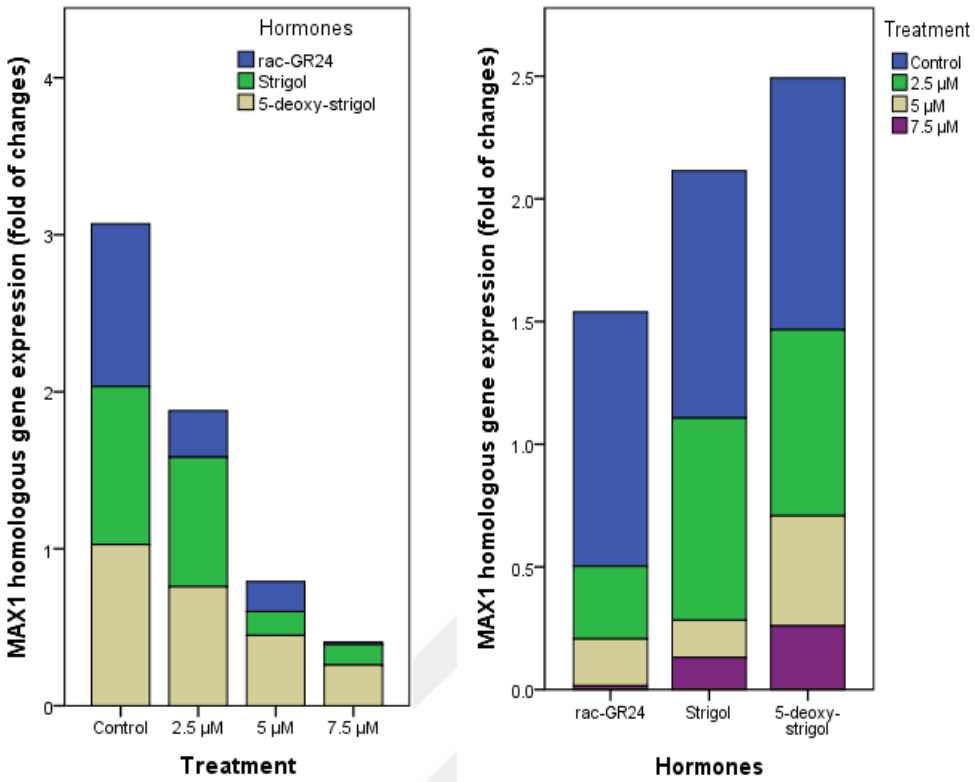


Figure 2.15. The effects of the hormones at different concentrations by their share on expression level. The figures clearly show each hormone's contribution to the expression level at the examined concentrations. a) It can be seen that *rac*-GR24 has the highest effect on the suppression of *MAX1* homologous gene expression. b) The highest level of suppression was recorded at the highest concentration of the hormones. The decrease in the expression level after hormone spraying was dose-dependent.

Result of qPCR and the other works done on the putative gene (*MAX1* homolog) in sugar beet for the first time demonstrated that most probably this gene involves in the synthesis pathway of strigolactones. The dose-dependent feedback effects of exogenously applied SLs and up regulated effect of exogenously applied inhibitor of SL biosynthesis (TIS108) suggested that the putative gene, which we studied, based on homology in NCBI can be involved in the biosynthetic pathway of strigolactone in sugar beet.

The association between the highest hormone concentration and the lowest gene expression was due to dose-dependent effect of this feedback regulation.

CHAPTER III

3 Evaluation of SLs' Effects on Salinity- and Drought-Stress Exposed Sugar Beet Plants

3.1 Introduction

Nowadays abiotic stresses including salinity and drought stresses are widespread and environmentally destructive to plants and crop production. Therefore, acquiring knowledge of physiological responses of plants to drought and salinity is highly significant. This understanding may form a basis for genetic studies and engineering in order to get plants more tolerant to salinity and drought stresses. Phytohormones can play a pivotal role in triggering appropriate responses to stress condition e.g. salinity and drought. The effects of well-known hormones on plants under salinity and drought condition are intensively investigated. Thus, in this thesis, one of the aims was to study any effects of newly discovered hormones, strigolactones (SLs), on sugar beet plants (cv. Serenada) under the mentioned stresses (i.e., salinity and drought).

3.2 Materials and Methods

3.2.1 Stress pre-experiments

3.2.1.1 Determination of soil electrical conductivity

For salinity stress, determination of soil electrical conductivity (EC), as an indicator of soluble ions (salt), seems necessary. The exact salinity stress can be

applied by evaluating primary EC (before adding salt). In this regard, first the primary EC (EC1) of the soil selected for the experiments was measured.

For EC analysis, a suspension of the soil was prepared in water with 1:2 ratio (soil: water). For each sample, 50 g air-dried-soil (≤1 mm) was transferred to a bottle. One hundred (100) ml deionized water was added to the soil. After shaking for 1 hour, the final suspension was passed through a filter paper (whatman #1).

3.2.1.2 Salinity stress pre-experiment

Based on standard definitions, soil with EC between 8 and 16 means moderate to high saline. However, sugar beet is relatively tolerant to salinity. Therefore, for examining the efficacy of the proposed amount of NaCl solution (200 mM) on increasing salinity in the soil, a pre-test was designed. In the test, 3 concentrations of NaCl including 150 mM, 200 mM, and 250 mM were prepared. For 10 days, 50 ml of the NaCl solutions was added to each pot on a daily basis. There were three replicates for each of the concentrations besides controls. The controls were irrigated with the same amount of distilled water (50 ml).

3.2.1.3 Drought stress pre-experiment

To evaluate the efficacy of drought stress duration (without irrigation) in the growth chamber condition (i.e., under controlled light and humidity), it was tried to monitor the plants' response to drought in following pattern:

When the plants wilted, one group of them was irrigated but the others were kept without watering. If any plant could recover from wilting, it was not considered as the threshold of tolerance. The process was continued with the other plants and when the irrigated one could not recover, it was considered the tolerance threshold of our sugar beet variety to drought stress, and the duration of drought was selected before it. The process in detail is as follows:

For the pre-experiment, 3-week-old plantlets were subjected to drought stress. The first pots were irrigated after 4 days and the second pots after 6 days. Both of the groups could recover themselves from wilting. However, the second group of pots (6-day drought) showed partly damaged leaves as compared with the first ones (4-day drought). The third group of pots were watered after 8 days. Therefore, the seedling underwent 4, 6 and 8 days of drought. After drought durations, they were irrigated with 50 ml filtered water to find the threshold of tolerance for sugar beet in the condition of the growth chamber.

3.2.2 Stress main experiments

3.2.2.1 Seedling production for salinity and drought Stresses

This part was done in the same manner for both of the experiments (salinity and drought). Firstly, sugar beet seeds were cultured after 24-hour imbibition period in the pots containing roughly the same amount of the soil. In each pot, five seeds were sown. After germination, only one healthy seedling in the same size with other pots' seedlings was kept in each pot. The treatment application was started with 10-day old plants. 10-day old seedlings were selected to work on because they were large enough to start hormone application.

3.2.3 Salinity experiment under hormonal treatments (*rac*-GR24, St, dSt, and control)

3.2.3.1 Hormonal treatments

After seedling production, the hormonal solutions were sprayed on the plant shoots. The concentration of the hormones (*rac*-GR24, St, and dSt) was 10 µM, and the amount was 5 ml. Besides, in the similar fashion, the plants in the control group were sprayed with water once per day. However, from the second day to the 8th day, spraying the hormones and water (control) were done twice a day. After that, like the

first day, the hormones and water were sprayed once a day until the last day of the hormonal duration.

3.2.3.2 Applying salinity stress

Salinity stress was applied after two weeks (14 days) of applying *rac*-GR24, St, dSt, and control (water). As the saline solution, 250 mM NaCl solution was prepared. Saline solution was used for 10 days instead of irrigation with water. Therefore, 50 ml of 250 mM NaCl solution was daily added to each pot for 10 days.

After completing the application of the salinity stress, the plants were again irrigated with distilled water for 4 days.

3.2.4 Drought experiment under hormonal treatments (*rac*-GR24, St, dSt, and control)

3.2.4.1 Hormonal treatments

In drought stress experiment, the concentration of the hormonal treatments (*rac*-GR24, St, dSt, and control) was 10 μM, the amount was 5 ml, and the duration was, as mentioned in the salinity part, 14 days in which the first day plus the 9th day to 14th day had one time spraying. However, the spraying was done twice a day from the 2nd day to 8th day.

3.2.4.2 Applying drought stress

To start drought stress, after applying the hormonal treatments, the plants underwent drought tension for 6 days. It means, they were not irrigated during those 6 days to undergo drought stress. The duration of drought stress was determined based on the preliminary experiment. Subsequently, after drought stress duration, the plants

were watered for three days to evaluate the presumed recovery potential of the hormone-treated plants as compared with controls (the water-treated ones).

3.2.5 Morphological assessment

At the end of both experiments' duration, firstly, photography was done in order to monitor any differences in the appearance of the treated plants as morphological assessment.

3.2.6 Collecting samples of salinity- and drought-stressed plants for further assessment

After taking the photos, leaf samples were collected from the treated plants. After snap freezing, the samples were kept in -80 ℃ for following experiments including catalase (CAT), chlorophyll content and malondialdehyde (MDA).

3.2.7 Physiological assessment of the treated plants under salinity and drought stresses

3.2.7.1 Chlorophyll content

Chlorophyll assay (for the samples of both salinity and drought stresses) was carried out as follows:

- 100 mg of each leaf tissue was finely ground by liquid nitrogen
- Then, was homogenized by 1 ml of 100 % dimethylformamide (DMF)
- Sonication was done for about 1 min
- Later, incubation was done for about 15 min at 4 ˚C
- Then, centrifuge was done for 10 min at 27000 ×g

- Next, supernatant was transferred to a new tube
- Absorbance was measured at 664.5 nm and 647
- Content of chlorophyll a and chlorophyll b, and subsequently total chlorophyll were calculated by following formulas (Moran, 1982):

Chl a = $(12.92\ A_{664.5}) - (2.12\ A_{647}) - (3.85\ A_{603})$

Chl b = $(-4.67\ A_{664.5}) + (26.09\ A_{647}) - (12.79\ A_{603})$

Total = $(8.24\ A_{664.5}) + (23.97\ A_{647}) - (16.64\ A_{603})$

3.2.7.2 Catalase activity (CAT)

Catalase activity (for the samples of both salinity and drought stresses) was carried out as follows:

- A standard curve was made for protein content. Therefore, Bovine Serum Albumin (BSA) was used for standard curve.

The soluble protein content was determined by the Lowry method (Lowry et al. 1951):

- Analytical reagents including "sodium carbonate mixed with NaOH solution" and "copper sulphate solution mixed with sodium potassium tartarate solution" were prepared
- Folin-Ciocalteau reagent solution: water reagent was prepared
- 2 ml of alkaline copper sulphate reagent (analytical reagent) was added to 200 µl protein solution (extraction)
- Mixed well
- The solution was incubated at room temperature for 10 mins
- 0.2 ml of Folin reagent was added
- The solution was incubated for 30 min
- Absorbance was measured at 660 nm by spectrophotometer against blank

By plotting the absorbance at 660 nm in standard calibration curve, protein content was obtained for each sample.

Catalase activity was determined according to the method of Lartillot et al. (1988):

- 3 ml of reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2 was added to tubes.
- Afterwards, 20 µl of enzyme extract was added to each tubes.
- The reaction was initiated by adding enzyme extract.
- The reaction was terminated by adding 0.5 ml 1 M HCl solution
- To detect the initial absorbance of H_2O_2 (As); absorbance of the solution containing 2.5 ml of reaction mixture and 0.5 ml of 1 M HCl was measured spectrophotometrically.
- To detect the absorbance reaction (Ar); absorbance of the solution containing 2.5 ml of reaction mixture and 20 µl of enzyme extract was measured spectrophotometrically
- In order to detect the protein absorbance (At); absorbance of the solution containing 20 µl of enzyme extract, 2.5 ml of buffer, and 0.5 ml of 1 M HCl was measured spectrophotometrically.
- CAT activity was determined by following consumption of H_2O_2 (extinction coefficient, 39 mM^{-1} cm^{-1}) at 240 nm over a 2 min interval.

The absorbance variation due to the enzymatic activity was calculated as follows:

$$A = (As + At) – Ar$$

For catalase activity, the formula below was used:

$$(A \times Vt) / (\varepsilon \cdot t \cdot V_e)$$

Vt; is the total reaction volume, V_e; the sample volume, ε; the specific absorbance coefficient of hydrogen peroxide, t; is the time interval.

3.2.7.3 Malondialdehyde (MDA)

Malondialdehyde (MDA) content assay (for the samples of both salinity and drought stresses) was carried out as follows (Zhang et al. 2012):

- 100 mg of each leaf tissue (similar age, and young-expanded leaf) was ground into powder with liquid nitrogen
- 1 ml of 0.1% (w/v) TCA was added to the tubes containing the powder and mixed by inverting them to homogenize the leaf tissue.
- Homogenized samples centrifuged at 10,000 x g for 10 min
- Then supernatants ware transferred to new tubes
- 4 ml of 20% TCA containing 0.5% TBA was added to the supernatants and mixed well
- The mixtures were boiled at 95 °C for 15 min
- Then immediately cooled on ice (TBA interacted with MDA and resulted into a red compound)
- The mixtures centrifuged at 10,000 x g for 5 min
- Supernatants were transferred into new tubes
- Content of MDA was calculated by measuring the density of the resulting red compound with spectrophotometer at 532 nm.

3.2.8 Statistical analysis

The data were analyzed using appropriate methods of analysis. Levene's test of homogeneity of variances and Shapiro–Wilk (S-W) and Lilliefors corrected Kolmogorov–Smirnov (K-S) tests of normality assumptions were used for the data. The data were subjected to one-way analysis of variance (ANOVA), and means were compared using Tukey's test, or Welch's adjusted *F* ratio for one-way ANOVA and Games–Howell post hoc tests were run to examine the differences between groups. The level of significance for the analyses was considered $p < 0.05$. A Windows™ based SPSS® program (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and MS-Office excel software (V.12) was used for graph drawing.

3.3 Results

3.3.1 Results of stress pre-experiments

3.3.1.1 Result of determination of soil electrical conductivity

The EC of the soil extracts before irrigating with saline solution was measured.

- EC1 (sample1): 9.08 mS/cm
- EC2 (sample2): 9.25 mS/cm
- EC3 (sample3): 9.19 mS/cm
- Mean EC of the samples: 9.17 mS/cm

3.3.1.2 Result of salinity stress pre-experiment

Based on phenotype, the plants under 250 mM salinity stress became shorter and smaller, and as a result stunted the seedlings growth (Figure 3.1). Therefore, 250 mM salinity was used for the main experiment.

Figure 3.1. Preliminary experiment on the effects of different concentrations of salinity on sugar beet seeding growth. The seedlings were irrigated with saline water (from left to right): Control, 150 mM, 200 mM, and 250 mM (millimoles) NaCl.

3.3.1.3 Result of Drought Stress Pre-Experiment

The seedling which experienced 4 and 6 days of drought revived themselves after irrigation, whereas 8 days of drought was lethal, which means that the seedling could not revive. Finally, the 6 days of drought stress seemed proper, because drought duration for 6 days could induce effective, observable and recordable wilting to evaluate the role of the hormones in revival of the stressed sugar beet plants (Figure 3.2).

Figure 3.2. Preliminary experiment on the effects of different drought durations on sugar beet seeding survival. The seedling underwent 4, 6 and 8 days of drought. After drought durations, they were irrigated with 50 ml filtered water. The seedling which experienced 4 and 6 days of drought revived themselves after irrigation, whereas 8 days of drought was lethal, which means that the seedling could not revive.

3.3.2 Result of morphological assessment

At the end of both the experiments' duration, firstly, photography was done in order to monitor any differences in the appearance of the treated plants as a morphological assessment.

Hormone-treated sugar beet plants in salinity condition are shown in Figure 3.3.

Figure 3.3. The hormone-treated sugar beet plants in salinity condition, 250 mM NaCl. The salinity-subjected plants were sprayed with a) water as control, b) *rac*-GR24, c) St, and d) dSt.

3.3.2.1 Hormone-treated sugar beet plants in drought condition

Hormone-treated sugar beet plants in drought condition are shown in Figure 3.4.

Figure 3.4. The hormone-treated sugar beet plants in drought condition, 6 days of water withholding followed by 4 days of watering. Before subjecting the plants to drought stress, they were sprayed with a) water as control, b) *rac*-GR24, c) St, and d) dSt.

3.3.3 Results of physiological assessment of treated plants under salinity and drought stresses

3.3.3.1 Chlorophyll content in salinity condition

The interactive effect of SLs hormones and salinity stress on chlorophyll (Chl) content was statistically analyzed based on $p < 0.05$ (ANOVA). In salinity condition, the effect of SLs on Chl a was statistically significant. The effect of control was not similar to any other treatments, i.e. *rac*-GR24 and dSt, and St. All the applied SL hormones statistically showed similar effect on Chl a. Therefore, it was shown that in salinity condition, the applied hormones could increase Chl a content in comparison with the control. In contrast, the applied SLs effect on Chl b was not statistically significant. Although based on the results it seemed that SLs effects on Chl b in salinity condition were different, but it was not confirmed with statistical analysis ($p < 0.05$). The total amount of Chl a+b (Chl T) was significantly changed by the application of the SLs. The effects of control on Chl T was statistically similar to *rac*-GR24 and dSt. However, the control effect on Chl T was different from St-treated plants in salinity condition (Figure 3.5).

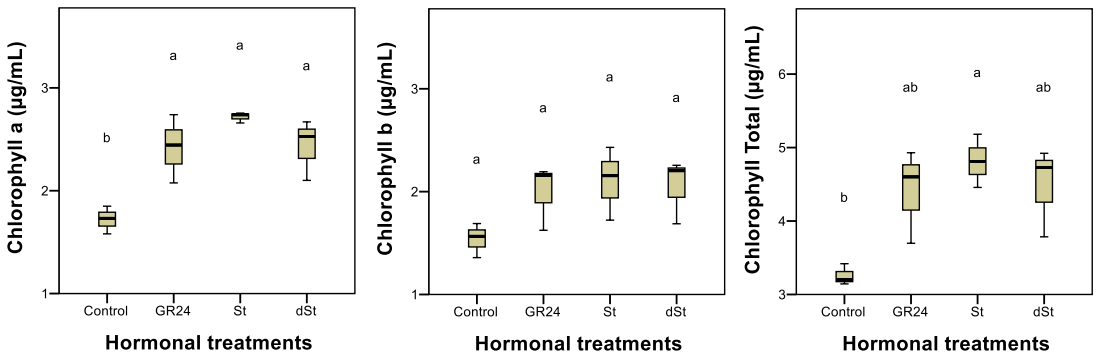


Figure 3.5. Effects of *rac*-GR24, Strigol, and 5-deoxystrigol on chlorophyll a, b and total of the sugar beet plants subjected to the salinity stress (irrigated by 250 mM NaCl). Bars show standard deviation (±SD).

3.3.3.2 Chlorophyll content in drought condition

The interactive effect of SLs hormones in drought stress on chlorophyll content was statistically analyzed based on $p < 0.05$ (ANOVA). In drought condition, the effect of SLs on Chl a was significant. The effect of control was similar to *rac*-GR24 and dSt, but was not similar to St. However, St and dSt show also similar effect in relation to Chl a. In contrast, the applied SLs effects on Chl b was not statistically significant. Although it seemed that SLs effects on Chl b in drought condition were different, but it was not confirmed with statistical analysis ($p < 0.05$). The total amount of Chl a+b (Chl T) was significantly changed by the application of the SLs. The effects of control on Chl T was statistically similar to *rac*-GR24 and dSt. However, the control effect on Chl T was different from St treated plants in drought condition (Figure 3.6).

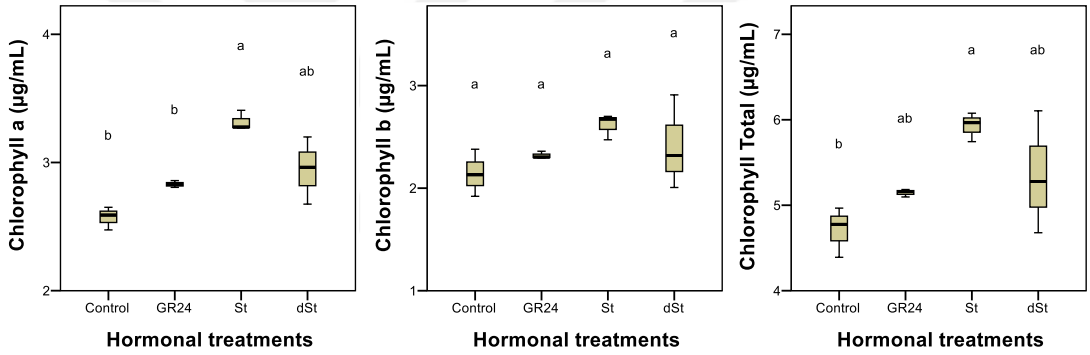


Figure 3.6. Effects of *rac*-GR24, Strigol, and 5-deoxystrigol on chlorophyll a, b and total of the sugar beet plants subjected to the drought stress (6 days without irrigation). Bars show standard deviation (±SD).

3.3.3.3 Catalase activity in salinity stress

Effects of the treatments on Catalase Enzyme Activity (CAT) in salinity condition was analyzed statistically. The applied treatments effects on CAT were not statistically significant ($p < 0.05$). However, it seemed that SL treated plants had more CAT activity in salinity stress condition. In addition, St treated plants showed the highest amount of CAT activity, which was followed by dSt and then by *rac*-GR24 (Figure 3.7).

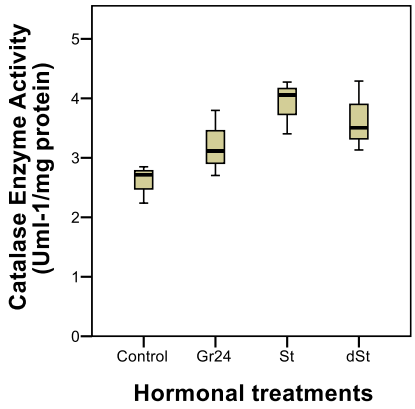


Figure 3.7. Effects of the SLs on Catalase (CAT) enzyme activity of the sugar beet plants subjected to the salinity stress (irrigated by 250 mM NaCl). Bars show standard deviation (±SD). The applied treatments effects on Catalase enzyme activity (CAT) were not statistically significant ($p < 0.05$).

3.3.3.4 Catalase activity in drought stress

Effects of the applied hormones on CAT in drought condition was analyzed statistically. The effects of the applied SLs on CAT activity were not statistically significant ($p < 0.05$). However, again, it seemed that SL treated plants had more CAT activity in drought stress condition. In addition, St treated plants showed the highest amount of CAT activity, which was followed by dSt and then by *rac*-GR24 (Figure 3.8).

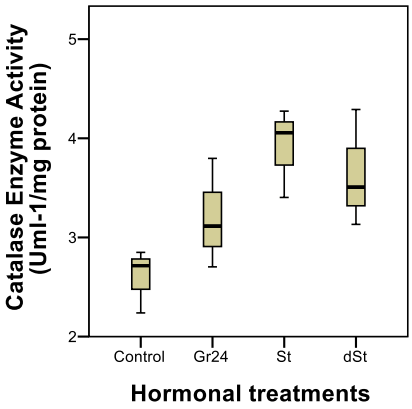


Figure 3.8. Effects of the SLs on Catalase (CAT) enzyme activity of the sugar beet plants subjected to the drought stress. Bars show standard deviation (±SD).

3.3.3.5 MDA in salinity stress

Effects of the SLs on the MDA content of the sugar beet plants in salinity condition were statistically significant in $p < 0.05$ (ANOVA). After appying the SLs, the MDA content of the St treated plants decreased significantly in comparison with the control. However, *rac*-GR24 and dSt treatments effects on MDA content were not significantly different from the control (Figure 3.9).

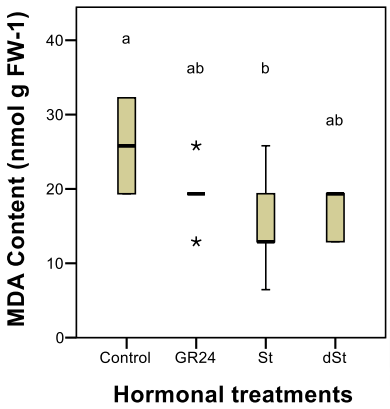


Figure 3.9. Effects of the SLs on Malondialdehyde (MDA) content of the sugar beet plants subjected to the salinity stress. Bars show standard deviation (±SD). The applied treatments effects on MDA content were statistically significant ($p < 0.05$).

3.3.3.6 MDA in drought stress

Effects of the SLs on the MDA content of the sugar beet plants in drought condition were statistically significant in $p < 0.05$ (ANOVA). After applying the SLs, the MDA content of the St treated plants decreased significantly in comparison with the control. However, *rac*-GR24 and dSt treatments effects on MDA content were not significantly different from the control (Figure 3.10).

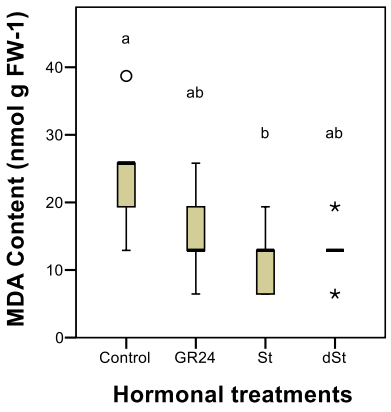


Figure 3.10. Effects of the SLs on malondialdehyde (MDA) content of the sugar beet plants subjected to the drought stress. Bars show standard deviation (±SD). The applied treatments effects on MDA content were statistically significant ($p < 0.05$).

3.4 Discussion

Alleviation of the side effects of the stresses after applying the hormonal treatments is evident in the morphological assessments of the sugar beet plants exposed to salinity and drought stresses. The number of turgid and erect leaves are more in the treated plants as compared with control under stress conditions. Exogenous application of phytohormones has proven to be effective for mitigating the adverse effects of salinity and drought conditions (Javid et al., 2011; Gomez et al., 2002; Hayat et al., 2010). This mitigation was observed through the SLs' application under salinity and drought stresses, which can demonstrate SLs roles in mediating morphological and physiological responses to external stresses.

Salinity and drought stresses decrease plant content of chlorophylls. The negative effects of salinity and drought on chlorophyll contents of the hormone treated sugar beet plants were decreased. In comparison with the control, Strigol produced the highest amounts of chlorophyll a, b and total. After Strigol, 5-deoxystrigol produced the highest amounts of chlorophyll a, b and total. The third rank was occupied by *rac*-GR24, which produced more chlorophyll a, b and total in comparison with the control. All the hormonal treatments increased chlorophyll a, b and total contents as compared with the control. Sairam et al. (1997) reported an increase in lipid peroxidation and a

decrease in the level of total chlorophyll in drought-subjected plants. Increased Thiobarbituric Acid Reactive Substances (TBARS) accumulation has been correlated to a reduction in the photosynthetic pigment content of leaves subjected to prolonged water deficit (Jiang and Huang, 2001). *Cucumis sativus* plants subjected to 100 mM NaCl produced lower amounts of chlorophyll a and b (Stępień and Kłbus, 2006). The amounts of chlorophyll a and b in *Fagus sylvatica* plants under severe drought stress decreased significantly (Gall and Feller, 2007). However, the interactions of SLs and salinity or drought stresses on plants have not been researched.

The hormonal treatments increased chlorophyll a and subsequently chlorophyll T contents in both stress conditions. Generally, environmental stresses, e.g. salinity can severely affect cell components, such as chloroplast (Nusrat et al., 2014), and result in a decrease in chlorophyll contents. It was showed that *rac*-GR24 treatment could limit the damages of salinity stress to the chloroplast (Ma et al., 2017), which was in parallel with the data obtained in the present research that indicated an increase in chlorophyll content under both salinity and drought stresses. The increased chlorophyll content was likely because of *rac*-GR24 positive effect on chloroplast and chlorophyll a.

To achieve salt tolerance, damage must be prevented or alleviated. The nature of the damage that high salt concentrations inflict on plants is not entirely clear. An important cause of the damage might be reactive oxygen species (ROS) generated by salt stress. Plants subjected to salt stress display complex molecular responses including the production of stress proteins. Many of the stress proteins with unknown functions probably detoxify plants by scavenging ROS or preventing them from cellular structures damage (Zhu, 2001). The Thiobarbituric Acid Reactive Substances (TBARS) assay is a well–established method for monitoring lipid peroxidation level. Catalase protects plant cell from the damaging effects of H_2O_2 accumulation. Varieties of cotton plants (*Gossypium hirsutura*) tolerant to salinity produced higher amounts of catalase enzyme than the sensitive ones in 150 mM NaCl in comparison with the control ones (Gossett et al., 1993). A very strong and positive correlation was reported to exist between levels of hydrogen peroxide and TBARS in the leaves of wheat plants grown under irrigated and rain–fed conditions (Singh et al., 2012). Similarly, the correlation between H_2O_2 and TBARS was positive in drought stressed wheat plants

(Tian and Lei, 2007). Therefore, it suggests that water availability has an effect on H_2O_2 level and consequently TBARS in plant tissues. Effects of Strigol (St) on increasing catalase enzyme activity was better than any other treatments. 5-deoxystrol was the second best hormonal treatment in improving catalase enzyme activity. *Rac*-GR24, a synthetic SL, increased the activity of catalase enzyme, but its inducing effect was lower than the two other SLs. In comparison with the control, all the hormonal treatments increased the activity of catalase enzyme (Figure 3.7 and Figure 3.8).

Stress-generated oxidative damages are due to the production of reactive oxygen species (ROS) e.g. hydrogen peroxide (H_2O_2) (Munns and Tester, 2008). In addition, salinity is one of the factors controlling acceleration in ROS generation (Gill and Tuteja, 2010). One of the strategies that stress-exposed plants adopt is the synthesis of antioxidants as ROS scavengers, e.g. CAT (Wang et al., 2014). Ma et al. (2017) indicated that *rac*-GR24 by increasing the amount of (peroxidase) POD and superoxide dismutase (SOD) as ROS scavengers positively influenced rapeseed plants in salinity conditions. Similarly, we observed that CAT, the other ROS scavenger in plant defense mechanisms, was enhanced in salinity-, drought-exposed sugar beet plants by all the hormonal treatments in comparison with control, however, the differences were not statistically significant.

An important side effect of high salt stress is generating ROS, which damages cellular membranes integrity (Zhu, 2001). The damages generally affect membrane phospholipids. The decomposition of lipid can readily produce aldehydes such as Malondialdehyde (MDA). MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. MDA level is commonly known as a marker of oxidative stress (Gaweł et al., 2004). Singh et al. (2012) showed that instability of biological membranes, as reflected by lipid peroxidation, was greater in drought–sensitive than in drought–tolerant wheat genotypes (*Triticum aestivum* L.). Similarly, it was observed that salinity tolerant varieties of cotton accumulated lower amounts of MDA in comparison with the sensitive ones (Gossett et al., 1993). Wheat plants subjected to salinity stress (195 mM of NaCl) contained more MDA contents. However, the salinity tolerant varieties of wheat accumulated lower amounts of MDA in comparison with the sensitive varieties (Zheng et al., 2009). Strigol-sprayed plants showed the lowest amounts of MDA

content, which was followed by 5-deoxystrigol-sprayed plants. *Rac*-GR24 decreased the amount of MDA in comparison with the control, but its effect on lowering the level of MDA was less than the two other SLs. All the applied SLs lowered the level of MDA in comparison with the control. It seems that SLs ameliorate the adverse effects of salinity and drought stresses (Figure 3.9 and Figure 3.10).

Abiotic stresses, e.g. salinity stress, can result in a greater amount of MDA as the final product of ROS-caused damages to polyunsaturated fatty acids in cell membranes (Jan et al., 2017; Kong et al., 2016). Ma et al. (2017) reported a decrease in MDA accumulation after application of GR24 in salinity-treated plants. Similarly, we observed that MDA content declined after treating by the SL hormones in the salinity and drought experiments. It shows that SLs, similar to the other hormones (Rady 2011; Stoparić, and Maksimović, 2008; Hasanuzzaman et al., 2017), can lessen adverse effects of the stresses on bio-membranes through decreasing lipid peroxidation, which can be mediated through increasing antioxidants like CAT activity.

CHAPTER IV

4 Evaluation of SLs' Effects on Germination of Sugar Beet Seeds

4.1 Introduction

Seed germination can be described as the emergence of the embryo from its enclosing coverings. Generally, among germination inducing or inhibiting factors, hormonal treatments are of high interest. The effect of gibberellins, brassinosteroids, auxins, cytokinins, ethylene, abscisic acid, and jasmonates or corresponding hormone biosynthesis inhibitors on sugar beet germination has been studied (Hermann et al., 2007). Strigolactones (SLs) are a new class of hormones, which recently attracted researchers' attention. SLs have shown inducing effects on parasitic plant seeds germination, but the effects of SLs on non-parasitic plants have not been thoroughly investigated. Moreover, SLs effect on sugar beet germination has never been investigated. Sugar beet, as one of the limited sugar producing species, is a commercially very important crop. Beet family includes many food and feed species among which germination rate is not (very) high. Commercial production of sugar beet is very dependent on seed germination. Therefore, it was designed to check whether SLs and SL inhibitor could affect crop seed germination or not.

4.2 Materials and Methods

4.2.1 Germination examination in media containing *rac*-GR24, St, dSt, and TIS

4.2.1.1 Medium preparation

For all the treatments (*rac*-GR24, St, dSt, and TIS stock) half-strength MS medium supplemented with 10 g L^{-1} sucrose was prepared. The pH was adjusted to 5.8. After autoclaving the media in bottles, they cooled down. Proper amounts of the hormones and the inhibitor were added to the bottles under a laminar flow hood and were swirled to mix well. The concentrations of the hormonal treatments were 0 as control, 2.5, 5 and 7.5 μM. The mixed media were poured into petri dishes.

4.2.1.2 Seed sterilization and sowing

Seeds of sugar beet cv. Serenada were sorted into (almost) the same size. The sorted seeds were sterilized using a hypochlorite solution (5%) for 1 h, after which they were rinsed five times with sterile distilled water.

Then, the sterilized seeds were cultured in the prepared media and germination of the seeds were recorded on 14th day for each set of the experiment separately.

4.2.2 Statistical analysis

The data were analyzed using appropriate methods of analysis. Levene's test of homogeneity of variances and Shapiro–Wilk (S-W) and Lilliefors corrected Kolmogorov–Smirnov (K-S) tests of normality assumptions were used for the data. The data were subjected to one-way analysis of variance (ANOVA), and means were compared using Tukey's test, or Welch's adjusted *F* ratio for one-way ANOVA and Games–Howell post hoc tests were run to examine the differences between groups.

The level of significance for the analyses was considered $p < 0.05$. A Windows™ based SPSS® program (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and MS-Office excel software (V.12) was used for graph drawing.

4.3 Results

4.3.1 Seed germination

Effects of the SLs and the inhibitor treatments on sugar beet seed germination were statistically insignificant. The results were analyzed by ANOVA. However, the germination rates were not significantly changed by SLs or its inhibitor treatment ($p < 0.05$) (Figure 4.1).

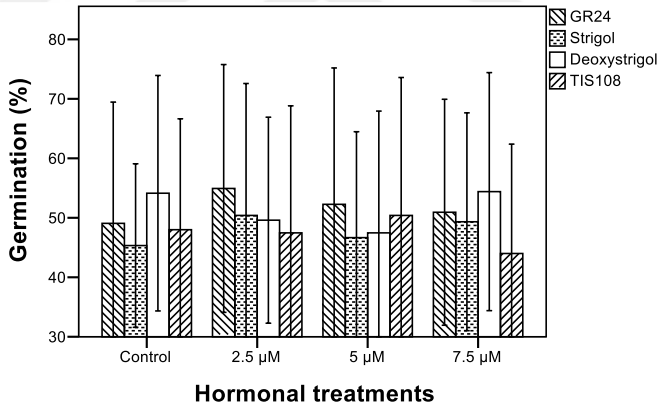


Figure 4.1. Effects of *rac*-GR24, strigol, 5-deoxy-strigol, and SL inhibitor (TIS108) on sugar beet seed germination. Bars show standard deviation (±SD). The applied treatment effects on sugar beet seed germination were not statistically significant ($p < 0.05$).

4.4 Discussion

Effects of the supplemented SLs and the inhibitor on sugar beet seed germination were insignificant. The obtained results did not show any significant and

consistent responses to the SLs and the inhibitor levels. Therefore, it seemed that the applied chemicals did not have any significant effects on sugar beet seed germination (Figure 4.1). SLs are generally inducing for parasitic plant seeds germination. It has been confirmed that SLs induce parasitic seeds germination from *Orobanche* and *Striga* spp. to recognize presence of an appropriate host plant (Matusova et al., 2005; Conn et al., 2015; Yoneyama et al., 2010; Brewer et al., 2013). However, the effects of SLs on non-parasitic seeds germination has not been confirmed yet.

CHAPTER V

5 Examination of SLs Effects on *In Vitro* Tissue Culture of Sugar Beet

5.1 Introduction

Plant tissue culture as one of fundamentals in plant science is significantly important. Shoot and root induction are of important steps in a successful production of *in vitro* new plant. Among various substances required for plant tissue culture, plant phytohormones can be mentioned as a crucial and effective factor (Gaspar et al., 1996). Phytohormones, individually/in cross-talk with other hormones, and directly/indirectly, can affect induction, growth and development of shoots and roots of an explant. Due to their huge impact on shooting and rooting of *in vitro* explants, in which it has been tried to study and clarify roles of the main plant hormones. Among the hormones, however, SLs have many undiscovered and unknown aspects that one of them is their effect on *in vitro* rooting and shooting of a non-model plant e.g. sugar beet. Therefore, the three SLs are chosen for assessing how they can affect *in vitro* shooting and rooting.

5.2 Materials and Methods

5.2.1 Preparation of a culture medium for seed germination

Firstly, a half-strength MS medium was prepared and adjusted to 5.8 and then was solidified a solidifying agent. The medium cooled down under a laminar flow hood after autoclaving and later was poured into sterilized petri dishes.

The sugar beet seeds were sorted into (almost) the same size. The sorted seeds were sterilized using a hypochlorite solution (5%) for 1 h, after which they were rinsed five times with sterile distilled water.

Next, the sterilized sugar beet seeds were cultured in the petri dishes containing hormone-free germination medium and kept in the growth chamber to germinate and produce small plantlets.

5.2.2 Preparation of media for examination of *rac*-GR24 effects on shooting and rooting

Full-strength MS medium supplemented with 30 g L^{-1} sucrose was prepared, then, divided up into four bottles. The pH was adjusted to 5.8. After autoclaving, they were allowed to cool down. Proper amounts of the hormone (*rac*-GR24) in different concentrations were added to the bottles under a laminar flow hood. The bottles were swirled to mix well. The concentrations of the hormonal treatment were 0 as control, 2.5, 5 and 7.5 μM. The mixed media were poured into sterilized magenta boxes.

5.2.3 Transferring small plantlets to the magenta boxes for shooting and rooting assessment

The small plantlets in almost the same size and good condition (healthy) were chosen from the germinated seeds in the petri dishes. After excising their roots, they were cultured in the magenta boxes. Three plantlets were cultured in each magenta box for each concentration and repetition for rooting assessment.

The healthy small plantlets in the same size were selected from the petri dishes. Then, their endings (radicles) were cut. After that, the plantlets with roughly the same height were transferred to the magenta boxes containing different concentrations of *rac*-GR24. 3 plantlets were cultured in each magenta box of the treatments (0, 2.5, 5 and 7.5 μM) for shooting assessment.

The shooting pattern and root growth were observed and the data were collected in one month.

5.2.4 Statistical analysis

The data were analyzed using appropriate methods of analysis. Levene's test of homogeneity of variances and Shapiro–Wilk (S-W) and Lilliefors corrected Kolmogorov–Smirnov (K-S) tests of normality assumptions were used for the data. Wherever appropriate, the data were subjected to one-way analysis of variance (ANOVA), and means were compared using Tukey's test, or Welch's adjusted *F* ratio for one-way ANOVA and Games–Howell post hoc tests were run to examine the differences between groups. Alternatively, the data were analyzed by the Kruskal–Wallis (K-W) test followed by the Bonferroni corrected Dunn's post hoc test or a stepwise step-down multiple comparison post-hoc test, adopted from Campbell and Skillings' method (1985). Comparisons between two independent samples were done with a two-sided Student's *t* test. The level of significance for the analyses was considered $p < 0.05$. A Windows™ based SPSS® program (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and graph drawing.

5.3 Results

The data for the number of leaves were analyzed by the K-W test, which indicated that there was a statistically significant difference between the effects of *rac*-GR24 on the number of leaves, χ^2 (3, N = 36) = 24.263; $p < .001$, with a mean rank of 31.17 for the control, 19.72 for 2.5 μM *rac*-GR24, 14.72 for 5 μM *rac*-GR24, and 8.39 for 7.5 μM *rac*-GR24. A step-down post hoc with the Bonferroni corrected Dunn's test indicated that the control had the highest number of leaves, while by increasing the concertation of *rac*-GR24 the number increased and resulted in the lowest number of leaves at the highest concentration of the hormone (Figure 5.1).

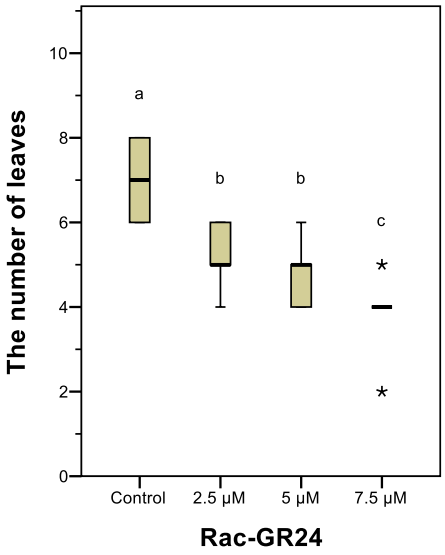


Figure 5.1. Effect of *rac*-GR24, a synthetic strigolactone hormone, on the number of leaves in sugar beet explants.

Since the effect of the treatments on the length was significantly different (*Welch's F*(3, 16.7) = 45.061, $p < .001$), the Games–Howell post hoc test was used for the comparison of means between total lengths of leaves in sugar beet explants treated with different concentrations of *rac*-GR24. The explants in the control medium produced the longest leaves, while with increasing the concentration of *rac*-GR24 the length of the leaves significantly decreased (Figure 5.2).

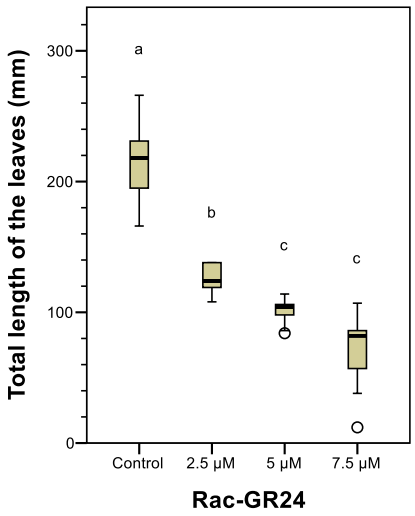


Figure 5.2. Effect of *rac*-GR24, a synthetic strigolactone hormone, on the total length of leaves in sugar beet explants.

An ANOVA indicated that the effect of *rac*-GR24 on the total area of leaves was significantly different ($F(3, 32) = 121.669$, $p < .001$). Tukey's post hoc test indicated that the control produced the largest area of leaves, and increasing the concentration of *rac*-GR24 significantly decreased the area (Figure 5.3).

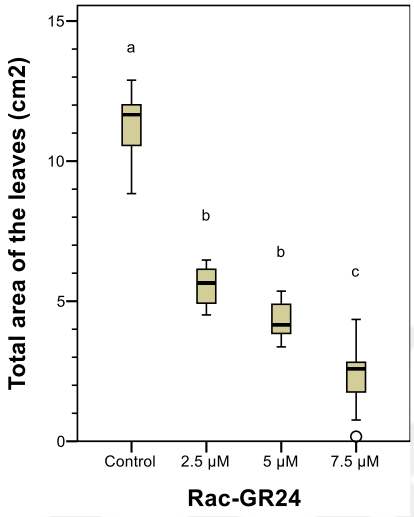


Figure 5.3. Effect of *rac*-GR24, a synthetic strigolactone hormone, on the total area of leaves in sugar beet explants.

The effect of the *rac*-GR24 treatments on the length of the roots was significantly different (*Welch's* $F(3, 16.9) = 4.846$, $p = .013$). The Games–Howell post hoc test was used for the comparison of means between total lengths of leaves in sugar beet explants treated with different concentrations of *rac*-GR24. The explants in the control medium produced the longest leaves, while with increasing the concentration of *rac*-GR24 the length of the leaves significantly decreased. Although based Games–Howell test indicated that the control and 5 µM *rac*-GR24 are not significantly different, further statistical analysis by independent t-test indicated that they are in fact statistically different at lower level of significance ($t(16) = 2.084$, $p = 0.053$). Similarly, *rac*-GR24 at 2.5 and 7.5 µM are in fact statistically different ($t(16) = 2.551$, $p = 0.021$) (Figure 5.4).

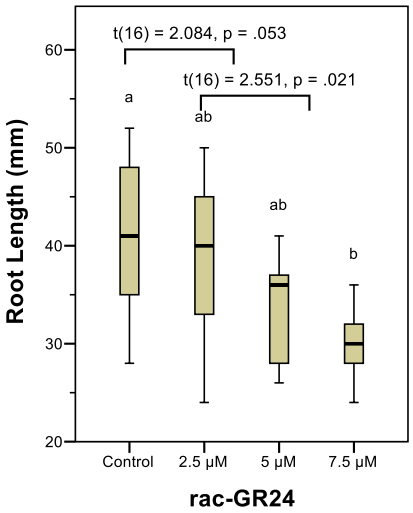


Figure 5.4. Effect of *rac*-GR24, a synthetic strigolactone hormone, on the length of root in sugar beet explants.

5.4 Discussion

One of the important roles of SLs is their considerable influence on modifying plant architecture through controlling growth and development (Brewer et al., 2013). Plants have low SL levels in normal and standard growing conditions; however, SL levels increase in any abnormal conditions to optimize plant growth and development for adaptation (Kohlen et al., 2011; Umehara et al., 2008). As is expected, by adding the SL hormones in tissue culture media, increasing the amount of the SLs affecting the sugar beet explants, changed their growth and development patterns. Principally, it has been observed that shoot branching can be inhibited by SLs (Sorefan et al., 2003; Umehara et al., 2008; Kohlen et al., 2011). In other words, bud activation is inhibited by SLs (Liang et al., 2010) and they can change the number of leaves. The number of sugar beet explant leaves decreased by increasing the concentration of *rac*-GR24 in the *in vitro* media, which is consistent with the results of an experiment done on onion var. BIOA-3 (Kopta et al., 2017). This observation can be explained by the inhibitory effect of SLs on bud outgrowth in plants (Brewer et al., 2009; Tan et al., 2019).

An inverse association was observed between the higher concentration of *rac*-GR24 with the total length and area of sugar beet explant leaves. Higher concentrations of the chemical decreased the total length and area of the explant leaves in comparison

with control. The data on the length of leaves do not confirm the results drawn from Kopta et al. (2017)'s experiment. This non-consistency in the obtained data can be described by the differences between growth patterns of plants, in which sugar beet is a rosette plant. The lower area of the leaves resulted from *rac*-GR24 treatment can be interpreted to mean that *rac*-GR24 in the media, around the basal part of the explants, signaled the nutrient deficiency. Therefore, by reducing the area of the leaves, the plantlets via reduction of leaf area directed the energy from the leaves toward the main stem and root system (Brewer et al., 2013).

Understanding phytohormone, such as SLs, cross-talks in root growth and development is complicated (Koltai, 2012). Adding *rac*-GR24 in the rooting media reduced the root length of the sugar beet plantlets as compared with control. This result corroborates the statement that SLs can only promote the length and density of root hairs in plants (Péret et al., 2011; Bates and Lynch, 2000).

CHAPTER VI

6 Examining the Interactions of SL with Auxin

6.1 Introduction

Crosstalk between plant hormones (in either synergistic or antagonistic interactions) makes notable and significant contributions to various aspects of a plant life (Verma et al., 2016) from plant growth and development till plant responses to different internal and external conditions. Studying a plant hormone without considering the roles of other hormones in its biosynthesis, transport, and transduction can be a gross oversimplification of the facts.

Regarding some common features observed for SLs and auxin, it can be assumed that there can be an interplay between them. For instance, their feedback loop may play a regulatory role in inhibition of axillary buds (Hayward et al., 2009). Finding of probable interactions among phytohormones can help clarify many still unclear points. Therefore, seeking a better understanding of interaction between SL and auxin hopefully can demonstrate more key functions of SLs. For performing a more accurate and detailed assessment, hormone inhibitors (TIS108, TIBA) were selected in addition to the hormones (*rac*-GR24, IAA) in different combinations.

6.2 Materials and Methods

6.2.1 Preparation of a culture medium for seed germination

It was done as previously explained manner: first, a half-strength MS medium was made and its pH was adjusted to 5.8 and then was solidified a solidifying agent.

After autoclaving, the medium cooled down under a laminar flow hood, and later was poured into sterilized petri dishes.

The sugar beet seeds were sorted into (almost) the same size. The sorted seeds were sterilized using a hypochlorite solution (5%) for 1 h, after which they were rinsed five times with sterile distilled water.

Next, the sterilized sugar beet seeds were cultured in the petri dishes containing hormone-free germination medium and kept in the growth chamber to germinate and produce small plantlets.

6.2.2 Preparation of media for examination of relation between SLs and auxin

After preparing a full-strength MS medium supplemented with 30 g L^{-1} of sucrose, it was divided up into the same amounts and poured in different bottles. Later, pH of them was adjusted to 5.8. Then, the proper amount of the solidifying agent was added to each bottle. After autoclaving, they were allowed to cool down under the laminar flow hood and soon after being enough cool, different amounts of the hormones (*rac*-GR24, IAA) and the inhibitors (TIS108, TIBA) from their newly prepared stocks were added to the bottles proportional to the specified concentrations and combinations as follows:

M0: Control

M1: 2 µM TIS108

M2: 2 µM TIS108 + 10 µM *rac*-GR24

M3: 10 µM indole-3-acetic acid (IAA) + 2 µM TIS108

M4: 10 µM 2,3,5-triiodobenzoic acid (TIBA) + 10 µM *rac*-GR24

M5: 10 µM TIBA + 10 µM *rac*-GR24 + 10 µM IAA

M6: 10 µM TIBA + 10 µM *rac*-GR24 + 10 µM IAA + 2 µM TIS108

The bottles were swirled to mix well. Then, the mixed media were poured into sterilized magenta boxes with corresponding labels.

6.2.3 Transferring small plantlets to the magenta boxes

The healthy small plantlets in the same size were selected from the petri dishes. Then, their endings (radicles) were cut. After that, the plantlets with roughly the same height were transferred to the magenta boxes containing different combinations of TIS108, TIBA, *rac*-GR24, and IAA. Three plantlets were cultured in each magenta box.

6.2.4 Statistical analysis

The data were analyzed using appropriate methods of analysis. Levene's test of homogeneity of variances and Shapiro–Wilk (S-W) and Lilliefors corrected Kolmogorov–Smirnov (K-S) tests of normality assumptions were used for the data. Wherever appropriate, the data were subjected to one-way analysis of variance using Welch's adjusted F ratio for one-way ANOVA and Games–Howell post hoc tests were run to examine the differences between groups. Alternatively, the data were analyzed by the Kruskal–Wallis (K-W) test followed by the Bonferroni corrected Dunn's post hoc test or a stepwise step-down multiple comparison post-hoc test, adopted from Campbell and Skillings' method (1985). The level of significance for the analyses was considered $p < 0.05$. A Windows™ based SPSS® program (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and graph drawing.

6.3 Results

The effects of the plant growth regulators on the number of leaves were compared using one-way ANOVA on ranks. The K-W test for the treatments was statistically significant (χ^2 (6, N = 63) = 43.438; p < .001), and the mean ranks of the variables were 45.39 for the control, 44.78 for TIS108, 32.33 for TIS108+GR24, 53.06

for TIS108+IAA, 11.67 for TIBA+GR24, 21.11 for TIBA+GR24+IAA, and 15.67 for TIBA+GR24+IAA+TIS108. A step-down post hoc with the Bonferroni corrected Dunn's test indicated that TIS108+IAA had the highest number of leaves, while the group with TIBA+GR24+IAA+TIS108 had the lowest number of leaves (Figure 6.1).

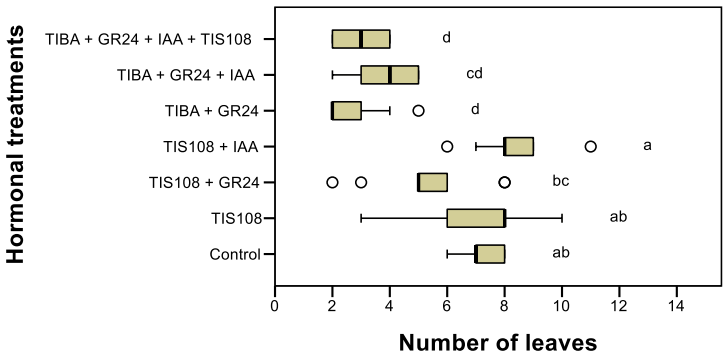


Figure 6.1. Effects of the interaction of different plant growth regulators on the number of leaves in sugar beet explants.

The effects of the plant growth regulators on the length of leaves were compared using ANOVA, which was statistically significant (*Welch's F*(6, 23.676) = 39.498, $p < .001$). The test was followed by the Games–Howell post hoc procedure to compare the means. While the control produced the longest leaves, TIBA+GR24, TIBA+GR24+IAA, and TIBA+GR24+IAA+TIS108 resulted in the shortest ones (Figure 6.2).

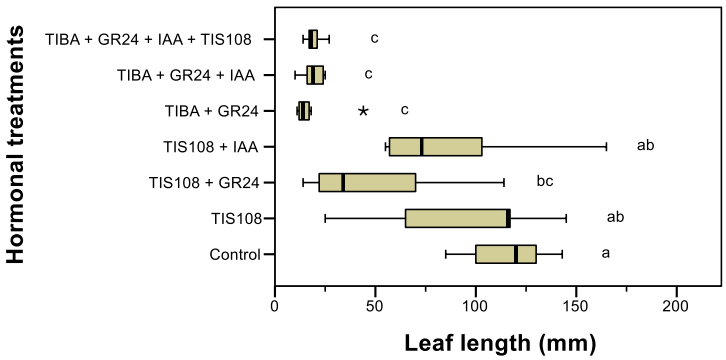


Figure 6.2. Effects of the interaction of different plant growth regulators on the length of leaves in sugar beet explants.

The effects of the plant growth regulators on leaf area were compared using the K-W test, which was statistically significant (χ^2 (6, N = 63) = 41.671; p < .001). The mean ranks of the treatment effects were 52.44 for the control, 47.94 for TIS108, 32.44 for TIS108+GR24, 41.89 for TIS108+IAA, 10.33 for TIBA+GR24, 18.44 for TIBA+GR24+IAA, and 20.50 for TIBA+GR24+IAA+TIS108. A step-down post hoc with the Bonferroni corrected Dunn's test indicated that the control had the largest area of leaves, while the group with TIBA+GR24 and two other similar treatments had the smallest area of leaves (Figure 6.3).

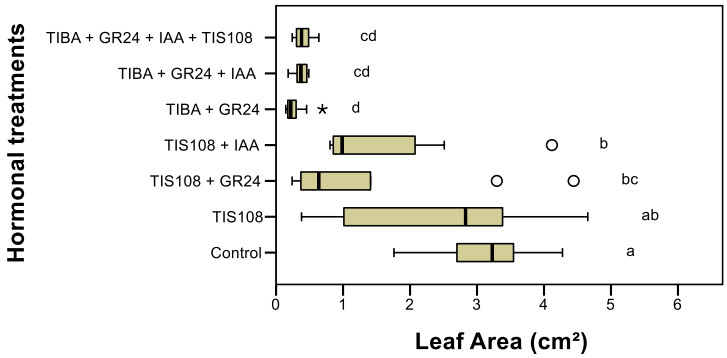


Figure 6.3. Effects of the interaction of different plant growth regulators on the area of leaves in sugar beet explants.

The effects of the plant growth regulators on length of the roots were statistically significant (χ^2 (6, N = 63) = 53.424; p < .001). The mean ranks of the treatments were 57.39 for the control, 48.83 for TIS108, 26.39 for TIS108+GR24, 40.39 for TIS108+IAA, 17.00 for TIBA+GR24, 17.00 for TIBA+GR24+IAA, and 17.00 for TIBA+GR24+IAA+TIS108. A step-down post hoc with the Bonferroni corrected Dunn's test indicated that the control produced the longest roots, while the group with TIBA+GR24 and two other similar treatments (TIBA+GR24+IAA and TIBA+GR24+IAA+TIS108) had the shortest roots (Figure 6.4).

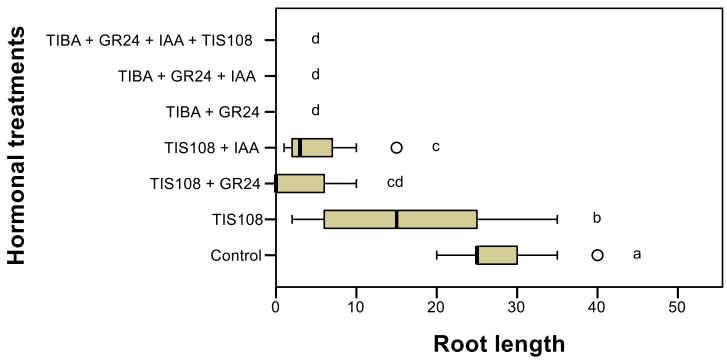


Figure 6.4. Effects of the interaction of different plant growth regulators on the length of the root in sugar beet explants.

The effects of the plant growth regulators on the number of the roots were statistically significant (χ^2 (6, N = 63) = 54.271; p < .001). The mean ranks of the treatments on the number of the roots were 51.83 for the control, 52.67 for TIS108, 23.61 for TIS108+GR24, 44.89 for TIS108+IAA, 17.00 for TIBA+GR24, 17.00 for TIBA+GR24+IAA, and 17.00 for TIBA+GR24+IAA+TIS108. A step-down post hoc with the Bonferroni corrected Dunn's test indicated that the control, TIS108, and TIS108+IAA produced the highest number of roots, while the treatments with TIBA+GR24, TIBA+GR24+IAA, and TIBA+GR24+IAA+TIS108 resulted in the lowest number of roots (Figure 6.5).

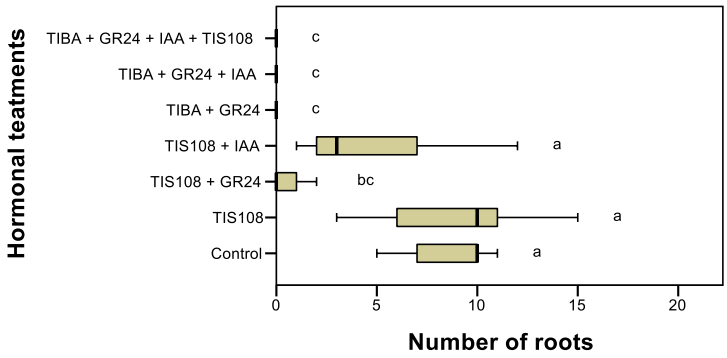


Figure 6.5. Effects of the interaction of different plant growth regulators on the number of the root in sugar beet explants.

6.4 Discussion

There are two major proposed hypotheses for the interaction between auxin and SLs. One proposes that there can be one or two second messengers, for instance SLs and CKs, mediating the auxin regulated processes. The other hypothesis is canalization hypothesis, which can connect buds to stem and the other parts of the plant through transporting and moving auxin away from auxin source (buds) (Sauer et al., 2006; Balla et al., 2011). Our findings demonstrated that for all the parameters evaluated in this part of the experiment, *rac*-GR24 in combination with TIBA (an auxin transport inhibitor) showed its highest and maximum inhibitory effects as compared with other treatments. It can be interpreted as the independency of SLs effects on plant architecture. The results are consistent and similar to the findings of Shinohara et al. (2013)'s work, which indicated that SLs could act directly and without apical auxin supply for inhibition of bud outgrowth.

CHAPTER VII

7 Conclusions

It is suggested that the putative gene (encoding Cytochrome P450 711A1) in sugar beet (*Beta vulgaris*) is most likely an ortholog of *MAX1* gene in *Arabidopsis* and the gene is involved in the biosynthesis pathway of SLs in sugar beet.

- SLs are effective in modifying sugar beet architecture in *in vitro* culture medium.
- The inhibitory effects of SLs have been demonstrated in the growth of the *in vitro* sugar beet explants.
- It has been revealed that SLs could alleviate the detrimental and adverse effects of salinity and drought conditions on the stress-exposed sugar beet plants.
- Regarding the interplay between auxin and SLs, it seems that SLs can act directly and independent of auxin.

Further detailed research studies can clarify more about the pathways and cross-talks involved in the processes and will further evaluate the obtained results. Any results which will come out from this thesis has a very likely potential to be extrapolated to many other important crop plants in Amaranthaceae family, and supposedly to almost all flowering plants.

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