DEVELOPMENT AND CHARACTERIZATION OF *SOLANUM LINNAEANUM* HAIRY ROOT LINES

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To my precious family…

ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF *SOLANUM LINNAEANUM* HAIRY ROOT LINES

Solanum linnaeanum, a wild relative of *Solanum melongena*, is native to several African countries and traditionally used for its therapeutic properties. This plant produces various secondary metabolites, such as phenolic compounds and glycoalkaloids, known for their antibiotic, antifungal, and antiviral activities. Hairy root culture, a common technique in plant tissue culture, is employed to produce these metabolites and study plant metabolic processes. In this study, an agropine strain of *Agrobacterium rhizogenes* (ATCC 43057) was used to induce hairy root cultures in *S. linnaeanum* and *S. melongena*. Transformation efficiency was higher in hypocotyl explants (22.22-31.92%) compared to cotyledon explants (18.67-26.11%), although the difference was not statistically significant. T-DNA integration was confirmed via PCR, and the transformed roots were grown in MS liquid medium for biochemical analysis. The study found that *S. linnaeanum* hairy root cultures had higher average total phenolic content (5.75 mg/g DW), flavonoid content (14.85 mg/g DW), and total antioxidant capacity (11.45 mg/g DW) compared to *S. melongena* hairy root cultures, which had 3.83 mg/g DW phenolic content, 9.32 mg/g DW flavonoid content, and 9.76 mg/g DW antioxidant capacity. Expression analysis of the *myb1*, *HQT*, and *Game9* genes showed higher expression levels in *S. linnaeanum* hairy roots than in hairy root cultures of *S. melongena*. These results indicate that *S. linnaeanum* hairy root cultures could be a promising source for secondary metabolite production, which could be further enhanced using elicitors or bioreactors.

ÖZET

SOLANUM LINNAEANUM TÜYLÜ KÖK HATLARININ GELİŞTİRİLMESİ VE KARAKTERİZASYONU

Solanum linnaeanum, birçok Afrika ülkesinde geleneksel olarak tedavi edici özellikleri nedeniyle sıkça kullanılır ve *Solanum melongena*'nın yabani bir akrabasıdır. Fenolik bileşikler ve glikoalkaloitler de dahil olmak üzere bu bitkinin ürettiği birçok ikincil metabolit, antibakteriyel, antifungal ve antiviral özellikler gösterir. Tüylü kök kültürleri ikincil metabolitlerin üretimi ve bitkinin metabolik yolaklarının çalışılması için sıkça kullanılan bir bitki doku kültürü yöntemidir. Bu çalışmada, *S. linnaeanum* ve *S. melongena* eksplantlarından tüylü kök hatları oluşturmak için *Agrobacterium rhizogenes*'in agropin suşlarından biri olan ATCC 43057 kullanıldı. İstatistiksel olarak anlamlı olmamasına rağmen, transformasyon verimliliği iki tür için de hipokotil eksplantlarında kotiledon eksplantlarına göre daha yüksek olarak bulundu. T-DNA bölgesinin bitki genomuna entegrasyonu PCR ile doğrulandıktan sonra, transforme edilen tüylü kökler sıvı MS besi yerinde büyütüldü ve biyokimyasal analizler yapıldı. Analizler sonucunda, *S. melongena* tüylü kök hatlarıyla karşılaştırıldığında, *S. linnaeanum* tüylü kök hatlarında total fenolik (5,75 mg/g kuru ağırlık) ve total flavonoid (14,85 mg/g kuru ağırlık) içeriğinin ve total antioksidan kapasitesinin (11,45 mg/g kuru ağırlık) daha yüksek olduğu gözlemlendi. Ayrıca, *myb1*, *HQT* ve *Game9* genlerinin ekspresyon analizi *S. linnaeanum* tüylü kök kültürlerinde daha fazla mRNA seviyeleri gösterdi. Bu sonuçlar, ikincil metabolit üretimi için *S. linnaeanum* tüylü kök kültürlerinin, elisitörler veya biyoreaktörler kullanılarak daha da geliştirilebilecek potansiyel bir kaynak olabileceğini göstermektedir.

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CHAPTER 1

INTRODUCTION

1.1. Plant Secondary Metabolites

Plants produce many important phytochemicals in addition to primary metabolites which they use for their growth and development. Secondary metabolites are the major class of phytochemicals that plants produce mainly to defend themselves against biotic and abiotic stress factors for their survival (Guerriero et al., 2018). Besides protecting the plant from various stress factors, secondary metabolites also have many significant functions such as attracting pollinators and establishing symbiosis (Ncube & Van Staden, 2015). In addition to benefiting plants in nature, these chemicals are used in various industrial fields namely in pharmaceutical, cosmetic and textile sectors. Therefore, plant secondary metabolites are well worth study in terms of both science and industry.

Plant secondary metabolites can be broadly classified into three groups: phenolics, nitrogen-containing compounds and terpenes (Saxena et al., 2013).

1.1.1. Phenolics

Phenolic compounds are a very common group of plant secondary metabolites, and the types of compounds vary between plants (Lattanzio, 2013). Phenolics are derived from benzene rings with one or more hydroxyl groups (-OH) (Velderrain-Rodríguez et al., 2014) and they can range from simple phenolic molecules to highly polymerized compounds. Higher plants are known to synthesize numerous phenolic compounds including flavonoids, chlorogenic acid, hydroquinone and xanthones (Tsimogiannis & Oreopoulou, 2019) through the phenylpropanoid pathway (Fraser & Chapple, 2011).

Due to their antioxidant properties, phenolic compounds have been commonly used as drugs to prevent and/or treat several diseases such as diabetes, cancer, and Alzheimer's disease (Aqil et al., 2006) since they can absorb and neutralize free radicals

and decompose peroxides (Osawa, 1994). For instance, Hussain et al. (2016) showed that reactive oxygen species (ROS) production decreased when fibroblast cells were exposed to phenolics, and Tanaka et al. (1993) demonstrated that treatment of fibroblast cells with phenolics increased collagen expression in the tissue.

1.1.2. Nitrogen-containing Compounds

Nitrogen-containing secondary metabolites are derived from common amino acids, and they contain a nitrogen atom in their structure (Jan et al., 2021). Nitrogen-containing compounds can be divided into three major groups: alkaloids, cyanogenic glycosides and glucosinates, with alkaloids being the largest family (Taiz & Zeiger, 2006).

Alkaloids play roles in germination and protection against predators in plants as well as having pharmacological effects such as anxiolytic and analgesic properties which act on the central nervous system (Twaij & Hasan, 2022). The first alkaloid which was isolated from a plant was morphine and it was first extracted from opium in 1803 by Friedrich Serturner (Courtwright, 2001). Many alkaloids including morphine, strychnine, quinine, and ephedrine are used for clinical purposes (Kurek, 2019). For example, it was shown that some alkaloids extracted from *Solanum khasianum* might be used against HIV infection along with the intestinal infection related to AIDS (Lewis & Elvin-Lewis, 1995).

1.1.3. Terpenes

Among plant secondary metabolites, terpenes are the largest and the most structurally diverse group which are derived from isoprene units (Anulika et al., 2016). Terpenes act as precursor molecules for numerous phytohormones, sterols and pigments which have a variety of functions in plants including attracting pollinators and providing defense against herbivores (Jan et al., 2021).

Terpenes have antimicrobial properties as it was shown that they induced cell rupture and inhibited the protein and DNA synthesis of both antibiotic-susceptible and antibiotic-resistant bacteria (Álvarez-Martínez et al., 2021). They are also widely used in the food industry as flavoring agents. For instance, 1-menthol, which is commonly used

as a flavoring chemical in cosmetics, cigarettes, toothpaste etc., is extracted from *Mentha arvensis* (Ninkuu et al., 2021).

To date, various studies have shown that terpenes also have therapeutic effects including antiviral, analgesic, and anti-inflammatory properties which make them very crucial for pharmaceutical and clinical applications (Xavier et al., 2023). For example, it was demonstrated that in lymphocyte cells, betulinic acid extracts from *Syzygium claviflorum* showed anti-HIV activity (Brahmkshatriya & Brahmkshatriya, 2013). Moreover, Jayakumar et al. (2018) showed that hinokitol, a natural monoterpenoid, which suppresses MMPs and induces the synthesis of several important antioxidant enzymes, also inhibited migration of A549 lung cancer cells.

1.2. Plant Families with Important Secondary Metabolism

Plants have always been essential for humankind throughout history due to their extraordinary properties. In ancient times, people used various plants for several purposes besides serving as a food source including as treatments, flavors, scents, and dyes. Plants and the phytochemicals that they produce are very important research subjects considering their potential uses in numerous fields. All plants produce phytochemicals to protect themselves in their habitat and to survive, although the types and the production levels of these chemicals may vary between different species. Nevertheless, there are several plant families that are well-known for their secondary metabolism including Amaryllidaceae, Asteraceae, Lamiaceae and Rosaceae (Bozyel et al., 2019).

1.2.1. Amaryllidaceae

Amaryllidaceae is a plant family which contains approximately 70-75 genera and 1,600 species (Christenhusz & Byng, 2016). They are mainly distributed in tropical regions and in warm climates such as southern Africa and the Mediterranean (Elgorashi, 2019). Many plant species belonging to the Amaryllidaceae family are known to be used to treat numerous diseases including headaches, wounds, and infertility in traditional medicine due to their rich alkaloid content (Hutchings et al., 1996).

To date, approximately 50 different alkaloids have been extracted from various species of Amaryllidaceae including buphanadrine, lycorine, and distichamine

(Elgorashi, 2019) with different biological activities such as antimicrobial, antitumor, and anti-inflammatory effects (Elgorashi & van Staden, 2010). For instance, lycorine and distichamine were shown to induce apoptosis in cancer cells (Nair & van Staden, 2018). Also, galanthamine extracted from different species of the Amaryllidaceae family was shown to inhibit the AChE enzyme (Elgorashi, 2019), which degrades acetylcholine at the central cholinergic synaptic junction and thus results in memory loss (Selkoe, 1992). Cherylline and epivittatine are two alkaloids extracted from Amaryllidaceae species which act as selective serotonin re-uptake inhibitor (SSRIs) suggesting that they can be used as antidepressants (Elgorashi et al., 2006). Furthermore, buphanidrine and distichamine extracted from *Boophone disticha* successfully inhibited the growth of both Gram-positive and Gram-negative bacteria strains (Cheesman et al., 2012). Finally, in a recent study it was shown that isoquinoline alkaloids from different species of the Amaryllidaceae family acted as antiviral agents by inhibiting DNA, RNA, and protein synthesis of various pathogens (Nair & van Staden, 2023).

1.2.2. Apiaceae

Apiaceae contains approximately 3,800 species including many vegetables and aromatic herbs and they are widely distributed throughout the world. Apiaceae is one of the most important plant families in terms of their secondary metabolite content with some of them having herbicidal, insecticidal, or antimicrobial activities which can be used in agriculture (Thiviya et al., 2022). Essential oil extracts, which are stored in Apiaceae plants, were shown to have insecticidal activity suggesting that they can be used to protect crops (Benelli et al., 2018). They can also be used as herbicides. For example, the monoterpene-rich essential oil extract of *Carum carvi* showed herbicidal activity against barnyard grass (Synowiec et al., 2019).

Besides their potential use in agriculture, the rich secondary metabolite content of Apiaceae species is also important for medicinal uses. Many vegetables belonging to the Apiaceae family including celery and carrot are rich in flavonoids (Wang et al., 2022). For instance, ferulic acid extracted from two different species of Apiaceae family was shown to have antitumor activity on breast cancer cells (Zhang et al., 2016). Moreover, Zhou et al. (2020) demonstrated that asiaticoside, a terpenoid, induced apoptosis in colorectal cancer cells via regulating various signaling pathways.

1.2.3. Asteraceae

With approximately 32,000 species, Asteraceae is one of the largest flowering plant families distributed in various regions in the world. Plants belonging to the Asteraceae family show a variety of biological activities such as antioxidant, antimicrobial, and anti-inflammatory effects; therefore, they are considered as important medicinal plants (Rolnik & Olas, 2021). Asteraceae species are shown to synthesize various phenolic compounds including chicoric acid, quercetin, caffeic acid, and chlorogenic acid (Petropoulos et al., 2019).

In an in-vivo study, phenolic acid extract of *Cynara scolymus* showed anti-inflammatory activity by increasing total leukocyte and lymphocyte cells (Hueza et al., 2019). Eruygur et al. (2019) demonstrated that total phenol and flavonoid extracts of *Achillea cucullata* inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Furthermore, anthocyanins extracted from *Cichorium intybus* leaves were shown to have antioxidant activity since they are able to neutralize free radicals (Mulabagal et al., 2009).

1.2.4. Leguminosae

Leguminosae is the third-largest terrestrial plant family which includes approximately 20,000 species (Benjamim et al., 2020). Members of this family are widely distributed throughout the world. These species produce a variety of important secondary metabolites with different biological activities; therefore, they are commonly used for therapeutic purposes.

de Araújo et al. (2014) revealed that *Libidibia férrea* produces many secondary metabolites including saponins, tannins, triterpenes, and phenolic acids like gallic and ellagic acid. It was shown that gallic and ellagic acid produced by *L. ferrea* can be used as an antiproliferative agent in colorectal cancer treatment (Guerra et al., 2017). Extracts of *Bauhinia purpurea* leaves also showed antiproliferative activity since they inhibited the proliferation of cancer cells without harming normal cells (Zakaria et al., 2011). Moreover, essential oil extracted from the seeds of *Pterodon emarginatus* successfully inhibited the growth of *Staphylococcus aureus,* thus demonstrating antibacterial activity (Dutra et al., 2009).

1.2.5. Rosaceae

Rosaceae is a flowering plant family which consists of approximately 5,000 species and 91 genera (Christenhusz & Byng, 2016) including *Rubus*, *Sorbus*, *Alchemilla* and *Prunus* that contains plums, cherries, and peaches (Bortiri et al., 2001). Although they are commonly found in the Northern Hemisphere, these species can be found almost everywhere in the world except for Antarctica. Plants from the Rosaceae family are economically very important since they have ornamental and therapeutic properties besides being important food sources (Kostikova & Petrova, 2021). Also, several genera from the Rosaceae family have a high capacity to produce important secondary metabolites such as flavonoids, alkaloids, and triterpenes. For instance, the *Spiraea* genus is shown to produce high amounts of flavonoids, lignans, and terpenes with various biological activities (Kostikova & Petrova, 2021).

Choudhary et al. (2009) revealed that quercetin extracted from *Spiraea canescens* has antioxidant activity since it acted as a scavenger of DPPH radicals. In an in vitro study, extracts from 14 different plant species of the genus *Spiraea* were used to test their antiviral activity on human influenza virus (H3N2) and avian influenza virus (H5N1), and it was demonstrated that all species had an antiviral effect (Kostikova et al., 2016). Ni et al. (2024) analyzed fruits of *Rosa roxburghii, Rosa sterilis, Rosa laevigata, Rosa davurica*, and *Rose sericea* and showed that extracts of all five *Rosa* fruits demonstrated antioxidant activity by decreasing the levels of malondialdehyde while increasing the activities of superoxide dismutase and glutathione peroxidase in the H_2O_2-induced HaCaT cell model.

1.2.6. Solanaceae

The Solanaceae family is also known as the nightshades and it contains nearly 3,000 species in more than 100 genera, some of which are very well-known such as *Solanum*, *Capsicum*, and *Physalis* (Biswas et al., 2023). Species belonging to this family have many therapeutic properties due to the secondary metabolites they produce. Solanaceae species produce various types of secondary metabolites such as terpenes, flavonoids, tannins, phenolics, and alkaloids (Chowański et al., 2016) which show a wide

range of biological activities including anti-inflammatory, antimicrobial, and antiseptic effects.

For instance, it was shown that glycoalkaloids such as solasonine, solanidine, and solamargine from *Solanum melongena* inhibited cell proliferation in liver cancer cell lines via cell cycle arrest and induced apoptosis (Fekry et al., 2019). Moreover, it was demonstrated that cinnamic acid produced by *Solanum* species has an antibacterial activity since it successfully inhibited the growth of *Staphylococcus aureus* (Kaunda & Zhang, 2019). Careaga et al. (2003) demonstrated that ethanolic extract of *Capsicum annuum* had antimicrobial activity against several species including *Micrococcus, Bacillus, Pseudomonas*, and *Citrobacter*.

1.3. Eggplant

Eggplants are one of the most widely consumed vegetables in the world and they are valued for their taste and nutrient content. Three cultivated eggplant species are known: the brinjal eggplant (*Solanum melongena*), the gboma eggplant (*Solanum macrocarpon*), and the scarlet eggplant (*Solanum aethiopicum*).

Solanum melongena is the most popular eggplant species which is commonly consumed as a vegetable, and it was first domesticated in Southeast Asia (Daunay, 2008). *S. melongena* contain many bioactive compounds including phenolic compounds and flavonoids besides nutrients; thus, it is an important source of phytochemicals (Gürbüz et al., 2018). Phenolic compounds are the major bioactive compounds showing antioxidant activity in eggplants (Sharma & Kaushik, 2021). Total phenolic content in different cultivars was studied by various researchers. For example, total phenolic content of American-type eggplant cultivar was 1512.5 mg/100 g, while it was 2049.8 mg/100 g for a Thai-type cultivar (Sharma & Kaushik, 2021). Anthocyanin is another bioactive compound which is very effective against several health problems including diabetes, cancer, and cardio-vascular disorders (Yousuf et al., 2016). It has additional biological activities such as anti-allergic, antioxidant, anti-inflammatory, antimicrobial and antiviral effects (Ghosh & Konishi, 2007). Anthocyanin content varies with different cultivars of eggplants. For instance, it was shown that anthocyanin content for a Thai cultivar was 3.9 mg/100 g, while it was 161.1 mg/100 g for a Philippine cultivar (Nino-Medina et al., 2014). Flavonoids are another group of bioactive compounds that eggplants contain, and

their total amount also varies between different cultivars. In a study carried out with different genotypes of Indian eggplant, the total flavonoid content varied from 3.23 to 25.96 mg quercetin equivalent/100 g (Kaur et al., 2014).

Solanum macrocarpon is another cultivated eggplant species which is commonly called "Gboma" (Dougnon et al., 2012). Its leaves and fruits are used as traditional medicine as well as consumed as a vegetable (Dougnon et al., 2012). It was shown that *S. macrocarpon* is a richer source of glycoalkaloids compared to the common eggplant *S. melongena* (Daunay, 2008). In another study, phytochemicals which *S. macrocarpon* produces were investigated, and it was revealed that leaves of *S. macrocarpon* contain alkaloids, tannins, and saponins while its fruits contain alkaloids, tannins, mucilages, and coumarins (Dougnon et al., 2012). Also, it was shown that the glycoalkaloid levels of *S. macrocarpon* fruits are much higher than to those of *S. melongena* (Sánchez-Mata et al., 2010).

Solanum aethiopicum, also known as the scarlet eggplant, is a cultivated eggplant species commonly consumed in Africa (Kamga et al., 2013). It is eaten as a vegetable due to its nutritional content. Besides its nutritional constituents, *S. aethiopicum* is also a rich source of valuable phytochemicals. It was shown that fruits of *S. aethiopicum* contain saponins, tannins, flavonoids, alkaloids, and steroids (Abubakar et al., 2020).

1.4. *Solanum linnaeanum*

Non-domesticated *Solanum* species are also known to be rich sources of valuable phytochemicals with various biological activities including antioxidant, antibacterial, antifungal, and anti-inflammatory effects. To date, many studies have indicated the antifungal features of wild *Solanum* species extracts (Ramanathan et al., 2018; Sunitha et al., 2017). For example, it was shown that the soilborne fungi *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Fusarium oxysporum* can be effectively controlled by diethyl ether extract of *Solanum trilobatum* and methanolic extract of *Solanum surattense* (Tuba et al., 2016).

Solanum linnaeanum, also known as devil's apple, is a wild *Solanaceous* species which is native to many African countries and southern Europe (Nefzi et al., 2018). *S. linnaeanum* has spiny leaves, purple flowers with small yellow stigmas. Its fruits, which are known to be poisonous, look like unripe tomatoes.

Elabbara et al. (2014) showed that *S. linnaeanum* is a rich source of alkaloids, steroids, and saponins. Also, it was shown that *S. linnaeanum* berries are rich in glycoalkaloids, especially solamargine and solasonine which are commonly used for cancer treatment and as a precursor of steroidal drugs (Gürbüz et al., 2015). In another study, it was revealed that *S. linnaeanum* leaf, stem, and fruit extracts have antifungal activity against *Fusarium oxysporum* (Nefzi et al., 2018).

1.5. Hairy Root Cultures

Plants are important sources of many valuable chemicals besides serving as nutritional sources. Phytochemicals like terpenes, alkaloids and phenolics produced by plants are commonly used in the pharmaceutical, food, cosmetic, and agrochemical industries (Abdulhafiz et al., 2022), thus they have enormous market potential (Dhiman et al., 2018). In nature, plants produce these phytochemicals via different metabolic pathways in various plant parts including leaves, seeds, floral buds, and roots (Gantait & Mukherjee, 2021). However, extraction of these phytochemicals from plants with conventional methods is challenging, time-consuming, and it may damage the plant's ecosystem and biodiversity. To overcome this problem, the production and extraction of important secondary metabolites can be achieved via different in vitro applications such as cell suspension culture and hairy root cultures (Gutierrez-Valdes et al., 2020).

Plant cell suspension culture is a simple and cost-effective method for large-scale production of valuable secondary metabolites (Gonçalves & Romano, 2018). In this method, cells from callus tissues divide and multiply in a liquid culture while producing metabolites. If the culture time is prolonged, cell suspension culture may become unstable due to the consumption of the nutrients in the culture media, and thus the quality and the quantity of produced secondary metabolites are decreased (Motolinía-Alcántara et al., 2021). Also, genetic variation may arise in the culture which affects the yield of secondary metabolite production (Ochoa-Villarreal et al., 2016). To date, several valuable secondary metabolites have been produced with plant cell suspension culture and some examples are given in Table 1.1.

Table 1.1. Plant-derived products produced from plant cell suspension culture and used in the pharmaceutical industry. (Source: Motolinía-Alcántara et al., 2021)

Species	**Product**	**Pharmaceutical use**
Coleus blumei	Rosmarinic acid	Anti-inflammatory
Echinacea purpurea	Echinacea polysaccharides	Immunostimulant, anti-inflammatory
Podophyllum spp.	Podophyllotoxin	Anticancer
Taxus baccata	Docetaxel	Ovarian cancer treatment
Lithospermum erythrorhizon	Shikonin	Anti-HIV, antitumor, anti-inflammatory

Hairy root culture is another in vitro approach for large-scale production of valuable secondary metabolites. This approach involves the use of *Agrobacterium rhizogenes*, which is a gram-negative soil bacterium (Gantait & Mukherjee, 2021). *A. rhizogenes* strains have a plasmid called the Ri-plasmid (root-inducing plasmid) which contains *rol* genes. As explained in section 1.6.2.1., these genes are integrated into the plant genome once the plant is infected. Upon infection and integration of the *rol* genes, hairy roots emerge from the infected tissues. These hairy roots have a very high growth rate without the need for any plant growth regulators. Also, hairy root cultures are physiologically and biochemically highly stable compared to plant cell suspension cultures (Hussain et al., 2022). This difference in stability of the cultures arises from the use of plant growth regulators in cell suspension cultures which may result in change in the chromosome number and somaclonal variation (Häkkinen et al., 2016). Also, plant cells are known to be genetically unstable when they are grown in an undifferentiated state like in cell suspension culture. In this case, rearrangements may occur at the chromosomal or gene level (Häkkinen et al., 2016).

1.5.1. Applications of Hairy Root Culture

Hairy root cultures can be used for several purposes including secondary metabolite production, recombinant protein production, and phytoremediation due to their advantages over other plant culture techniques.

1.5.1.1. Secondary Metabolite Production

Hairy root cultures are capable of producing secondary metabolites at levels that are either comparable to or higher than those of normal roots (Dhiman et al., 2018), therefore they provide an efficient approach for large-scale production of valuable secondary metabolites. Further manipulations including genetic engineering, elicitation and optimization of physical parameters can also be applied to hairy root cultures in order to enhance the production of secondary metabolites (Gerszberg & Wiktorek-Smagur, 2022). To date, various secondary metabolites have been produced by hairy root cultures with some examples given in Table 1.2.

Table 1.2. Examples of secondary metabolites produced by hairy root cultures. (Source: Gutierrez-Valdes et al., 2020)

Species	**Products**	**Usage**
Astragalus membranaceus	Isoflavonoid	Antioxidant, antimutagenic, anti-carcinogenic, antiproliferative
Echinacea pupurea	Caffeic	Anti-inflammatory, anticancer, antiviral
Fagopyrum tataricum	Rutin, quercetin	Anti-inflammatory, anti-carcinogenic, antioxidant
Papavar orientale	Morphine	Analgesic
Salvia castanea	Tanshinone	Anti-tumor, anti-inflammatory, antioxidant, neuroprotective
Scopolia parviflora	Scopolamine	Anticholinergic

1.5.1.2. Recombinant Protein Production

Plants are increasingly used to produce therapeutic proteins for drug and/or vaccine production (Gerszberg & Hnatuszko-Konka, 2022). For example, taliglucerase alfa, a drug for Gaucher's disease, is successfully produced in carrot cell cultures and is the first such plant-produced pharmaceutical approved by the U.S. Food and Drug Administration (Owczarek et al., 2019). Hairy root cultures can be used to produce recombinant proteins that are therapeutically important at satisfactory levels once an appropriate expression cassette is designed (Gerszberg & Wiktorek-Smagur, 2022). To date, various recombinant proteins have been produced by hairy root cultures (Table 1.3).

Table 1.3. Examples of recombinant proteins produced in hairy root cultures. (Source: Gerszberg & Wiktorek-Smagur, 2022)

Species	Protein	Usage	Concentration
Helianthus tuberosus	Human interferon (HuINFa_2b)	Antiviral activity	54,500 IU/g FW
Solanum tuberosum	Hepatitis B surface antigen (HBsAg)	Hepatitis B vaccine	97.1 ng/g FW
Nicotiana tabacum	IpaD antigen	Shigellosis treatment (vaccine)	0.94 ng per µg TSP
Nicotiana benthamiana	Human glucocerebrosidase (GCase)	Enzyme activity, Gaucher disease treatment	1 µg/g
Solanum lycopersicum	Antifungal antibody scFvFc 2G8	*Candida albicans* treatment	68 g/kg

1.5.1.3. Phytoremediation

Phytoremediation is a process in which plants are used to extract and remove pollutants in soil since plants can absorb ionic compounds via their roots (Yan et al., 2020). Phytoremediation is very advantageous since it is economically practical, eco-friendly, and easily applicable. For example, a recent study showed that *Artemisia annua* can act as an arsenic or cadmium hyperaccumulator (Pandey et al., 2021). Moreover, hairy root cultures of various species including *Alyssum bertoloni, Solanum nigrum, Brassica juncea*, and *Thlaspi caerulescens* were used to accumulate cadmium, zinc, uranium, and nickel, respectively (Moola et al., 2021).

1.6. *Agrobacterium rhizogenes*

Agrobacterium rhizogenes is a gram-negative, rod-shaped soil bacterium which causes hairy root disease in dicotyledonous plants and belongs to the Rhizobiacea family (Nartop, 2018). *A. rhizogenes* is attracted to wounded plant tissues due to the phenolic compounds that plants produce (Veena & Taylor, 2007). *A. rhizogenes* possesses a large Ri (root-inducing) plasmid and the integration of the T-DNA region of this plasmid into the plant DNA leads to emergence of hairy roots from the infected tissues. Wild *A. rhizogenes* strains can be classified into three groups in terms of opine type: agropine (Figure 1.2), mannopine (Figure 1.1), and cucumopine. There are different regions in the

Ri plasmid, and each has various roles including transfer and integration of T-DNA, opine synthesis, and root induction and development.

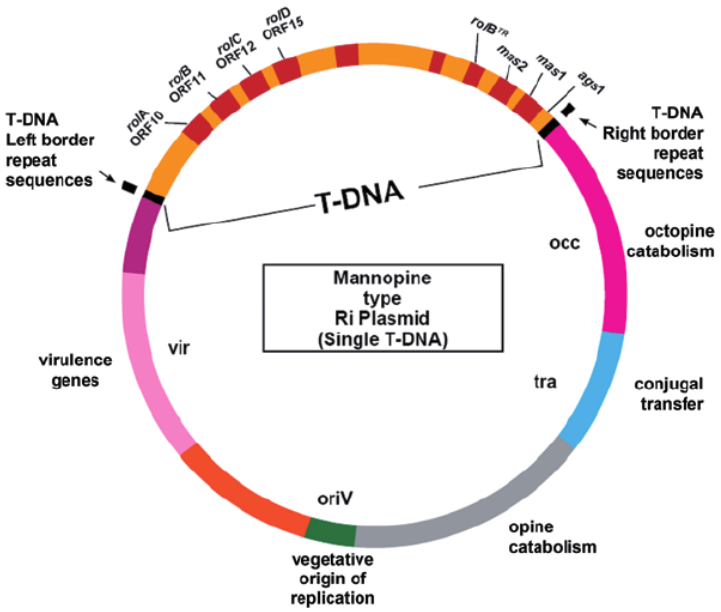

Figure 1.1. Schematic representation of Mannopine type Ri-plasmid of *A. rhizogenes*. (Source: Ozyigit et al., 2013)

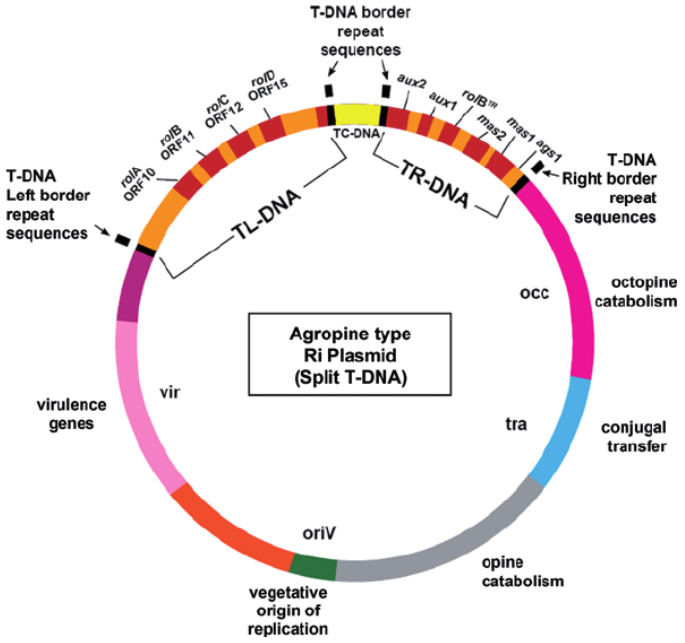

Figure 1.2. Schematic representation of Agropine type Ri-plasmid of *A. rhizogenes* showing the split TL and TR-DNA regions. (Source: Ozyigit et al., 2013)

1.6.1. Virulence (*vir*) Genes of Ri Plasmid

The virulence genes in the Ri plasmid of *A. rhizogenes* play roles in the mobilization and transfer of the T-DNA into the host plant genome (Bahramnejad et al., 2019). These genes are not integrated into the plant genome; however, they are crucial in terms of successful transformation of the T-DNA region to the plant genome since it is known that any mutation in these *vir* genes results in failure of T-DNA transfer (Toro et al., 1989).

1.6.2. T-DNA of Ri Plasmid

The structure of the T-DNA region in the Ri plasmid differs between strains of *A. rhizogenes*. Mannopine and cucumopine type strains have only one T-DNA region. However, agropine type strains are characterized by a split T-DNA region in which there are two T-DNA regions: TL-DNA and TR-DNA, with a spacer sequence between them (White et al., 1985).

1.6.2.1. The *rol* (root oncogenic loci) Genes

The *rol* genes are located on the T-DNA region of the Ri plasmid of mannopine and cucumopine type strains, and on the TL-DNA region of the Ri plasmid of agropine type strains of *A. rhizogenes* (Slightom et al., 1986; White et al., 1985). These *rol* genes play roles in rapid, hormone independent growth and increased branching of Ri transformed roots (Tepfer, 1990). To date, four *rol* genes have been identified which are *rolA*, *rolB*, *rolC*, and *rolD*; however, their molecular mechanism of action is not yet clearly understood (Bahramnejad et al., 2019).

The *rolA* gene has varying length depending on the strain of *A. rhizogenes* and encodes a small protein (Meyer et al., 2000). The *rolA* gene is found on all Ri plasmids and its product functions as a regulatory transcription factor (Veena & Taylor, 2007). Although the molecular mechanism of action is not known, *rolA* gene has several functions including inhibiting cell elongation, decreasing hormone concentrations, modulating hormone physiology of gibberellic acid, and interfering with polyamine metabolism (Ozyigit et al., 2013).

The *rolB* gene is also found on all Ri plasmids and it encodes a protein which is localized in the plasma membrane (Meyer et al., 2000; Veena & Taylor, 2007). *rolB* is thought to have an important role in hairy-root induction (Bellincampi et al., 1996) since root induction is decreased when the *rolB* gene is silenced in the Ri plasmid on kalanchoe leaves (White et al., 1985). Besides contributing to hairy root induction, the *rolB* gene has several important functions such as stimulating new meristem formation, inducing secondary metabolism, and modulating auxin signaling (Ozyigit et al., 2013).

Like the *rolA* and *rolB* genes, the *rolC* gene is found on all Ri plasmids. The product of the *rolC* gene has several important functions including reducing cell size; reducing abscisic acid (ABA), polyamine and ethylene levels; and regulating sugar metabolism and transport (Ozyigit et al., 2013). It also enhances the production of important secondary metabolites like tropane alkaloids (Bonhomme et al., 2000), pyridine alkaloids, indole alkaloids (Palazon et al., 1998), and ginsenosides (Bulgakov et al., 1998) in transgenic plants.

The *rolD* gene is only found on the TL-DNA region of agropine type Ri plasmids and it is not able to induce root formation on its own (Mauro et al., 1996). The *rolD* gene encodes a cytosolic protein which plays a role in conversion of ornithine to proline (Trovato et al., 2001).

1.6.2.2. The *aux* and Opine Genes

Genes that are involved in auxin and opine biosynthesis are found on the TR-DNA region of agropine type Ri plasmids (Christey, 2001). These genes are *aux1*, *aux2*, $rolB^{TR}$, *mas1*, *mas2*, and *ags* (Veena & Taylor, 2007). These genes are found only on Ri plasmids of agropine type strains of *A. rhizogenes* suggesting that the presence of these genes is not necessary to generate hairy root disease, although they contribute to the hairy root phenotype and help extend the host range of the bacterium (White et al., 1985; Cardarelli et al., 1987; Hansen et al., 1991; Sevón & Oksman-Caldentey, 2002).

1.6.3. Transfer of T-DNA

The T-DNA transfer process (Figure 1.3) starts with the attachment of *A. rhizogenes* to the plant cell walls with the help of the *Agrobacterium* chromosomal genes

chvA and *chvB* products which enhance the formation of cyclic β-1,2 glucan which is translocated into the periplasm (Chandra, 2012). Following attachment to the plant cell wall, *Agrobacterium* virulence (vir) proteins VirD1 and VirD2 nick the Ri plasmid at the T-DNA border repeat sequences. Then, VirD2 protein binds to the 5' end of the T-strand and this complex leaves the bacterium via a Type IV protein secretion system (T4SS) which consists of 11 VirB proteins and VirD4 protein (Chandra, 2012). Once this complex gets into the cytoplasm, virulence effector proteins, which contain nuclear localization signal (NLS) sequences, assist the T-DNA strand to target the nucleus. In addition to virulence effector proteins, a plant protein, importin α, is also involved in nuclear targeting of the T-DNA strand. Finally, with the assistance of VirF protein all vir effector proteins and plant proteins are removed from the complex and integration of the T-DNA strand into the plant chromosome takes place (Chandra, 2012).

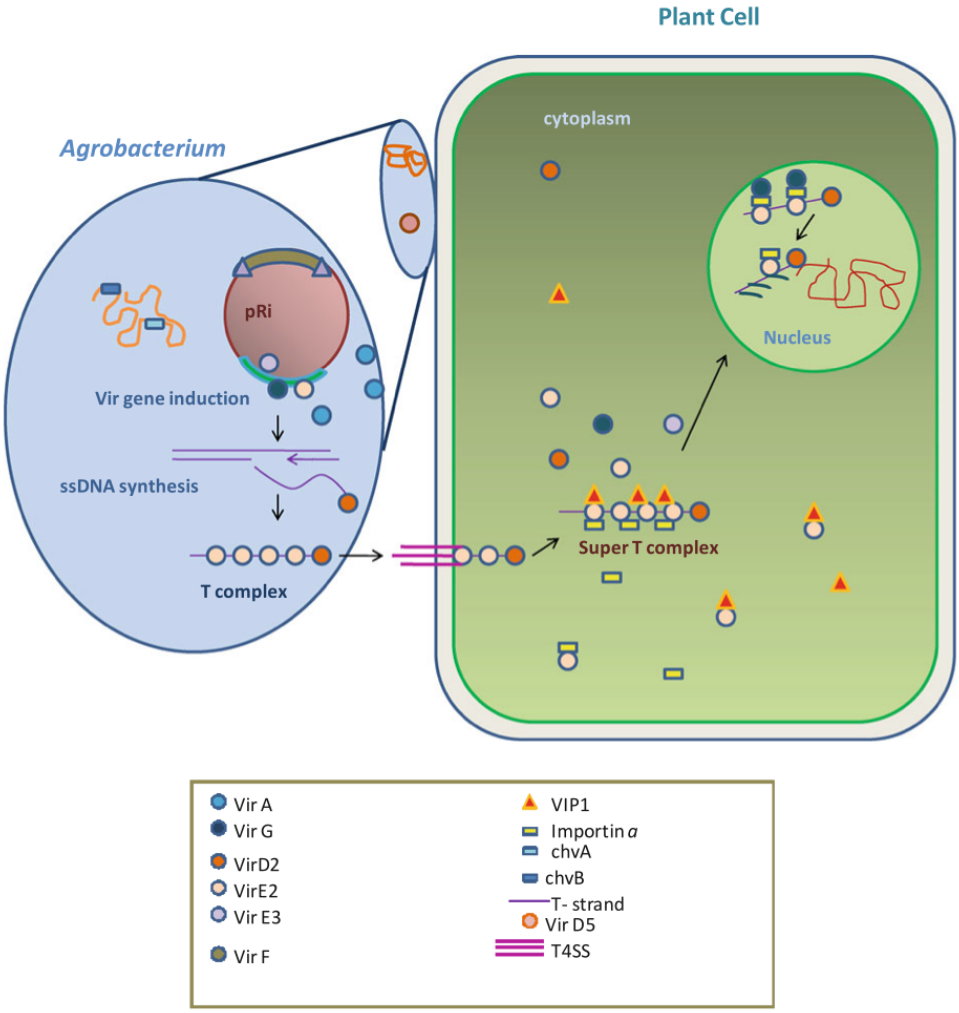

Figure 1.3. *Agrobacterium* Ri plasmid-based T-DNA transfer for plant genetic transformation. (Source: Chandra, 2012)

1.7. Aim of the Study

Secondary metabolites are compounds which are produced by plants but are not directly involved in growth, development, or reproduction. They are usually produced as a part of the defense mechanism of plants in response to stress. Many of these secondary metabolites have various biological activities including antibiotic, antiviral, anti-inflammatory, and antioxidant effects. Due to these features, secondary metabolites produced by plants are good sources of therapeutic compounds and for other industries such as cosmetics and textiles. In this research, the metabolic potential of hairy root cultures of two eggplant species, *Solanum melongena* and *Solanum linnaeanum,* were explored. Thus, the present study focused on developing hairy root cultures of these two species via *Agrobacterium*-mediated transformation. Total phenolic and total flavonoid contents and total water-soluble antioxidant activities of the cultures were analyzed. In addition, expression levels of three different genes, involved in various secondary metabolite production pathways, were determined. The resulting information can help improve our understanding of the mechanisms of secondary metabolite production in eggplant hairy root cultures and be used to enhance the production of these important phytochemicals with the use of elicitors or bioreactors in future studies.

CHAPTER 2

MATERIALS & METHODS

2.1. Materials

2.1.1. Plant Materials

Solanum melongena Kemer seeds were provided by Antalya Agriculture, Inc. and *Solanum linnaeanum* (MM195) seeds were originally provided by Marie-Christine Daunay (INRA) and subsequently collected from fruits of plants grown in the greenhouse.

2.1.2. Bacterial Strain

Agrobacterium rhizogenes strain ATCC 43057 was obtained from American Type Culture Collection (ATCC), Manassas VA, USA.

2.2. Methods

2.2.1. Seed Sterilization and Germination

S. melongena and *S. linnaeanum* seeds were kept at 4°C for 2 days and then pretreated by addition of enough 25 mg/ml gibberellic acid to cover all the seeds. Seeds were incubated at room temperature for one day with the gibberellic acid. After the pretreatment solution was discarded, seeds were surface sterilized with 70% (v/v) ethanol for 1 min. After 1 min, the ethanol was discarded, and seeds were gently shaken for 25 min with 50% (v/v) sodium hypochlorite (NaClO) solution which contained 2-3 drops of Tween-20. Then, seeds were rinsed with sterile double distilled water at least three times. After sterilization, seeds were sown onto ½ MS0 medium supplemented with 15 g/L sucrose and 8 g/L agar in jars. The seeds were incubated at 25°C under dark conditions

for two days. After two days, the seeds were incubated under 16h light/8h dark photoperiod.

2.2.2. Preparation of *A. rhizogenes*

ATCC 43057 strain was cultured in Yeast Mannitol Agar (YMA) medium containing 10 mg/L rifampicin. After two days, a single colony was taken from solid medium and transferred to a small volume of liquid YMA medium containing 10 mg/L rifampicin. The bacterial culture was incubated at 28°C on an orbital shaker at 150 rpm overnight. The culture was sub-cultured to a larger volume of liquid YMA medium containing 10 mg/L rifampicin and 100 mM acetosyringone and incubated at 28°C on an orbital shaker at 150 rpm overnight. Bacterial cells were harvested by centrifugation at 4750 rpm for 10 min, the supernatant was discarded, and the bacterial pellet was resuspended in MS liquid medium containing 100 mM acetosyringone to an OD_{600}: 0.5-0.6.

2.2.3. Induction and Co-cultivation of Hairy Roots

Hypocotyl and cotyledon explants from 21-day-old *S. melongena* and *S. linnaeanum* seedlings were used for hairy root induction. Before co-cultivation with *A. rhizogenes* ATCC 43057 strain, explants were wounded with scalpel. Hypocotyls were cut into 3-4 pieces and cotyledons were cut into 2 pieces. Explants were incubated with bacteria culture at 37°C for 5 min, then at 25°C for 20 min. After this incubation, explants were transferred to co-cultivation medium which was hormone-free MS medium supplemented with 20 g/L sucrose, 7 g/L agar, and 100 mM acetosyringone. Explants were incubated on co-cultivation medium for 2 days in total darkness at 25°C. After two days, explants were transferred to MS basal medium supplemented with 300 mg/L timentin for control of *A. rhizogenes* growth. After three weeks, newly emerged hairy roots were excised and transferred to MS basal medium supplemented with 300 mg/L timentin. Hairy root cultures were incubated at 25°C under dark conditions and subcultured every three weeks.

2.2.4. PCR Verification of Ri Plasmid Integration

In order to verify the integration of Ri T-DNA in established hairy root lines, genomic DNA was isolated from the cultures by CTAB method (Stewart, 1997). Samples consisting of 200 mg of hairy root tissue were used to extract genomic DNA from each culture. Plasmid DNA of *A. rhizogenes* ATCC 43057, which was used as a positive control, was isolated using Monarch Plasmid Miniprep (NEB) kit.

Two sets of primers were used to detect T-DNA integration. The primers and their sequences are given in Table 2.1. The reaction volume was 25 µl for both PCR reactions. The components and the PCR conditions are given in Table 2.2 and Table 2.3.

Table 2.1. Sequences of two sets of primers used to detect T-DNA integration.

rolA	Forward	5'-CAG AAT GGA ATT AGC CGG ACT AA-3'
	Reverse	5'-CGT ATT AAT CCC GTA GGT TTG TTT-3'
rolB2	Forward	5'-TCG TCG ACA TCC AAC TCA CAT CAC AAT GG-3'
	Reverse	5'-AAG GTA CCC TAC AAC TCC CAA GGT TCT GTG-3'

Table 2.2. PCR components and conditions for *rolA* gene amplification.

10 mM dNTP	1 µl	94°C → 4 minutes
10 µM Forward primer	2.5 µl	94°C → 1 minutes ┐
10 µM Reverse primer	2.5 µl	71°C → 1 minutes ├ 18 cycles
10X Buffer	2.5 µl	72°C → 2 minutes ┘
25 mM $MgCl_2$	1.5 µl	94°C → 1 minutes ┐
Taq polymerase	0.5 µl	62°C → 1 minutes ├ 17 cycles
Total volume	25 µl	72°C → 2 minutes ┘
		72°C → 10 minutes
		4°C → ∞

Table 2.3. PCR components and conditions for *rolB2* gene amplification.

10 mM dNTP	0.5 µl	94°C → 4 minutes
10 µM Forward primer	2.5 µl	94°C → 1 minutes ┐
10 µM Reverse primer	2.5 µl	67°C → 1 minutes ├ 18 cycles
KCl Buffer	5 µl	72°C → 2 minutes ┘
25 mM $MgCl_2$	3 µl	94°C → 1 minutes ┐
Taq polymerase	0.2 µl	58°C → 1 minutes ├ 17 cycles
Total volume	25 µl	72°C → 2 minutes ┘
		72°C → 10 minutes
		4°C → ∞

2.2.5. Liquid Hairy Root Culture

After the verification of Ri T-DNA integration into the plant genome, hairy root clusters of 4 cm in diameter from the selected hairy root cultures were transferred to liquid MS medium (50 ml) supplemented with 300 mg/L timentin. Liquid hairy root cultures were incubated in 66 cl jars at 25°C on an orbital shaker at 100 rpm in the dark. Liquid medium was renewed every week by replacing old liquid medium with fresh MS liquid medium (50 ml). After sufficient growth was achieved, hairy root tissues were harvested, and their fresh weight was recorded. From each line, 50 mg tissue was collected for RNA extraction. Remaining hairy root tissues were lyophilized for 3 days. After lyophilization, dry weight of tissues was recorded, and tissues were ground in a ceramic mortar and stored at -80°C for phytochemical analysis.

2.2.6. Phytochemical Analysis

2.2.6.1. Sample Preparation and Extraction

Extraction was performed from non-transformed root cultures, hairy root cultures (4 weeks after the establishment of liquid cultures), leaves (2nd leaves from the top), fruits and peels (commercially mature fruits of *S. melongena* and physiologically mature fruits of *S. linnaeanum*) of *S. melongena* and *S. linnaeanum* plants. For each sample, 25 mg of lyophilized tissue powders were weighed and transferred to 2 ml Eppendorf tubes. For

each sample, three replicates were prepared. Then, 1 ml 80% methanol was added to each tube and mixed by vortexing. Samples were incubated at room temperature using a gyratory shaker at 200 rpm for 30 min for extraction. After the incubation, tubes were centrifuged at 10,000 rpm for 20 min and supernatant was transferred to new tube. Obtained extracts were stored at -80°C.

2.2.6.2. Total Phenolic Analysis

Total phenolic content was analyzed by the Folin-Ciocalteu method which was modified by Wu et al. (2006). Firstly, methanolic extracts of samples were diluted and 50 µl of diluted extract was mixed with 50 µl Folin-Ciocalteu reagent and 1.25 ml of distilled water. The mixture was incubated at room temperature for 6 min under dark conditions. Then, 250 µl of 20% sodium carbonate (Na_2CO_3) was added to the mixture and incubated at room temperature for 30 min under dark conditions. After the incubation, absorbance was measured at 760 nm using a spectrophotometer. Different concentrations of gallic acid (10-320 mg/L) were used to plot a standard curve and the results were expressed as mg of gallic acid equivalent (GAE) per gram of dry roots.

2.2.6.3. Total Flavonoid Analysis

Total flavonoid content was analyzed with the aluminum chloride colorimetric method as reported by Wu et al. (2006). For the reaction, 250 µl methanolic extracts of samples were mixed with 1.25 ml of distilled water. Then, 75 µl of 5% sodium nitrite ($NaNO_2$) was added and mixture was incubated for 6 min. After the incubation, 150 µl of 10% aluminum chloride ($AlCl_3$) was added and the mixture was incubated for an additional 5 min. Then, 0.5 ml of 1M NaOH was added, and the absorbance was measured at 510 nm using a spectrophotometer. Different concentrations of (+)-catechin (0.01-0.64 mg/ml) were used to plot a standard curve and the results were expressed as mg of (+)-catechin equivalent per gram of dry roots.

2.2.7. Evaluation of Total Antioxidant Capacity

For determining the total water-soluble antioxidant capacity of hairy roots, non-transformed roots, leaves, fruit flesh, and fruit peels of *S. melongena* and *S. linnaeanum,* 100 mg powdered tissue of each sample was incubated in 12 ml methanol: dH_2O (1:1, v/v) mixture. After incubation, samples were centrifuged at 5000 g for 15 min and supernatant was collected. Total antioxidant capacity was evaluated by ABTS radical cation decolorization assay modified by Deng et al. (2012). The $ABTS^{\bullet+}$ stock solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate in a volume ratio of 1:1, and the prepared stock solution was incubated in the dark for 16 h at room temperature to allow the mixture to reach a stable oxidative state. The working solution of $ABTS^{\bullet+}$ was prepared by diluting the stock solution with ethanol to an absorbance value of 0.70 ± 0.05 at 734 nm. For spectrophotometric measurement, 10 µl of each sample was mixed with 190 µl of $ABTS^{\bullet+}$ working solution and absorbance was measured at 734 nm immediately after mixing, then after 6 min of incubation at 30°C. For blank measurement, 10 µl of dH_2O was used instead of sample. Different concentrations of Trolox (0.25-1.5 mM) were used as standard. The percentage of absorbance inhibition was calculated with the following formula $1 – (A_f/A_0) \times 100$ where A_f and A_0 indicates absorbance measured after 6 min and absorbance of uninhibited radical, respectively. The results were expressed as mg of Trolox equivalent (TE) per g of dry weight (mg/g).

2.2.8. mRNA Expression Analysis

Total RNA was extracted from leaves and PCR-verified hairy root cultures using the Plant/Fungi Total RNA Purification Kit from Norgen following the kit's instructions. After the RNA extraction, cDNA synthesis was performed using GoScript™ Reverse Transcriptase kit by Promega. Then, mRNA expression level was analyzed by Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) performed on a Light Cycler 480. RT-qPCR reaction components and conditions are given in Table 2.4. Four sets of primers were used in RT-qPCR which amplify *cyclophilin*, *myb1*, *Game9*, and *HQT* genes and the relative expression levels were calculated by the Livak method (Livak & Schmittgen, 2001). The primers that were used in the reactions and their sequences are given in Table 2.5.

Table 2.4. RT-qPCR components and conditions.

GoTaq qPCR Master Mix (Promega)	5 µl	95°C → 2 min 95°C → 15 s 60°C → 1 min 72°C → 10 s } 40 cycles
Forward primer	0.75 µl	
Reverse primer	0.75 µl	
cDNA template	2 µl	
dH_2O	1.5 µl	
Total	10 µl	

Table 2.5. Sequences of four sets of primers used in RT-qPCR.

CyclophilinRT	Forward	5'-ACA GCC TCG GCC TTC TTA ATC ACA-3'
	Reverse	5'-GCG CCA AAT TCA ACG AGG AGA ACT-3'
KT27965rt4 (*myb1*)	Forward	5'-GCA AAG AAA TAA CAA GTG ACA AGC AAA C-3'
	Reverse	5'-TCT CCT TCA ACA GCG TCG TCA-3'
Sme2.5_05213.1_rt (*Game9*)	Forward	5'-AAG ATT GGA GGC GGT TCA TAG-3'
	Reverse	5'-TTC ATA AGT TCC CAG CCA CAG-3'
KT259042.1rt (*HQT*)	Forward	5'-GGA TTT CAT AAG TGC TAC CCT CGT-3'
	Reverse	5'-TCC TCC ACC TAC CCT CAA CTC-3'

The ΔCt value, ΔΔCt value, and relative expression were calculated with the equations given below.

ΔCt = Ct (*myb1*, *Game9*, or *HQT*) – Ct (Reference gene, *Cyclophilin*)

ΔΔCt = ΔCt (hairy root) - ΔCt (non-transformed root)

Relative expression = $2^{-\Delta\Delta Ct}$

2.2.9. Correlation Analysis

Correlations between *myb1*, *Game9*, and *HQT* gene expression and total phenolic content, total flavonoid content and total antioxidant capacity were calculated using SPSS.

2.2.10. Statistical Analysis

The data were analyzed by Student's t-test. For comparisons, a P value less than 0.05 was considered as statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Induction of Hairy Roots

Two sets of transformations were performed for both *S. melongena* and *S. linnaeanum*, and 200 seeds were planted for each transformation set. The germination efficiency of each set is given in Table 3.1. Figure 3.1 shows an example of seedlings that were used for transformation experiments.

Table 3.1. Seed germination efficiency for each transformation set.

Transformation Set	**Germination Efficiency**
S. melongena – Set 1	52.5%
S. melongena – Set 2	65.0%
S. linnaeanum – Set 1	44.0%
S. linnaeanum – Set 2	39.5%

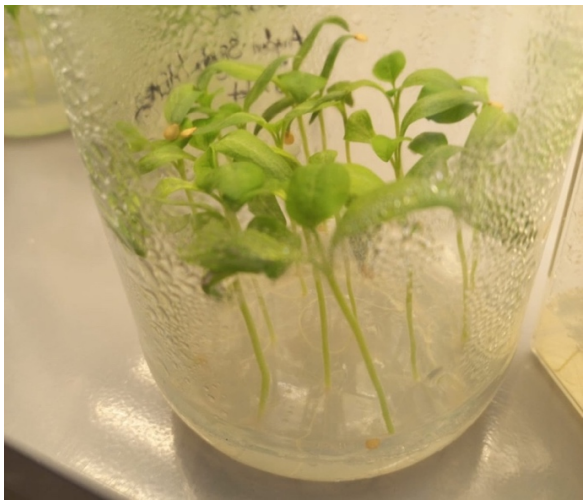

Figure 3.1. Germinated seeds of *S. melongena*.

For transformation both hypocotyl and cotyledon explants of 21-day-old seedlings were used. After co-cultivation with *A. rhizogenes*, explants were cultured on MS basal medium supplemented with 300 mg/L timentin. Emergence of hairy roots was observed

10-12 days after the transformation (Figure 3.2). Three weeks after the transformation, each newly emerged hairy root was excised and cultured on MS basal medium supplemented with 300 mg/L timentin. Transformation efficiency varied in each set and with explant type (Table 3.2). Regardless of species, in all transformation sets, transformation efficiency of hypocotyl explants (22.22 to 31.92%) was higher than cotyledon explants (18.67 to 26.11%); however, this difference was not statistically significant.

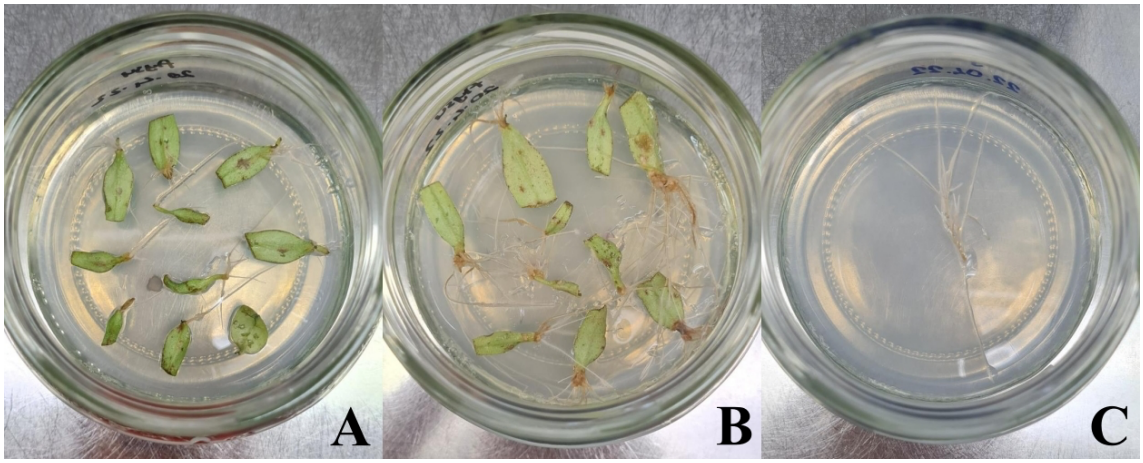

Figure 3.2. Induction of hairy root cultures from cotyledon explants of *S. linnaeanum* by *A. rhizogenes* ATCC 43057. (A) Cotyledon explants 12 days after the transformation, (B) cotyledon explants 3 weeks after the transformation, (C) single hairy root excised from cotyledon explants.

Table 3.2. Transformation efficiency for each transformation set and explant type.

Transformation Set	**Transformation Efficiency**	
S. melongena – Set 1	Hypocotyl	31.92%
	Cotyledon	26.11%
S. melongena – Set 2	Hypocotyl	28.96%
	Cotyledon	25.26%
S. linnaeanum – Set 1	Hypocotyl	22.22%
	Cotyledon	18.67%
S. linnaeanum – Set 2	Hypocotyl	24.10%
	Cotyledon	20.77%

3.2. PCR Verification of Ri Plasmid Integration

In order to verify the integration of Ri-plasmid into the plant genome, PCR amplification of *rolA* and *rolB2* genes was carried out. In total, 25 out of 61 hairy root cultures of *S. melongena* and 25 out of 45 hairy root cultures of *S. linnaeanum* were tested for these two *rol* genes. The Ri-plasmid of *A. rhizogenes* was used as a positive control, while DNA isolated from non-transformed roots was used as a negative control. Also, a non-template control, which was the corresponding PCR mixture without any DNA template, was used. PCR amplification of the *virD2* gene was carried out to see if hairy root cultures were free from *A. rhizogenes* contamination since the *virD2* gene is only present in the Ri-plasmid and not integrated into the plant genome.

All hairy root cultures of *S. melongena* tested positive for the *rolA* and *rolB2* genes, producing 310 bp and 900 bp amplicons, respectively (Figure 3.3 and Figure 3.4). As expected, there was no amplification in the non-template and non-transformed root samples. Amplification of *virD2* gene showed that in 4 out of 25 (16%) *S. melongena* hairy root cultures, growth of *A. rhizogenes* had not been inhibited. Because these cultures were contaminated with *A. rhizogenes,* they were not used in the following experiments (Figure 3.5).

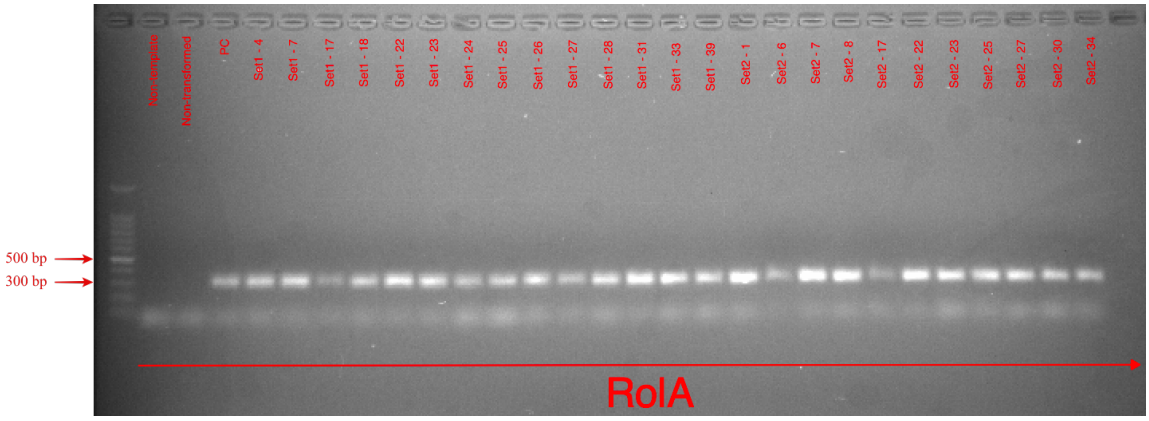

Figure 3.3. PCR amplification of *rolA* gene in *S. melongena* hairy root cultures. Lanes: 100 bp DNA ladder, non-template sample which contained distilled water instead of DNA template, non-transformed root which contained DNA sample extracted from root tissues of *Solanum melongena* Kemer seedlings, PC (positive control) which was the Ri plasmid of *A. rhizogenes* ATCC 43057.

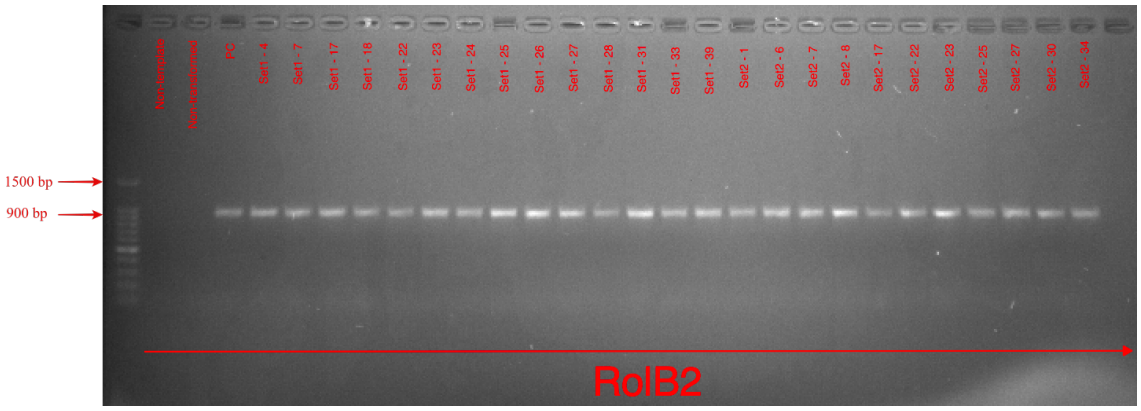

Figure 3.4. PCR amplification of *rolB2* gene in *S. melongena* hairy root cultures. Lanes: 100 bp DNA ladder, non-template sample which contained distilled water instead of DNA template, non-transformed root which contained DNA sample extracted from root tissues of *Solanum melongena* Kemer seedlings, PC (positive control) which was the Ri plasmid of *A. rhizogenes* ATCC 43057.

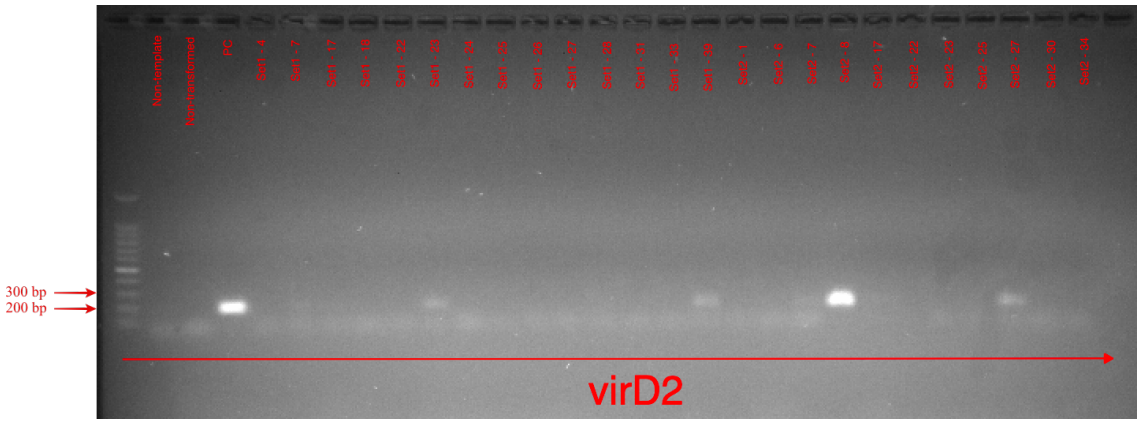

Figure 3.5. PCR amplification of *virD2* gene in *S. melongena* hairy root cultures. Lanes: 100 bp DNA ladder, non-template sample which contained distilled water instead of DNA template, non-transformed root which contained DNA sample extracted from root tissues of *Solanum melongena* Kemer seedlings, PC (positive control) which was the Ri plasmid of *A. rhizogenes* ATCC 43057.

Similar to those of *S. melongena*, all hairy root cultures of *S. linnaeanum* tested positive for the *rolA* and *rolB2* genes, again giving 310 bp and 900 bp amplicons, respectively (Figure 3.6 and Figure 3.7). As expected, there was no amplification in the non-template and non-transformed root samples. Amplification of *virD2* gene was also carried out with *S. linnaeanum* hairy root cultures and it was seen that 8 out of 25 (32%)

cultures were contaminated with *A. rhizogenes* (Figure 3.8) despite the fact that the medium contained 300 mg/L timentin and cultures were subcultured to fresh medium every three weeks.

As explained in Section 1.6.2.1., *rol* genes are essential to induce the hairy root phenotype because they stimulate root differentiation and morphogenesis (Chriqui et al., 1996). In addition to inducing hairy root phenotype, *rol* genes, especially the *rolB* gene, also increase secondary metabolite production (Bulgakov, 2008).

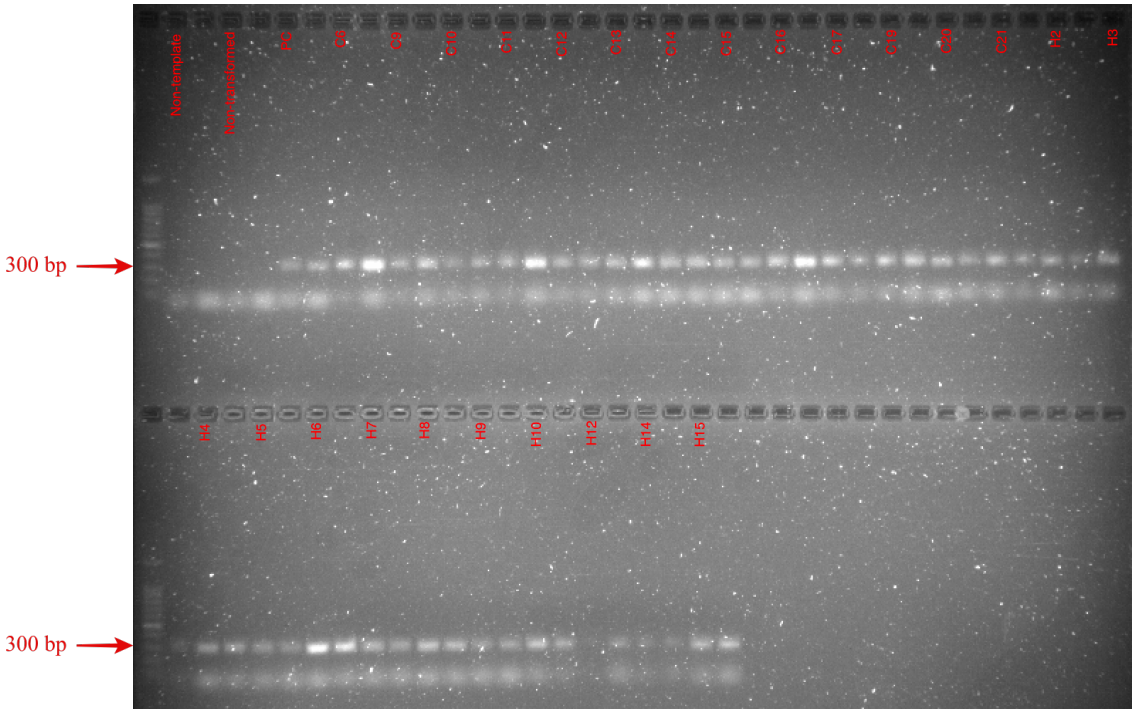

Figure 3.6. PCR amplification of *rolA* gene in *S. linnaeanum* hairy root cultures. Lanes: 100 bp DNA ladder, non-template sample which contained distilled water instead of DNA template, non-transformed root which contained DNA sample extracted from root tissues of *Solanum linnaeanum* seedlings, PC (positive control) which was the Ri plasmid of *A. rhizogenes* ATCC 43057.

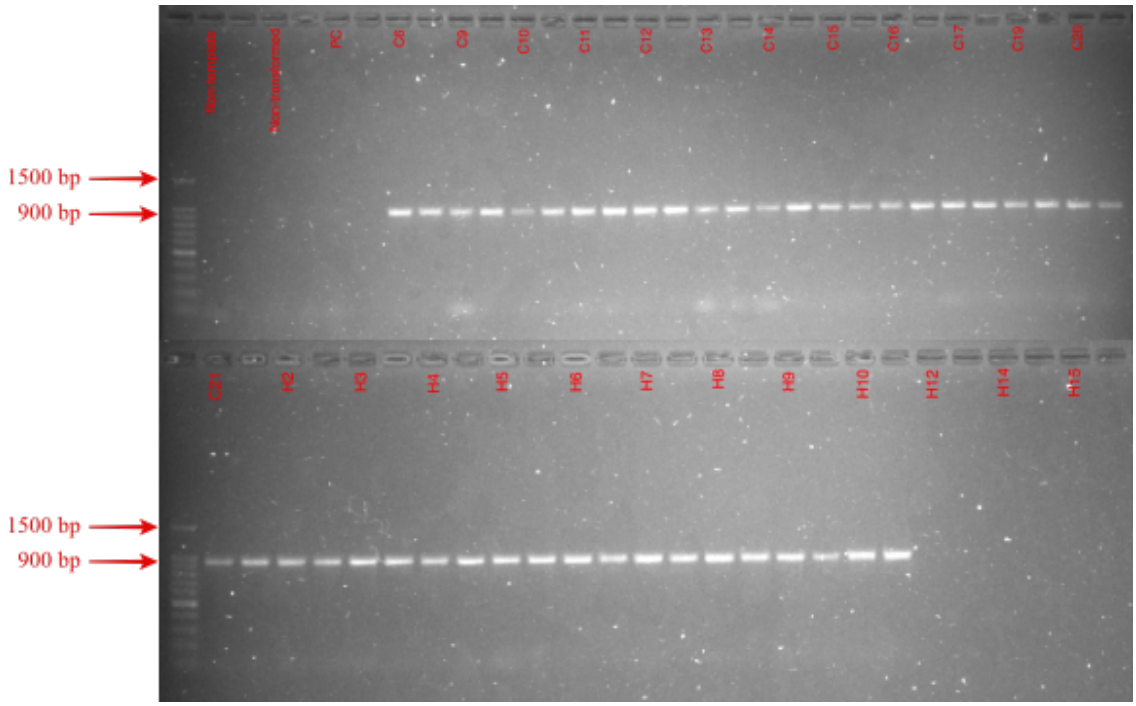

Figure 3.7. PCR amplification of *rolB2* gene in *S. linnaeanum* hairy root cultures. Lanes: 100 bp DNA ladder, non-template sample which contained distilled water instead of DNA template, non-transformed root which contained DNA sample extracted from root tissues of *Solanum linnaeanum* seedlings, PC (positive control) which was the Ri plasmid of *A. rhizogenes* ATCC 43057.

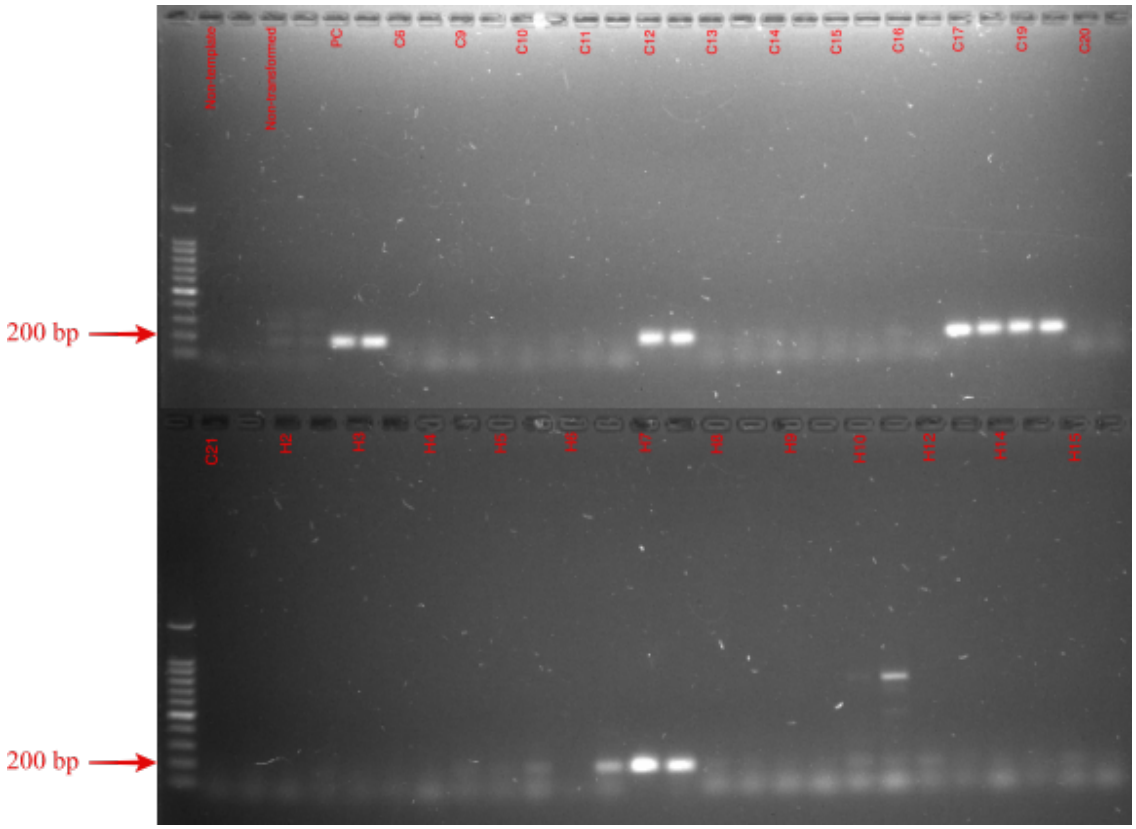

Figure 3.8. PCR amplification of *virD2* gene in *S. linnaeanum* hairy root cultures. Lanes: 100 bp DNA ladder, non-template sample which contained distilled water instead of DNA template, non-transformed root which contained DNA sample extracted from root tissues of *Solanum linnaeanum* seedlings, PC (positive control) which was the Ri plasmid of *A. rhizogenes* ATCC 43057.

3.3. Liquid Hairy Root Culture

After verification of *rol* gene integration, three hairy root cultures of *S. melongena* and four hairy root cultures of *S. linnaeanum* were selected and used in the following experiments. Selected hairy root cultures were transferred to MS liquid medium containing 300 mg/L timentin after they showed sufficient growth on solid MS medium (Figure 3.9 and Figure 3.10).

Figure 3.9. Growth of *S. melongena* hairy roots in MS liquid medium.

Figure 3.10. Growth of *S. linnaeanum* hairy roots in MS liquid medium.

3.4. Phytochemical Analysis

3.4.1. Total Phenolic Analysis

Total phenolic content was measured by the Folin-Ciocalteu method in hairy roots which were cultured in liquid MS medium. To compare total phenolic content in hairy

root cultures, total phenolic content of non-transformed root, leaf, fruit flesh and fruit peel of *S. melongena* and *S. linnaeanum* were also analyzed. Different concentrations of gallic acid (10-320 mg/L) were used to plot a standard curve and the results were expressed as mg of gallic acid equivalent (GAE) per gram of dry roots.

3.4.1.1. Non-transformed Tissues of *S. melongena*

Average total phenolic content in *S. melongena* non-transformed roots, leaves, fruit flesh and fruit peels were 2.15, 2.48, 5.06, and 8.63 mg/g, respectively, with small variation among the different plants (Figure 3.11). The total phenolic content in *S. melongena* non-transformed roots and leaves were similar, while in fruit flesh and fruit peels of *S. melongena*, the total phenolic content was approximately 2- and 3.5-fold higher than in non-transformed roots and leaves.

Eggplant is known to be a good source of important secondary metabolites, namely phenolic compounds (Okmen et al., 2009). When fruits of 25 Turkish and one foreign eggplant cultivar were analyzed, total phenolic contents ranged from 0.615 mg/g to 1.389 mg/g with an average of 0.992 mg/g (Okmen et al., 2009). Nisha et al. (2009) analyzed total phenolic contents in four different varieties of *S. melongena* which ranged from 0.490 mg/g to 1.069 mg/g. In our study, the total phenolic content in eggplant fruit (Kemer cultivar) ranged from 4.81 mg/g to 5.19 mg/g with an average of 5.06 mg/g which indicates a higher level of total phenolics in eggplant fruits compared to previous studies.

In a study carried out with different organs of eggplant, total phenolic content in ethanolic extract of eggplant peels and leaves was found to be 55.19 mg/g and 37.86 mg/g, respectively (Jung et al., 2011). In our study, the average total phenolic content in methanolic extract of eggplant peels and leaves was much lower, 8.63 mg/g and 2.48 mg/g, respectively. The developmental stage of the fruits and leaves and the environmental conditions under which the plants were grown may give rise to this difference between our study and the literature in total phenolic content. Also, our results showed that total phenolic content in peels of *S. melongena* was almost 2-fold than in fruit flesh which was also demonstrated by Huang et al., (2004) who found that total phenolic content was 0.118 mg/g in eggplant fruit, while in peel, total phenolic content was 0.267 mg/g.

3.4.1.2. Hairy Root Cultures of *S. melongena*

Total phenolic content in hairy root cultures of *S. melongena* ranged from 3.20 to 4.71 mg/g with an average of 3.83 mg/g. The highest phenolic content was observed in sample Set1-26 with a value of 4.71 mg/g which had 2.2-fold higher phenolic content than the non-transformed root (Table 3.3). The lowest phenolic content was observed in sample Set1-17 with a value of 3.20 mg/g. Two hairy root cultures (Set1-26 and Set2-22) showed statistically higher levels of total phenolics than the non-transformed root ($p \leq 0.05$) (Figure 3.11).

When the total phenolic contents in leaves and hairy root cultures of *S. melongena* were compared, one hairy root culture (Set2-22) showed statistically higher levels of total phenolics than the leaves ($p \leq 0.05$) with a value of 3.58 mg/g. When the total phenolic content in *S. melongena* fruit flesh and fruit peels were compared with the hairy root cultures of *S. melongena*, it was seen that fruit flesh and fruit peels had significantly higher levels of total phenolics than the hairy root cultures of *S. melongena* ($p \leq 0.05$).

In our study, established hairy root cultures of *S. melongena* produced significantly higher levels of total phenolics than non-transformed roots and leaves, however, the level of total phenolics was lower than for fruits and peels.

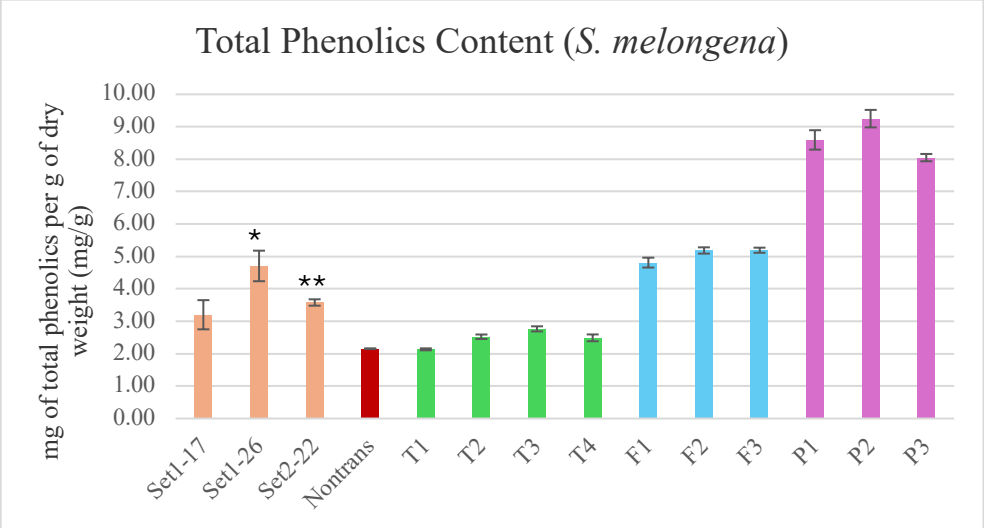

Figure 3.11. Total phenolics content in *Solanum melongena* hairy root cultures, non-transformed root, leaves (T1-T4), fruit flesh (F1-F3) and fruit peels (P1-P3). Asterisk indicates statistically significant differences between hairy root culture and non-transformed root ($p \leq 0.05$). Error bars indicate standard error.

Table 3.3. Total phenolics fold change in *Solanum melongena* hairy root cultures compared to non-transformed root.

Culture ID	Fold Change	P value
SM-Set1-17	1.5X	0.1979
SM-Set1-26	2.2X	0.0474
SM-Set2-22	1.7X	0.0065

3.4.1.3. Non-transformed Tissues of *S. linnaeanum*

Average total phenolic content in *S. linnaeanum* non-transformed roots, leaves, fruit flesh and fruit peels were 4.46, 2.76, 5.01, and 3.51 mg/g, respectively, with small variation among the different plants (Figure 3.12).

Wild species are known to be rich sources of important secondary metabolites since these species must survive in harsh environments by producing phytochemicals (Picchi et al., 2020). *S. linnaeanum* was shown to produce a wide range of important phytochemicals including steroids, saponins, and glycoalkaloids (Elabbara et al., 2014; Gürbüz et al., 2015). Chivodze et al. (2022) analyzed total phenolic content in root bark, stem bark, and leaves of *S. linnaeanum* and showed that the total phenolic content in these tissues ranged from 1.581 to 49.53 mg/g. Also, in another study carried out with methanolic extract of *S. linnaeanum* fruits, the total phenolic content was 152.8 mg/g indicating a higher level of total phenolics than in our study (Mahomoodally & Ramcharun, 2015).

3.4.1.4. Hairy Root Cultures of *S. linnaeanum*

Total phenolic content in hairy root cultures of *S. linnaeanum* ranged from 5.03 to 6.49 mg/g with an average of 5.75 mg/g. The highest phenolic content was observed in sample H5 with a value of 6.49 mg/g which had 1.5-fold higher phenolic content than the non-transformed root (Table 3.4). The lowest phenolic content was observed in sample H14 with a value of 5.03 mg/g which was similar to untransformed roots. Three hairy root cultures (H5, C12, and C19) showed statistically higher levels of total phenolics than the non-transformed root ($p \leq 0.05$) (Figure 3.12).

When the total phenolic contents of leaves and hairy root cultures of *S. linnaeanum* were compared, four hairy root cultures (H5, H14, C12, and C19) showed statistically higher levels of total phenolics than the leaves ($p \leq 0.05$). When the total phenolic content in *S. linnaeanum* fruit flesh were compared with the hairy root cultures of *S. linnaeanum*, it was seen that one hairy root culture (H5) showed significantly higher levels of total phenolic content (1.3-fold) than the fruit flesh of *S. linnaeanum*. Also, four hairy root cultures (H5, H14, C12, and C19) showed significantly higher levels of total phenolic content compared to peels of *S. linnaeanum* fruits ($p \leq 0.05$).

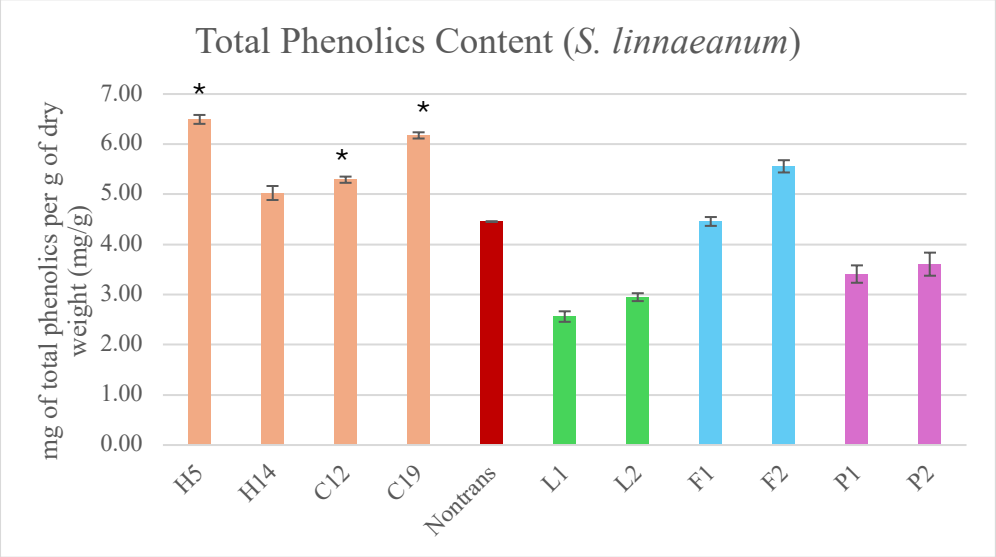

Figure 3.12. Total phenolics content in *Solanum linnaeanum* hairy root cultures, non-transformed root, leaves (L1-L2), fruit flesh (F1-F2) and fruit peels (P1-P2). Asterisk indicates statistically significant differences between hairy root culture and non-transformed root ($p \leq 0.05$). Error bars indicate standard error.

Table 3.4. Total phenolics fold change in *Solanum linnaeanum* hairy root cultures compared to non-transformed root.

Culture ID	Fold Change	P value
H5	1.5X	0.0379
H14	1.1X	0.0513
C12	1.2X	0.0206
C19	1.4X	0.0465

3.4.1.5. *S. melongena* vs *S. linnaeanum*

When total phenolic contents in different tissues of untransformed *S. melongena* and *S. linnaeanum* were compared, it was observed that, although *S. linnaeanum* leaves and fruit flesh had high values (5.06, and 5.01 mg/g, respectively, vs. 2.48, and 2.76 mg/g for *S. melongena*), when the values of different individual plants were averaged, this difference was not statistically significant. When the total phenolic content in peels of *S. melongena* and *S. linnaeanum* fruits were compared, it was seen that peels of *S. melongena* fruits had 2.5-fold higher total phenolic content than the peels of *S. linnaeanum* fruits with an average of 8.63 mg/g. In normal roots of *S. melongena* and *S. linnaeanum*, total phenolics content was statistically different. *S. linnaeanum* roots (4.46 mg/g) had 2.1-fold higher total phenolic content than the roots of *S. melongena* (2.15 mg/g). When hairy root cultures of *S. linnaeanum* were compared with those of *S. melongena,* the wild species had significantly higher levels (1.5-fold higher) of total phenolics.

3.4.2. Total Flavonoid Analysis

Total flavonoid content was measured by the aluminum chloride method in hairy roots which were cultured in liquid MS medium. To compare total flavonoid content in hairy root cultures, total flavonoid content of non-transformed root, leaf, fruit flesh and fruit peels of *S. melongena* and *S. linnaeanum* were also analyzed. Different concentrations of (+)-catechin (0.01-0.64 mg/ml) were used to plot a standard curve and the results were expressed as mg of (+)-catechin equivalent per gram of dry roots.

3.4.2.1. Non-transformed Tissues of *S. melongena*

Average total flavonoid content in *S. melongena* non-transformed roots, leaves, fruit flesh and fruit peels were 7.19, 7.88, 16.14, and 20.0 mg/g, respectively (Figure 3.13). The total flavonoid content in *S. melongena* non-transformed roots and leaves were similar, while in fruit flesh and fruit peels of *S. melongena*, the total flavonoid content was approximately 2- and 2.5-fold higher than in non-transformed roots and leaves.

Fidrianny et al. (2017) reported that total flavonoid content in ethanolic extract of *S. melongena* leaves, fruits and stems was 19.1, 7.2, and 3.8 mg QE/g, respectively. In a study carried out with total fruits of five different varieties of *S. melongena*, total flavonoid contents ranged from 0.226 mg CE/g to 1.02 mg CE/g (Nayanathara et al., 2016). Kaur et al. (2014b) reported that the total flavonoid content ranged from 0.032 to 0.259 mg QE/g among fruits of 34 different genotypes of eggplants and its wild relatives. In our study, the total flavonoid content in eggplant fruit (Kemer cultivar) ranged from 14.85 to 17.62 mg CE/g with an average of 16.14 mg CE/g while in leaves the average total flavonoid content was 7.88 mg CE/g. Thus, the total flavonoid content of *S. melongena* fruit flesh in our study was considerably higher than mentioned studies.

3.4.2.2. Hairy Root Cultures of *S. melongena*

Total flavonoid content in hairy root cultures of *S. melongena* ranged from 8.03 to 10.37 mg/g with an average of 9.32 mg/g (Figure 3.13). The highest flavonoid content was observed in sample Set1-17 with a value of 10.37 mg/g which had 1.4-fold higher flavonoid content than the non-transformed root (Table 3.5). The lowest flavonoid content was observed in sample Set2-22 with a value of 8.03 mg/g. Two hairy root cultures (Set1-26 and Set2-22) showed statistically higher levels of total flavonoids than the non-transformed root ($p \leq 0.05$).

When the total flavonoid content in leaves and hairy root cultures of *S. melongena* was compared, one hairy root culture (Set1-26) showed statistically higher levels of total flavonoid than the leaves ($p \leq 0.05$). When the total flavonoid content in *S. melongena* fruits and peels were compared with the hairy root cultures of *S. melongena*, it was seen that fruits and peels had significantly higher levels of total flavonoid than the hairy root cultures of *S. melongena* ($p \leq 0.05$). This dramatic difference in total flavonoid content between hairy root cultures and peels is most likely the result of high anthocyanin content in eggplant peels.

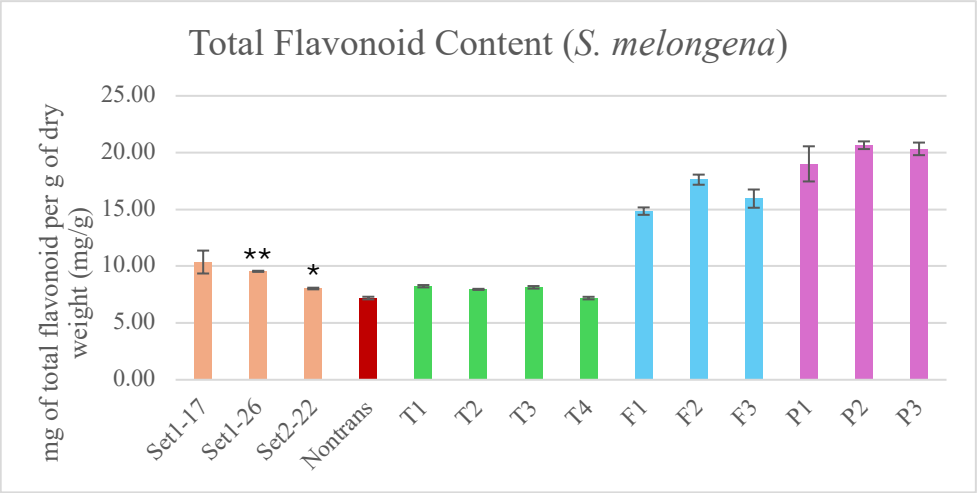

Figure 3.13. Total flavonoid content in *Solanum melongena* hairy root cultures, non-transformed root, leaves (T1-T4), fruit flesh (F1-F3) and fruit peels (P1-P3). Asterisk indicates statistically significant differences between hairy root culture and non-transformed root ($p \leq 0.05$). Error bars indicate standard error.

Table 3.5. Total flavonoid fold change in *Solanum melongena* hairy root cultures compared to non-transformed root.

Culture ID	Fold Change	P value
SM-Set1-17	1.4X	0.1222
SM-Set1-26	1.3X	0.0011
SM-Set2-22	1.1X	0.0119

3.4.2.3. Non-transformed Tissues of *S. linnaeanum*

Average total flavonoid content in *S. linnaeanum* non-transformed roots, leaves, fruit flesh and fruit peels were 16.14, 6.64, 21.03, and 12.67 mg/g, respectively (Figure 3.14).

Chivodze et al. (2022) reported that in root bark, stem bark, and leaves of *S. linnaeanum* the total flavonoid content ranged from 0.973 to 28.743 mg QE/g with highest total flavonoid content in leaves. This result is in contrast to our study in which the lowest total flavonoid content was detected in leaves. In another study carried out with methanolic extract of *S. linnaeanum* fruits, the total flavonoid content was 0.174 mg rutin equivalent (RE)/g (Mahomoodally & Ramcharun, 2015).

3.4.2.4. Hairy Root Cultures of *S. linnaeanum*

Total flavonoid content in hairy root cultures of *S. linnaeanum* ranged from 11.38 to 17.25 mg/g with an average of 14.85 mg/g (Figure 3.14). The highest flavonoid content was observed in sample H5 with a value of 17.25 mg/g which had 1.1-fold higher flavonoid content than the non-transformed root (Table 3.6). The lowest flavonoid content was observed in sample H14 with a value of 11.38 mg/g. When the flavonoid content of hairy root cultures of *S. linnaeanum* were compared to the normal roots, no significant differences were observed.

When the total flavonoid content in leaves and hairy root cultures of *S. linnaeanum* were compared, four hairy root cultures (H5, H14, C12, and C19) showed statistically higher levels of total flavonoids than the leaves ($p \leq 0.05$). When the total flavonoid content in *S. linnaeanum* fruit flesh was compared with the hairy root cultures of *S. linnaeanum*, it was seen that two hairy root cultures (H14 and C12) showed statistically higher levels of total flavonoids than the fruit flesh of *S. linnaeanum*. Also, total flavonoid content of H5 was significantly higher than those of fruit peels of *S. linnaeanum* fruits ($p \leq 0.05$).

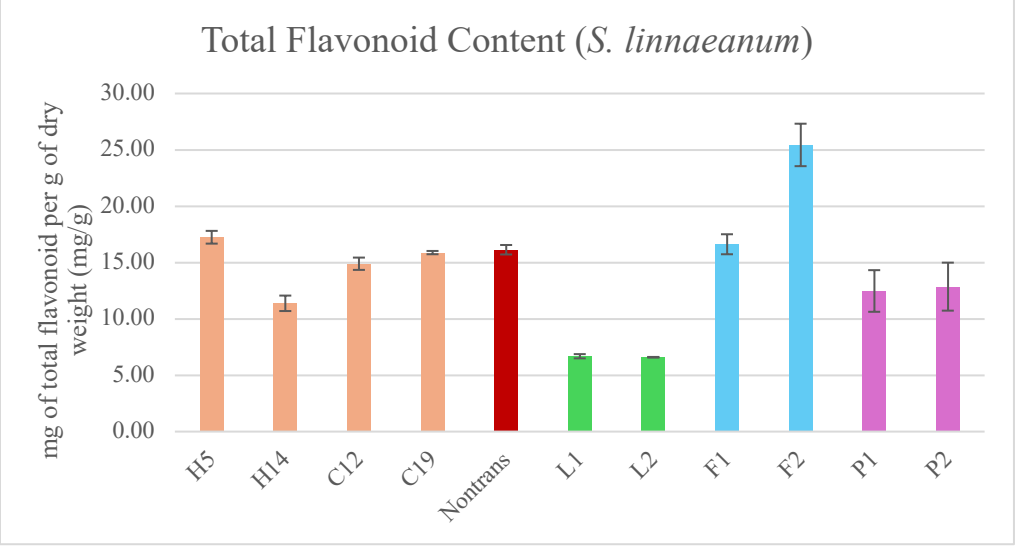

Figure 3.14. Total flavonoid content in *Solanum linnaeanum* hairy root cultures, non-transformed root, leaves (L1-L2), fruit flesh (F1-F2) and fruit peels (P1-P2). Asterisk indicates statistically significant differences between hairy root culture and non-transformed root ($p \leq 0.05$). Error bars indicate standard error.

Table 3.6. Total flavonoid fold change in *Solanum linnaeanum* hairy root cultures compared to non-transformed root.

Culture ID	Fold Change	P value
H5	1.1X	0.2738
H14	0.7X	0.0134
C12	0.9X	0.1734
C19	1.0X	0.6788

3.4.2.5. *S. melongena* vs *S. linnaeanum*

When total flavonoid contents in different tissues of *S. melongena* and *S. linnaeanum* were compared, it was observed that, total flavonoid content in *S. melongena* leaves was significantly higher than the leaves of *S. linnaeanum.* However, there was no significant difference in total flavonoid content in the fruits of the two species. When the total flavonoid content in peels of *S. melongena* and *S. linnaeanum* fruits were compared, it was seen that peels of *S. melongena* fruits had 1.6-fold higher flavonoid content than the peels of *S. linnaeanum* fruits probably due to the presence of high levels of anthocyanin in *S. melongena* fruit which are purple compared to the green fruit of *S. linnaeanum*.

In normal roots of *S. melongena* and *S. linnaeanum*, total flavonoid content was statistically different. *S. linnaeanum* roots (16.14 mg/g) had 2.2-fold higher total flavonoids than the roots of *S. melongena* (7.19 mg/g). Hairy root cultures of *S. linnaeanum* had significantly higher levels (1.6-fold higher) of total flavonoid than the hairy root cultures of *S. melongena*.

3.5. Total Antioxidant Capacity

Total antioxidant capacity was analyzed by the ABTS radical cation decolorization assay in hairy roots which were cultured in liquid MS medium. To compare total antioxidant capacity of hairy root cultures, total antioxidant capacity of non-transformed root, leaf, fruit flesh and fruit peel of *S. melongena* and *S. linnaeanum* were also analyzed. Different concentrations of Trolox (0.25-1.5 mM) were used to plot a standard curve and the results were expressed as mg of Trolox equivalent (TE) per g of dry weight (mg/g).

3.5.1. Non-transformed Tissues of *S. melongena*

Average total antioxidant capacity in *S. melongena* non-transformed roots, leaves, fruit flesh and fruit peels were 6.91, 8.32, 9.45, and 13.10 mg TE/g, respectively (Figure 3.15). The highest total antioxidant capacity was observed in fruit peels of *S. melongena* which was 1.9-, 1.6-, and 1.4-fold higher than the those of non-transformed root, leaves, and fruit flesh of *S. melongena*, respectively.

Ahmed et al. (2016) showed that radical scavenging activity of fruits of seven different *S. melongena* cultivars ranged from 80.79% to 87.64%. In another study which analyzed radical scavenging activity of different parts of *S. melongena*, it was shown that fruit peel and fruit flesh of *S. melongena* had approximately 25% and 30% scavenging activity, respectively (Sultana et al., 2013). In our study, fruit peels and fruit flesh of *S. melongena* had lower scavenging activity than the mentioned studies which were approximately 7.82% and 5.66%, respectively.

3.5.2. Hairy Root Cultures of *S. melongena*

Total antioxidant capacity of hairy root cultures of *S. melongena* ranged from 9.01 to 10.48 mg TE/g with an average of 9.76 mg TE/g. The highest antioxidant capacity was observed in sample Set1-26 with a value of 10.48 mg TE/g which had 1.5-fold higher antioxidant capacity than the non-transformed root (Table 3.7). The lowest antioxidant capacity was observed in sample Set2-22 with a value of 9.01 mg TE/g. Two hairy root cultures (Set1-17 and Set1-26) showed statistically higher levels of antioxidant capacity than the non-transformed root ($p \leq 0.05$) (Figure 3.15).

When the antioxidant capacity of leaves and hairy root cultures of *S. melongena* were compared, two hairy root cultures (Set1-17 and Set1-26) showed statistically higher levels of antioxidant capacity than the leaves ($p \leq 0.05$). When the total antioxidant capacity of *S. melongena* fruit flesh and fruit peels were compared with the hairy root cultures of *S. melongena*, it was seen that fruit flesh and fruit peels had significantly higher levels of total antioxidant capacity than the hairy root cultures of *S. melongena* ($p \leq 0.05$).

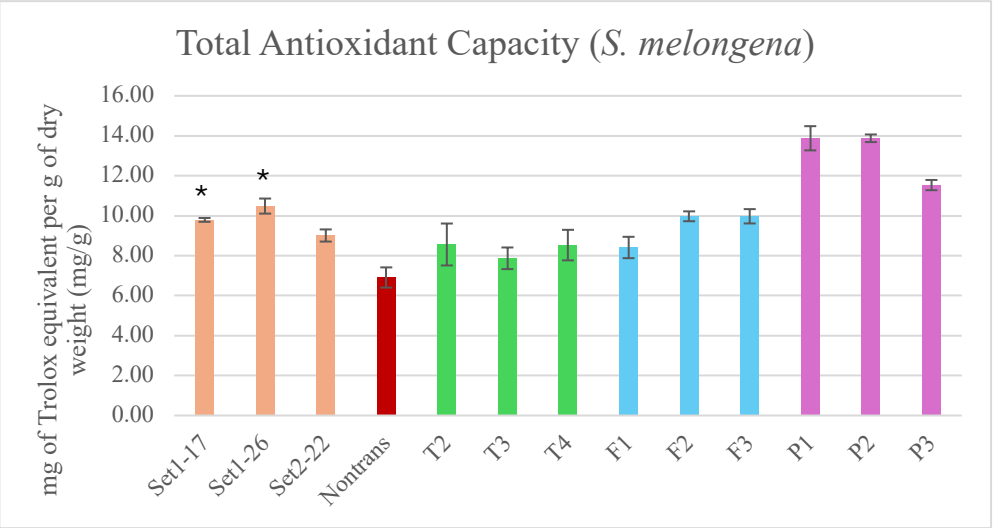

Figure 3.15. Total antioxidant capacity of *Solanum melongena* hairy root cultures, non-transformed root, leaves (T2-T4), fruit flesh (F1-F3) and fruit peels (P1-P3). Asterisk indicates statistically significant differences between hairy root culture and non-transformed root ($p \leq 0.05$). Error bars indicate standard error.

Table 3.7. Total antioxidant capacity fold change in *Solanum melongena* hairy root cultures compared to non-transformed root.

Culture ID	Fold Change	P value
SM-Set1-17	1.4X	0.0388
SM-Set1-26	1.5X	0.0117
SM-Set2-22	1.3X	0.0559

3.5.3. Non-transformed Tissues of *S. linnaeanum*

Average total antioxidant capacity in *S. linnaeanum* non-transformed roots, leaves, fruit flesh and fruit peels were 10.69, 8.56, 11.64, and 9.67 mg TE/g, respectively (Figure 3.16). The highest total antioxidant capacity was observed in fruit flesh of *S. linnaeanum*.

Chivodze et al. (2022) demonstrated that leaves, stem bark, and root bark of *S. linnaeanum* had 35.09%, 22.96%, and 20.92% scavenging activity, respectively. In our study, the scavenging activity was found as 4.56% for leaves of *S. linnaeanum*.

3.5.4. Hairy Root Cultures of *S. linnaeanum*

Total antioxidant capacity of hairy root cultures of *S. linnaeanum* ranged from 9.28 to 13.82 mg/g TEAC with an average of 11.45 mg TE/g. The highest antioxidant capacity was observed in sample H5 with a value of 13.82 mg TE/g which had 1.3-fold higher antioxidant capacity than the non-transformed root (Table 3.8). The lowest antioxidant capacity was observed in sample H14 with a value of 9.28 mg TE/g. Two hairy root cultures (H5 and C12) showed statistically higher levels of antioxidant capacity than the non-transformed root ($p \leq 0.05$) (Figure 3.16).

When the antioxidant capacity of leaves and hairy root cultures of *S. linnaeanum* were compared, three hairy root cultures (H5, C12 and C19) showed statistically higher levels of antioxidant capacity than the leaves ($p \leq 0.05$). When the total antioxidant capacity of *S. linnaeanum* fruit flesh and hairy root cultures were compared, one hair root culture (H5) showed statistically higher levels of total antioxidant capacity while two hairy root cultures (H5 and C12) had significantly higher antioxidant capacity than the fruit peels of *S. linnaeanum* ($p \leq 0.05$).

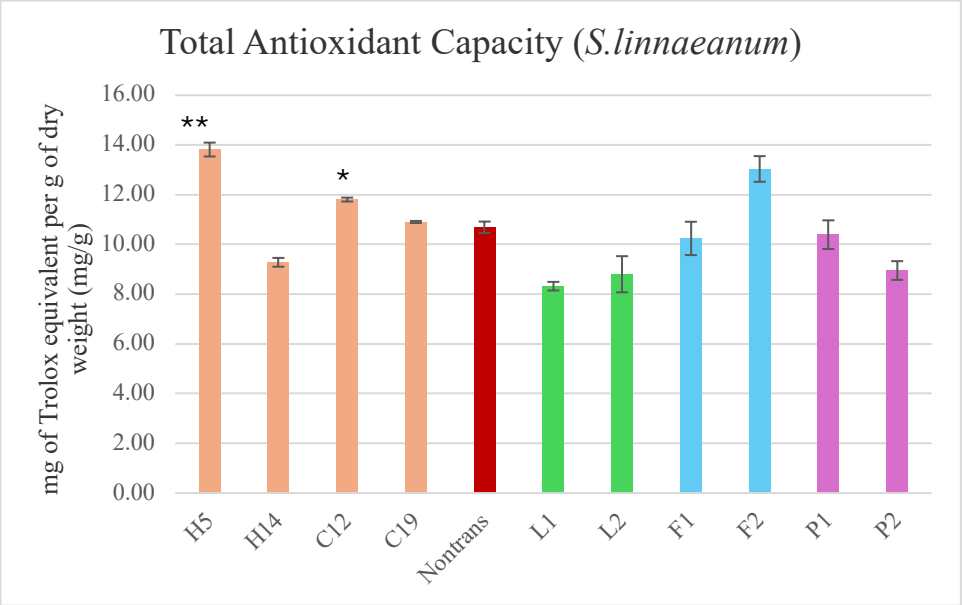

Figure 3.16. Total antioxidant capacity of *Solanum linnaeanum* hairy root cultures, non-transformed root, leaves (L1-L2), fruit flesh (F1-F2) and fruit peels (P1-P2). Asterisk indicates statistically significant differences between hairy root culture and non-transformed root ($p \leq 0.05$). Error bars indicate standard error.

Table 3.8. Total antioxidant capacity fold change in *Solanum linnaeanum* hairy root cultures compared to non-transformed root.

Culture ID	Fold Change	P value
H5	1.3X	0.0024
H14	0.9X	0.0190
C12	1.1X	0.0435
C19	1.0X	0.5101

3.5.5. *S. melongena* vs *S. linnaeanum*

When total antioxidant capacity in different tissues of *S. melongena* and *S. linnaeanum* were compared, it was observed that total antioxidant capacity of *S. melongena* and *S. linnaeanum* leaves were not significantly different while, fruit peels of *S. melongena* had significantly higher total antioxidant capacity than those of *S. linnaeanum* perhaps due to their high anthocyanin content. When the total antioxidant capacity of *S. melongena* and *S. linnaeanum* fruit flesh were compared, it was seen that fruit flesh of *S. linnaeanum* had 1.2-fold higher total antioxidant capacity than the fruit flesh of *S. melongena*.

Total antioxidant capacity of normal roots of *S. melongena* and *S. linnaeanum* was statistically different. *S. linnaeanum* roots had 1.5-fold higher total antioxidant capacity than the roots of *S. melongena*. Also, hairy root cultures of *S. linnaeanum* had significantly higher levels (1.2-fold higher) of total antioxidant capacity than the hairy root cultures of *S. melongena*.

In our study, it was shown that one hairy root culture of *S. melongena* (Set1-26) and two hairy root cultures of *S. linnaeanum* (H5 and C12) might potentially be used in further research since they showed enhanced levels of total phenolics and total flavonoid content and total antioxidant capacity. Also, this study showed that hairy root cultures are potentially a better system than whole plants for phytochemical production because they can be grown in bioreactors. By using bioreactors, hairy root cultures can be kept contaminant-free, and the large-scale production of important phytochemicals can be achieved. For instance, shikonin, which is a naphthoquinone-derivative compound having antibacterial, antitumor and anti-inflammatory activities, was produced in a two-phase bubble column reactor at a constant level of 10.6 mg/L per day during 54-days-culture

period (Sim & Chang, 1993). Yazaki (2017) stated that a 750 L-culture tank which contains 600 L medium can produce 2 g/L shikonin in 2 weeks. When grown in the field, the same level of production would require the cultivation of *Lithospermum erythrorhizon* plants at a density of 3.5 plants/m^2 in a field of 17.6 hectares for 4 years. There are many examples of important secondary metabolites which have been successfully produced from hairy root cultures in different bioreactor types, and they are given in Table 3.9.

Table 3.9. Secondary metabolites produced from hairy root cultures in various bioreactors (Source: Mishra & Ranjan, 2008).

Plant species	**Secondary metabolite**	**Bioreactor type**
Duboisia leichhardtii	Scopolamine	Stirred tank reactor
Nicotiana rustica	Nicotine	Air lift reactor
Beta vulgaris	Betacyanins	Trickle bed reactor
Tagetes patula	Thiophene	Nutrient mist reactor
Hyoscyamus muticus	Tropane alkaloids	Bubble column reactor

3.6. mRNA Expression Analysis

Expression analysis of three genes, *myb1*, *HQT*, and *Game9*, in leaves and hairy roots of *S. melongena* and *S. linnaeanum* was performed using RT-PCR. The relative mRNA expression in different tissues were compared with each other, and the *cyclophilin* gene was used as a reference gene.

The mRNA expression level of the *myb1* gene was compared between the same tissues of different species and different tissues in the same species. When expression level of *myb1* gene in *S. melongena* hairy root cultures was compared to leaves, it was seen that in two hairy root cultures, Set1-26 and Set2-22, the *myb1* gene was expressed approximately 3- and 4-fold higher than the leaves, respectively (Figure 3.17). When the same comparison was made between *S. linnaeanum* hairy roots and leaves, it was seen that in all four hairy root cultures, the *myb1* gene was expressed 2- to 4-fold higher than the leaves (Figure 3.18).

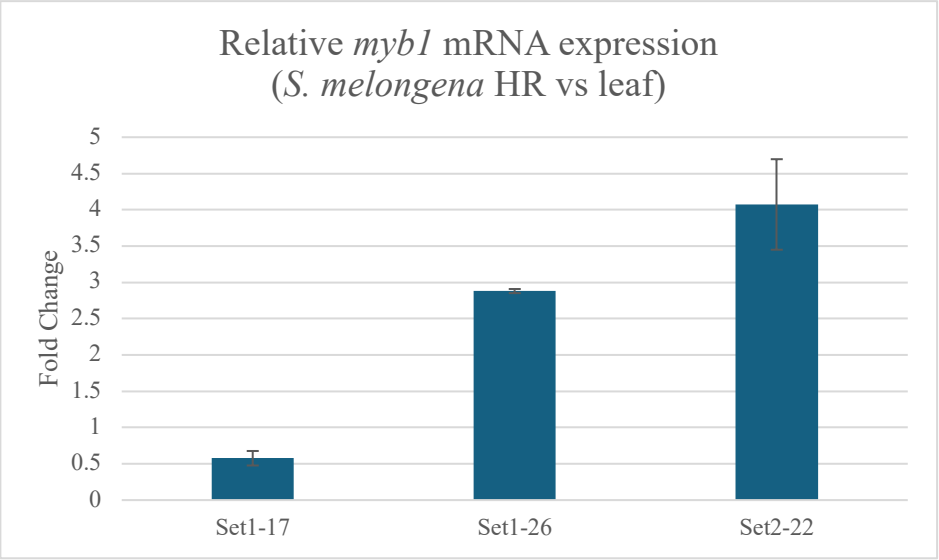

Figure 3.17. mRNA expression level change of *myb1* gene in *S. melongena* hairy root cultures compared to S. melongena leaves. Error bars indicate standard error.

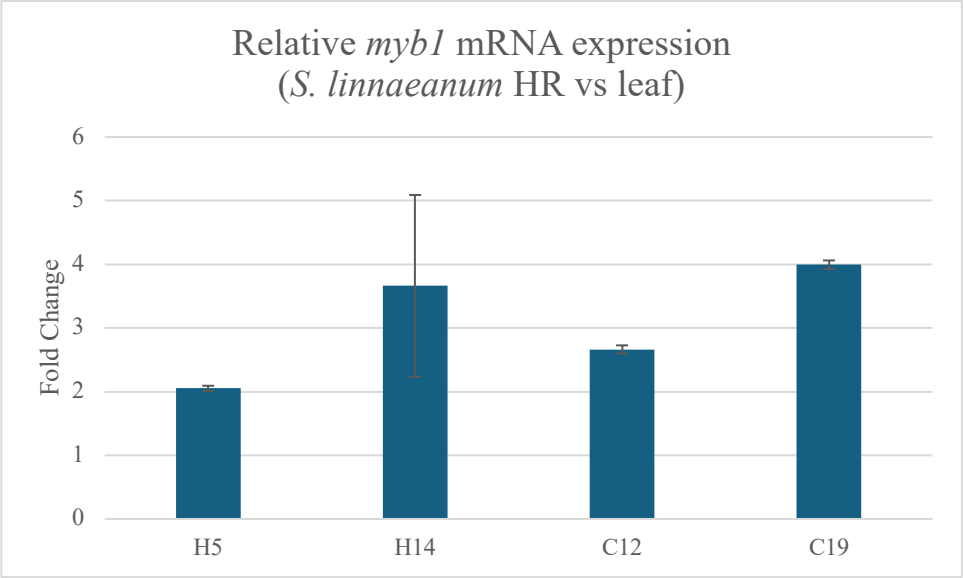

Figure 3.18. mRNA expression level change of *myb1* gene in *S. linnaeanum* hairy root cultures compared to S. linnaeanum leaves. Error bars indicate standard error.

The expression level of *myb1* gene was at least 2-fold higher in *S. linnaeanum* hairy root cultures and leaves compared to hairy root cultures and leaves of *S. melongena* (Figure 3.19 and 3.20).

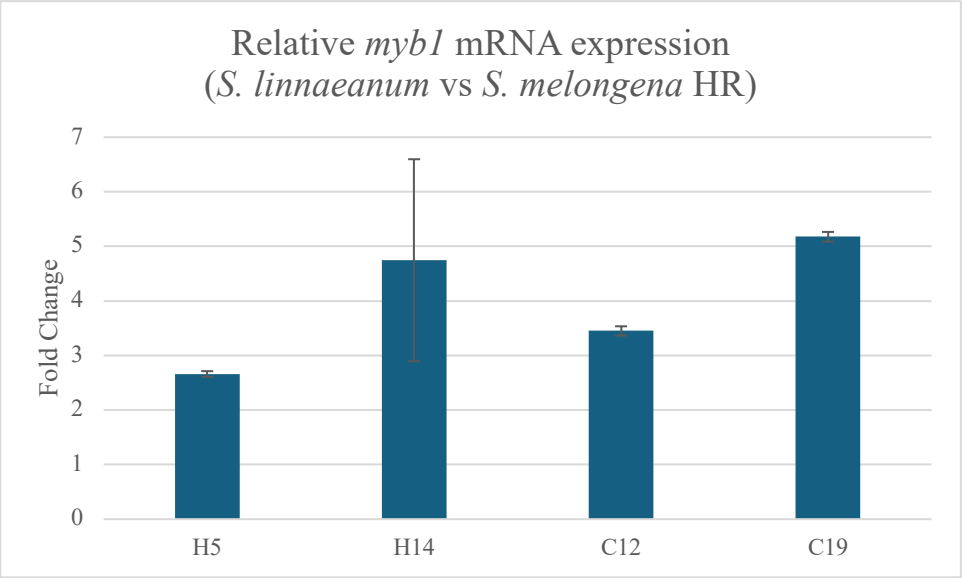

Figure 3.19. mRNA expression level change of *myb1* gene in *S. linnaeanum* hairy root cultures compared to S. melongena hairy root cultures. Error bars indicate standard error.

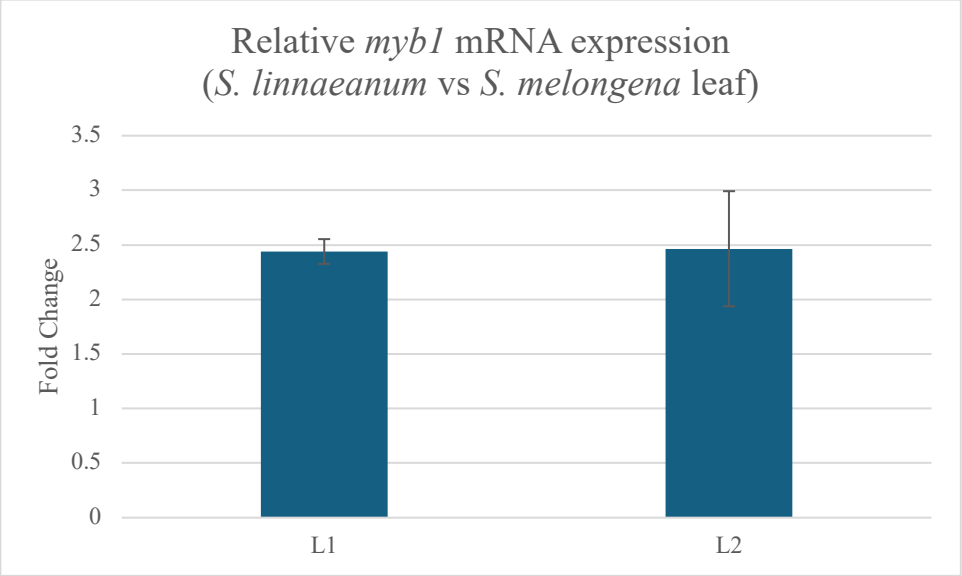

Figure 3.20. mRNA expression level change of *myb1* gene in *S. linnaeanum* leaves compared to S. melongena leaves. Error bars indicate standard error.

The *Myb1* gene encodes a transcription factor which is known to regulate several biosynthetic pathways (Yang et al., 2012). For example, in *Allium cepa*, transcription factor *myb1* was shown to regulate anthocyanin production along with other several MYB regulators which were involved in regulating flavonoid and phenylpropanoid pathways (Schwinn et al., 2016). Also, another study reported that three *myb* genes, *SbMYB1,*

SbMYB2, and *SbMYB3*, were involved in biosynthesis of flavonoids in *Selaginella bryopteris* (Kumar et al., 2018). In *S. melongena*, *myb1* transcription factor was shown to be involved in the accumulation of anthocyanins and chlorogenic acid (Zhang et al., 2016; Docimo et al., 2016). In our study, it was shown that, *myb1* expression increased at least 2-fold in hairy root cultures compared to leaves regardless of the species. Also, in *S. linnaeanum* leaves and hairy root cultures, *myb1* gene expression was higher than in the same tissues of *S. melongena* suggesting that *S. linnaeanum* might be a better source of important secondary metabolites. Our findings were consistent since total phenolic and total flavonoid content were higher in hairy root cultures than in leaves.

In addition to *myb1*, the expression level of the *Game9* gene was also analyzed in leaves and hairy root cultures of *S. melongena* and *S. linnaeanum*. In two hairy root cultures of *S. melongena*, Set1-26 and Set2-22, the *Game9* gene was expressed 4- and 13-fold higher than leaves (Figure 3.21). Similarly, in hairy root cultures of *S. linnaeanum*, the *Game9* expression was 2- to 8-fold higher than leaves (Figure 3.22).

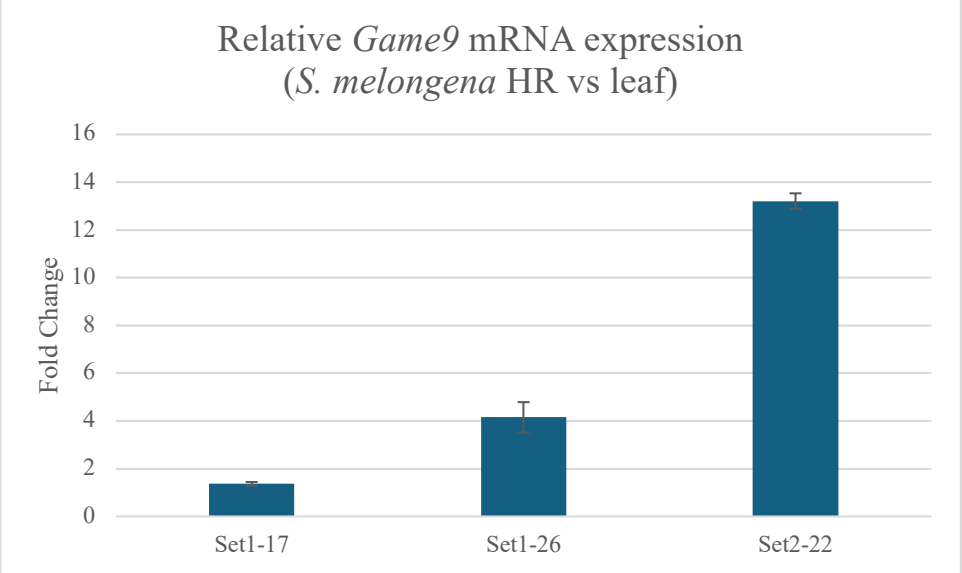

Figure 3.21. mRNA expression level change of *Game9* gene in *S. melongena* hairy root cultures compared to S. melongena leaves. Error bars indicate standard error.

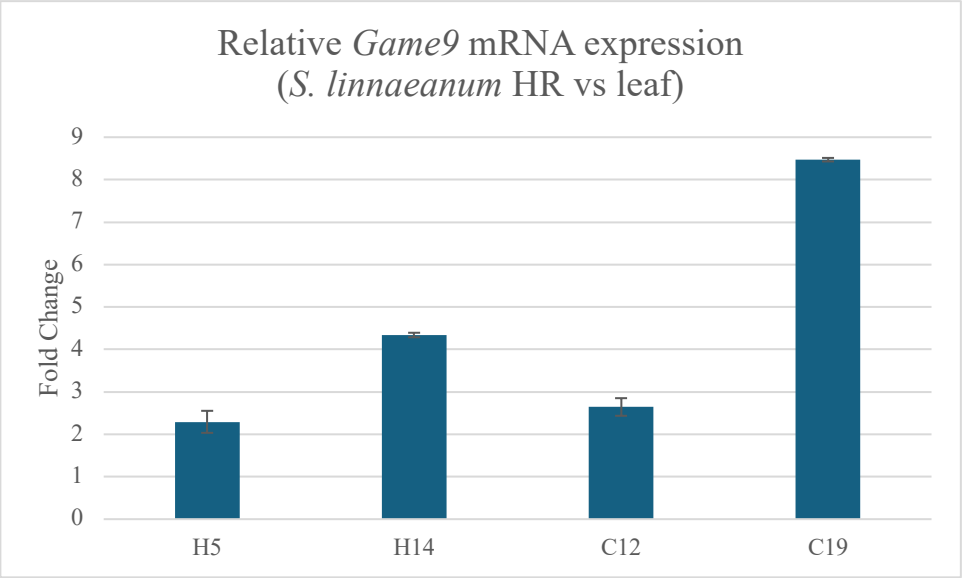

Figure 3.22. mRNA expression level change of *Game9* gene in *S. linnaeanum* hairy root cultures compared to S. linnaeanum leaves. Error bars indicate standard error.

The mRNA expression levels of the *Game9* gene were higher in both hairy root cultures and leaves of *S. linnaeanum* than those of *S. melongena*. In hairy root cultures of *S. linnaeanum*, the expression of *Game9* gene was 2- to 9-fold higher than *S. melongena* hairy root cultures, while *S. linnaeanum* leaves showed 3- to 5-fold higher *Game9* expression than the leaves of *S. melongena* (Figure 3.23 and Figure 3.24).

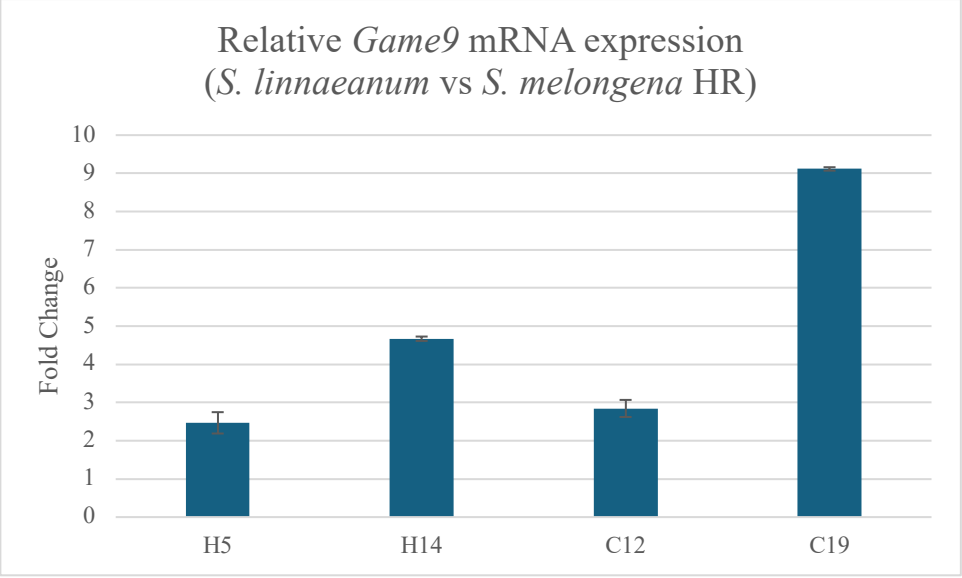

Figure 3.23. mRNA expression level change of *Game9* gene in *S. linnaeanum* hairy root cultures compared to S. melongena hairy root cultures. Error bars indicate standard error.

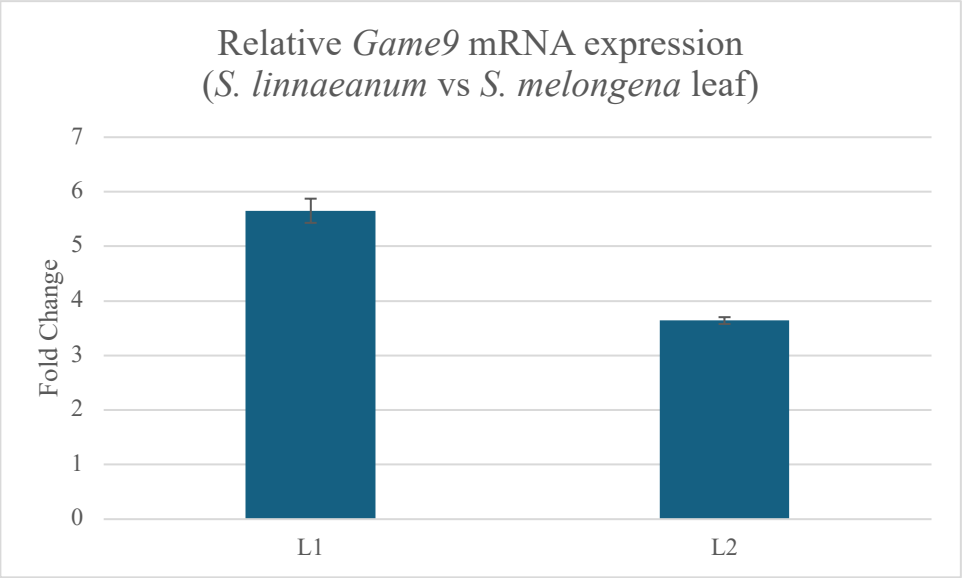

Figure 3.24. mRNA expression level change of *Game9* gene in *S. linnaeanum* leaves compared to S. melongena leaves. Error bars indicate standard error.

Game genes were reported to be involved in the biosynthesis pathway of steroidal glycoalkaloids in both tomato and potato (Itkin et al., 2013). The *Game9* gene encodes a transcription factor which was shown to regulate the biosynthesis of steroidal alkaloids as well as several upstream mevalonate and cholesterol precursor pathway genes in Solanaceae plants (Cárdenas et al., 2016). In our study, we analyzed the expression level of *Game9* gene in leaves and hairy root cultures of *S. melongena* and *S. linnaeanum*. Similar to *myb1* gene expression, *Game9* was also expressed in higher levels in hairy root cultures than in leaves regardless of the species. Also, the expression level of *Game9* gene was higher in leaves and hairy root cultures of *S. linnaeanum* than in those of *S. melongena*.

HQT expression levels in hairy root cultures of both *S. melongena* and *S. linnaeanum* was lower than those of their leaves (Figure 3.25 and Figure 3.26). Similarly, *HQT* expression level was lower in *S. linnaeanum* hairy root cultures compared to hairy root cultures of *S. melongena* (Figure 3.27). In one of the *S. linnaeanum* leaf sample, *HQT* expression was roughly equal to those of *S. melongena*, while in the other leaf sample of *S. linnaeanum*, the expression was 5-fold higher than *S. melongena* leaves (Figure 3.28).

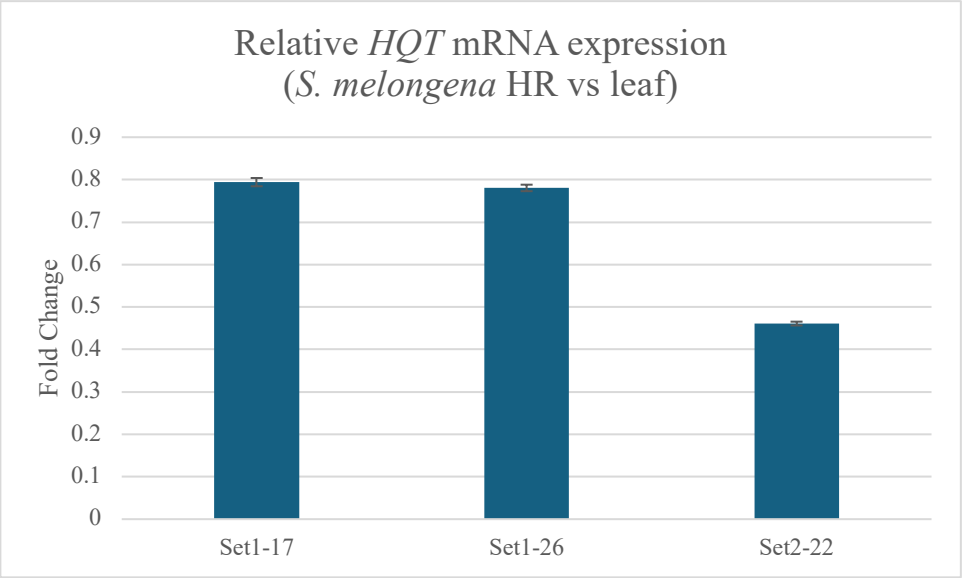

Figure 3.25. mRNA expression level change of *HQT* gene in *S. melongena* hairy root cultures compared to S. melongena leaves. Error bars indicate standard error.

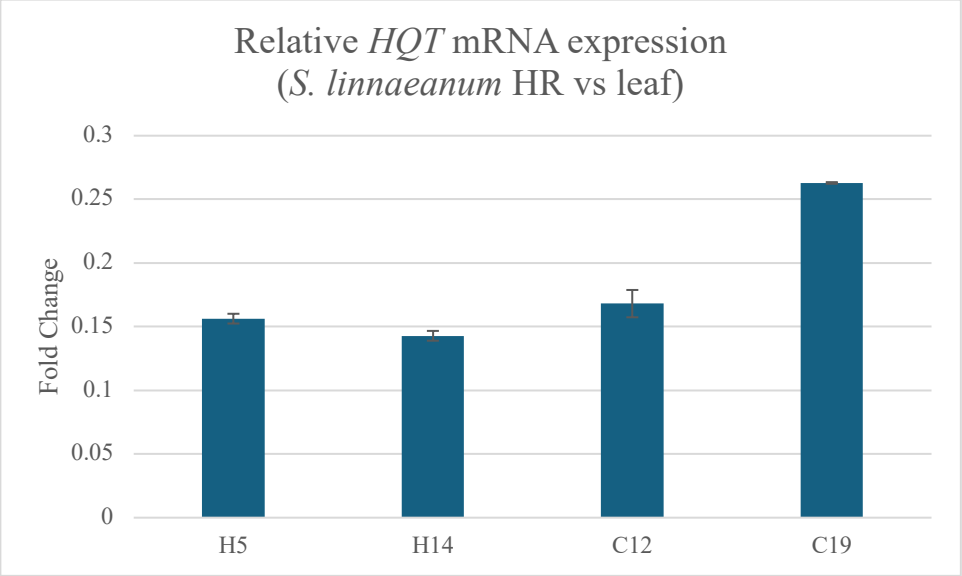

Figure 3.26. mRNA expression level change of *HQT* gene in *S. linnaeanum* hairy root cultures compared to S. linnaeanum leaves. Error bars indicate standard error.

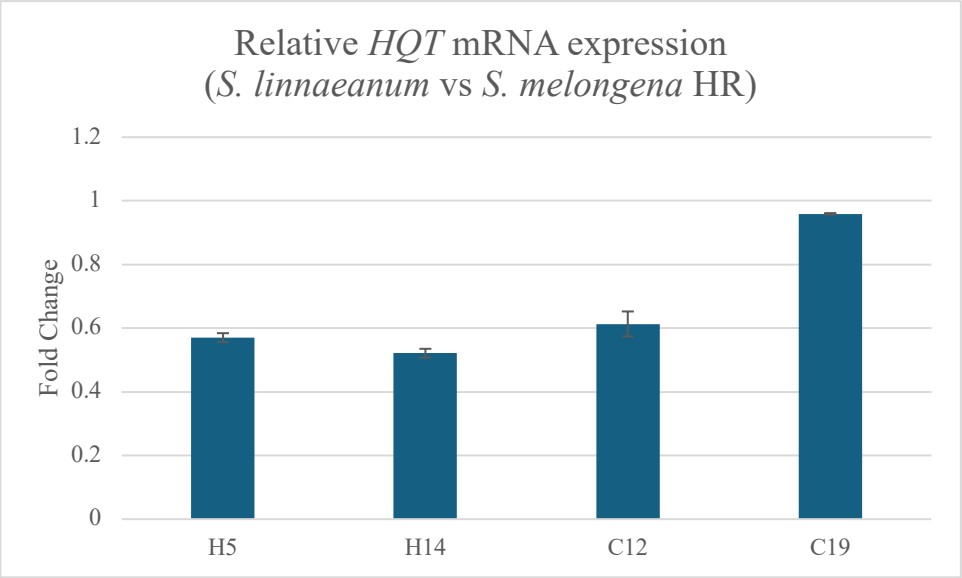

Figure 3.27. mRNA expression level change of *HQT* gene in *S. linnaeanum* hairy root cultures compared to S. melongena hairy root cultures. Error bars indicate standard error.

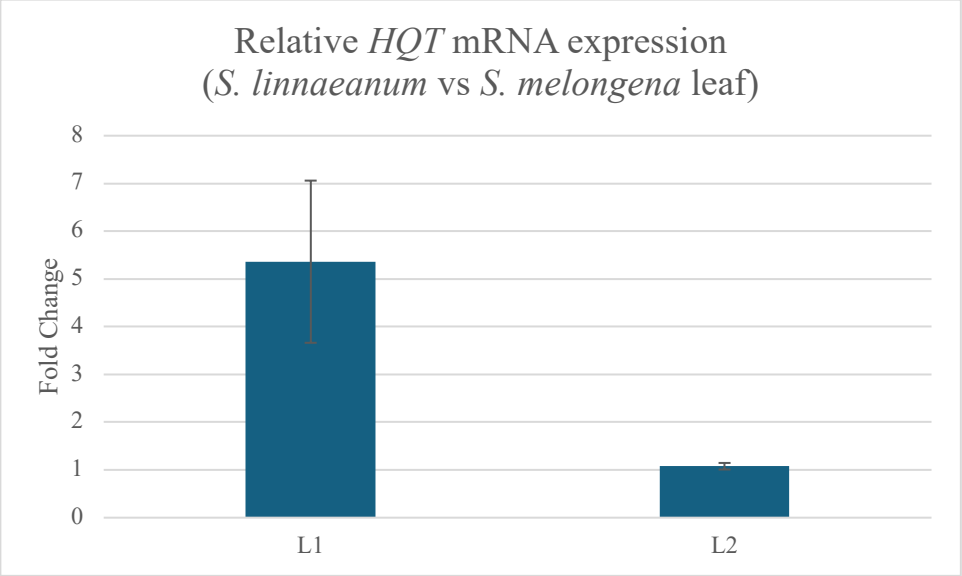

Figure 3.28. mRNA expression level change of *HQT* gene in *S. linnaeanum* leaves compared to S. melongena leaves. Error bars indicate standard error.

Chlorogenic acid, the primary phenolic compound in Solanaceous plants, is synthesized via the HQT pathway, which is facilitated by the enzyme hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (D'Orso et al., 2023). Niggeweg et al. (2004) reported that silencing of *HQT* gene led to a significant decrease in chlorogenic acid, while overexpression of *HQT* gene resulted in accumulation of chlorogenic acid in

tomato. In our study, when *HQT* expression levels were analyzed in different tissues of *S. melongena* and *S. linnaeanum*, it was observed that *HQT* expression was lower in hairy root cultures than in leaves. Also, in hairy root cultures of *S. linnaeanum*, *HQT* expression was lower than those of *S. melongena*. *HQT* expression in one of the *S. linnaeanum* leaf sample was almost equal to the expression level in *S. melongena* leaves, while the other leaf sample had 5-fold higher expression than *S. melongena* leaves. This dramatic difference may be a result of an experimental error. Overall, *HQT* expression levels were not significantly different between tissues or species suggesting that there might be additional mechanisms such as post-transcriptional or post-translational which affect the expression of *HQT* gene.

3.7. Correlation Analysis

Correlation analysis was performed to investigate the relationship between relative expression of *myb1*, *Game9*, and *HQT* genes and total phenolic content, total flavonoid content and total antioxidant capacity (Table 3.10). Results showed that there was a significant positive correlation between the relative expression of *myb1* gene and total phenolic and total flavonoid content since *myb1* transcription factor is involved in the accumulation of anthocyanins and chlorogenic acid in *S. melongena* (Zhang et al., 2016; Docimo et al., 2016). Similarly, the relative expression of *Game9* gene was found to be significantly positively correlated with total phenolic and total flavonoid content. However, the relative expression of the *HQT* gene was negatively correlated with total phenolic and total flavonoid content in our study. This negative correlation between the expression of the *HQT* gene and total phenolic and total flavonoid content may be observed because the *HQT* gene encodes the last enzyme involved in chlorogenic acid biosynthesis. No significant correlation between total antioxidant capacity and the relative expression of *myb1*, *Game9*, and *HQT* genes was observed. The most probably explanation for this finding is that there are many genes involved in the synthesis of the numerous plant secondary metabolites that contribute to the total antioxidant capacity.

Table 3.10. Correlation analysis carried out between relative expression of myb1, Game9, and HQT genes and total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC).

	TPC	**TFC**	**TAC**
Relative expression of *myb1*	0.821**	0.687**	0.439
Relative expression of *Game9*	0.769**	0.681*	0.424
Relative expression of *HQT*	-0.588*	-0.636*	-0.509
** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).			

CHAPTER 4

CONCLUSION

Non-domesticated *Solanum* species are known to be rich sources of valuable phytochemicals with various biological activities including antioxidant, antibacterial, antifungal, and anti-inflammatory effects. *Solanum linnaeanum* is a non-domesticated nightshade species which produces many secondary metabolites including phenolic compounds and glycoalkaloids. With this current study, a protocol for hairy root induction of *S. linnaeanum* was developed to characterize the secondary metabolites produced by the plant. Hairy root cultures of *S. linnaeanum* were successfully established via transformation with *A. rhizogenes* strain ATCC 43057 and T-DNA integration was confirmed. Phytochemical analyses showed that hairy root cultures of *S. linnaeanum* were more potent than the hairy root cultures of *S. melongena* in terms of total phenolic and total flavonoid contents. Hairy root cultures of *S. linnaeanum* also showed higher total antioxidant capacity than those of *S. melongena*. Expression analysis of *myb1* and *Game9* genes, which are involved in production of various phytochemicals, demonstrated that these genes were expressed at higher levels in *S. linnaeanum* hairy root cultures than in *S. melongena* cultures. Our study showed that hairy root cultures of *S. linnaeanum* are promising in terms of production of important secondary metabolites and can be improved to produce enhanced levels of these phytochemicals by using bioreactor systems and elicitors.

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