AGROBACTERIUM-MEDIATED TRANSFORMATION OF TURKISH SWEETPOTATO CULTIVAR USING A MARKER GENE

by

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ABSTRACT

AGROBACTERIUM-MEDIATED TRANSFORMATION OF TURKISH SWEETPOTATO CULTIVAR USING A MARKER GENE

Biotechnological studies are gaining importance due to climate change, population growth and the difficulties in meeting the increasing food demand. Sweetpotato [*Ipomoea batatas* (L.) Lam.] which is highly nutritious and beneficial for health is consumed worldwide as a functional food. In Turkey, the increasing interest in *I. batatas* has led to an increased production. Nowadays, sweetpotato is generally grown in the south part of the country, mainly in Hatay region. While local varieties are yet to be improved agriculturally to supply economic gain, it is difficult to perform traditional hybridization for *I. batatas*. Therefore, by using transgenic plant technology, it might be possible to develop varieties with desired traits including high nutritional value, resistance to diseases and tolerance to stress conditions. In this study, *Agrobacterium*-mediated gene transfer system in *I. batatas* local variety Hatay Red was optimized by using the *β*-glucuronidase (GUS) gene as a marker in pCAMBIA1301 plasmid carried by *Agrobacterium* LBA4404 strain. Calli formation from *I. batatas* Hatay Red internode explants were optimized with an average callus formation efficiency of 79 per cent. YEB medium was optimized at 30°C for growth of LBA4404 strain and for efficient single colony formation. The co-cultivation duration of *Agrobacterium* and *I. batatas* were optimized as 3 days. Putative transgenic calli were confirmed by GUS assay and PCR identification of GUS gene. The results showed that efficiency of *Agrobacterium*-mediated transformation is negatively influenced by many factors including the plant genotype, type of *Agrobacterium* strain, the age of explant, bacterial density, time for inoculation and co-cultivation. To the best of our knowledge, this was the first plant transformation study conducted with a Turkish *I. batatas* variety and has a great potential to provide new insights for transformation studies in local cultivars of *I. batatas*. Besides, optimizing plant tissue culture protocols are crucial to overcome viral disease problems in Turkish sweetpotato cultivars. In the second part of this study, 7 different South Korean *I. batatas* cultivars were micropropagated with an established protocol for future agricultural applications and biotechnological research studies.

ÖZET

TÜRK TATLI PATATES ÇEŞİDİNDE *AGROBACTERIUM* ARACILIĞI İLE MARKÖR GEN TRANSFERİ

İklim değişikliği, nüfus artışı ve artan gıda talebinin karşılanmasındaki zorluklar nedeniyle biyoteknolojik çalışmalar önem kazanmaktadır. Besleyiciliği yüksek ve sağlığa yararlı olan tatlı patates [*Ipomoea batatas* (L.) Lam.], tüm dünyada fonksiyonel bir gıda olarak tüketilmektedir. Türkiye'de tatlı patatese artan ilgi sonucu üretiminde artış görülmektedir. Günümüzde tatlı patates genellikle ülkenin güney kesimlerinde, ağırlıklı olarak Hatay bölgesinde yetiştirilmektedir. Yerel çeşitler ekonomik kazanç sağlamak için henüz tarımsal olarak geliştirilmemiş olmakla birlikte tatlı patateste geleneksel melezleme yöntemlerinin uygulanması zordur. Bu nedenle, transgenik bitki teknolojisi kullanılarak besin değeri yüksek, hastalıklara dayanıklı ve stres koşullarına toleranslı yeni özelliklere sahip çeşitler geliştirilebilir. Bu çalışmada, tatlı patates yerel çeşidi Hatay Kırmızı'da *Agrobacterium* aracılığı ile gen transferi, *Agrobacterium* LBA4404 suşu tarafından taşınan pCAMBIA1301 plazmidinde *β*-glukuronidaz (GUS) geni markör olarak kullanılarak optimize edilmiştir. Hatay Kırmızı çeşidinde boğum arası eksplantlar kullanılarak optimize edilmiş ve ortalama yüzde 79 kallus oluşturma oranı gözlenmiştir. LBA4404 suşunun büyümesi ve koloni oluşumu, YEB besiyerinde 30°C'de sağlanmıştır. *Agrobacterium* ile tatlı patatesin birlikte ko-kültür süresi 3 gün olarak optimize edilmiştir. Varsayılan transgenik kallus, GUS aktivitesi ve GUS geninin PCR tayini ile doğrulanmıştır. Sonuçlar, *Agrobacterium* aracılığı ile transformasyon başarısının genotip, eksplant yaşı, *Agrobacterium* suşu, bakteriyel konsantrasyon, inokülasyon süresi ve ko-kültür süresi gibi birçok faktörden etkilendiğini göstermiştir. Bilgimiz dahilinde, bu çalışma, tatlı patates yerel çeşidinde yapılan ilk bitki transformasyon çalışmasıdır ve yerel tatlı patates çeşitlerinde transformasyon çalışmaları için yeni bilgiler sağlayacaktır. Bunun yanı sıra, Türkiye'ye özgü tatlı patates çeşitlerinde viral hastalıkların üstesinden gelmek için bitki doku kültürü protokollerinin optimize edilmesi önemlidir. Çalışmanın ikinci bölümünde, 7 farklı Güney Kore tatlı patates çeşidinde mikroçoğaltım yapılmıştır. Optimize edilen mikroçoğaltım protokolü Türkiye'deki tatlı patates bitkisel gen kaynaklarının korunmasını ve gelişmiş ıslah uygulamalarında kullanılabilmesini sağlayacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

I. batatas	*Ipomoea batatas* (L.) Lam.
α	Alpha
β	Beta
C	Celsius
µg	Microgram
µl	Microliter
µmol	Micromolar
ANOVA	Analysis of variance
AS	Acetosyringone
bp	Base pairs
BAP	Benzylaminopurine
CAK2M	Plant ortholog of cyclin-dependent kinase-activating kinases
CaMV	Cauliflower Mosaic Virus
Cb	Carbenicillin
Chv	Chromosomal virulence
CIM	Callus Induction Medium
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAOSTAT	The Food and Agriculture Organization Corporate Statistical Database
g	Gram
GA_3	Gibberellic acid
GUS	*β*-glucuronidase
GFP	Green Fluorescence Protein
Hg	Hygromycin
Hr	Hour

2,4-D	2,4-Dichlorophenoxyacetic acid
Kan	Kanamycin
kB	Kilobase pair
$KH_2PO.3H_2O$	Potassium phosphatedibasic trihydrate
l	Liter
LB	Left border
LUC	Luciferin
mg	Milligram
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
min	Minute
ml	Milliliter
Mm	Millimolar
MS	Murashige and Skoog
mmHg	millimeter of mercury
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
nm	Nanometer
NPC	Nuclear pore complex
nptII	Neomycin phosphotransferase
OD	Optical Density
ori	Origin of Replication
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRs	Plant growth regulators
PPM	Plant preservative mixture
pH	Power of Hydrogen
Rm	Rifampicin
RB	Right border
RM	Regeneration Medium

Rpm	Revolutions per minute
Ss	Single stranded
TAE	Tris acetate- EDTA
TBP	TATA-box binding protein
Tc	Tetracycline
T-DNA	Transfer DNA
T-HCl	Thiamine hydrochloride
Ti	Tumor-inducing
T4SS	Bacterial type IV secretion system
UPP	Unipolar polysaccharide
Vir	Virulence
VIP 1	VirE2-interacting protein 1
YEB	Yeast extract broth
X-gluc	5-bromo-4-chloro-3-indolyl glucuronidase

1. INTRODUCTION

Climate change, human population growth and increased food consumption threaten existing agricultural production systems. Due to the difficulty in meeting the food demand for the growing world population and the inequality of agricultural productivity, studies in the field of plant genetic resources and agricultural practices gain importance throughout the years. Sweetpotato [*Ipomoea batatas* (L.) Lam.] is a dicot plant belonging to the *Convolvulaceae* family. When considering its global consumption, *Ipomoea batatas* is the most important crop as a functional food after wheat, corn, potato, rice and barley [1]. With consumption, it reduces the risk of liver, skin and prostate cancer. Thus, it is also known to be effective in reducing the risk of a heart attack. In addition, it strengthens the immune system and fights against the negative effects of cancer [2].

The interest in *I. batatas* grown in the Hatay region in our country is increasing due to its consumption as a functional food. Depending on the interest, in addition to Hatay, there is an increase in the production and cultivation of *I. batatas* in Iskenderun, Kahramanmaraş, Gaziantep and Adana regions. However, the lack of knowledge about virus problems and limited number of local varieties prevents the increase of sweetpotato production in our country [3]. Field and molecular studies are insufficient for trait improvement in the varieties known as Hatay Native, Hatay Red, Carrot, Pencil and Yellow Potato. With genetic studies in *I. batatas*, this valuable functional food can be improved agriculturally to supply economic gain to our country. Due to the incompatibility with other species, it is difficult to perform traditional hybridization for *Ipomoea batatas* [4]. Besides, the spread of diseases is easy in vegetatively propagated plants and the seeds can deteriorate in a short time due to viral and fungal diseases. Black rot disease is a negative impact caused by the fungus *Ceratocystis Fimbriata* that considered the most dangerous disease of *I. batatas* in many places of the world. This disease agent can develop on the plants in the tuber or the roots during storage [5]. As a result of this disease, plant growth and yield are significantly decreased. Also, it reduces production in the agricultural field.

The development of a new variety of plants is a significant success of transgenic biotechnology to overcome traditional breeding problems.By using transgenic plant technology, it might be possible to achieve varieties with high nutritional value, resistance to the virus and tolerance to stress conditions [6]. Therefore, among different gene transfer techniques, the use of the *Agrobacterium*-mediated transformation system is preferred by many researchers as it does not involve complex equipment and procedures [7]. Reporter genes are used as suitable markers to visualize gene expression and protein localization in vivo across a broad spectrum of prokaryotes and eukaryotes. Commonly used reporter genes can be listed as chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) luciferase (LUC), and *β*-glucuronidase (GUS) for *Agrobacterium*-mediated gene transfer [8].

In this study, *Agrobacterium*-mediated gene transfer system in *I. batatas* was optimized by using the *β*-glucuronidase (GUS) gene as a marker. Turkey originated Hatay Red variety has been used for *Agrobacterium* mediated transformation. To our knowledge, this was the first plant transformation study conducted with a Turkish *Ipomoea batatas* variety. For this reason, using Turkey-specific variety is a significant pioneer for the biotechnological applications of sweetpotato in our country. In addition, the regeneration capacity of South Korean *I. batatas* varieties has been optimized under tissue culture conditions for future agricultural applications and biotechnological research studies. Thus, by obtaining pathogen-free plants via plant tissue culture techniques, both the sweetpotato germplasm in Turkey will be preserved and its agricultural production will be increased.

2. LITERATURE REVIEW

2.1. Ipomoea batatas (L.) Lam. (Sweetpotato

Ipomoea batatas (L.) Lam. is a dicotyledonous, tuberous plant to be a member of *Convolvulaceae* family. In 1753 this perennial plant was named as *convolvulus batatas* by Linnaeus. Then, in 1971, according to its surface of the pollen grains and stigma shape, this hexaploid herbaceous plant was classified as *Ipomoea* by Lamarck [9]. Although it shares a similar name, it is not related to the potato plant (*Solanum tuberosum*) [10].

Figure 2. 1. *Ipomoea batatas* (L.) Lam. tuberous plant [11].

Sweetpotato is one of the most essential nutritional products regarding to global consumption. It is consumed as a functional food in the world due to its rich fiber, protein and carbohydrate source [1]. This perennial herb rich in carotenoids, potassium, iron and calcium sources is grown as an annual crop to store nutrient reserves in its roots.

The storage roots of *I. batatas* contain 70 per cent starch, 10 per cent sugar, and 5 per cent protein [3]. As the differences between *I. batatas* varieties increase, differences may occur in the quality and balance of ingredients such as ascorbic acid, carotene, protein B and minerals [12]. Depending on the environmental conditions, *I. batatas* have different shapes, colors and sizes. Its edible tubers appear in white, yellow, orange and purple colors. Consumed as one of the most nutritious vegetables and basic food product, the starchy tubers of *I. batatas* are an important consumption product. In addition to tuber consumption, the leaves and shoots are also edible [11].

According to the research on its content, the dry mass of *I. batatas* is between 16-40 per cent. The carbohydrates (starch, sugar, cellulose, pectin and hemicellulose) constitute between 75-90 per cent of this weight. In addition to sucrose, small amounts of glucose and fructose are the plenteous sugars in raw *I. batatas* [13]. Consumed as a functional food, *I. batatas* has a regulating effect on blood sugar in terms of health. Its consumption reduces the risk of liver, skin and prostate cancer. It is established to be capable in reducing the risk of a heart attack. In addition, it strengthens the immune system and fights against the effects of cancer and aging [14].

In terms of development, this heat-preferred plant is very sensitive to cold and froze. It matures between 25-35°C degrees. For this reason, it is affected by cold at temperatures below 10º C and its growth slows down. In our country, it is mostly preferred to be planted at the beginning of April in terms of adaptation. To support root development during the plant growth period, it is ideal that the daytime temperatures are between 30-35ºC and the nighttime temperatures are around 20ºC [15].

Since *I. batatas* is a tuberous root crop, the soil to be used must be suitable for root development (Figure 2.1). The crop grows best in sandy, well drained, loam soil with a pH of 5.0-6.7 [3]. By providing this environment, sweetpotato is easily cultivated and propagated with high efficiency.

In addition to development, irrigation time is sufficient for its growth. To prevent the plant from rotting from a high amount of water, irrigation is ended 3 weeks before the harvest time and the soil is allowed to dry well [16].

In our country, there has been an increase in the interest in *I. batatas* cultivation in recent years. However, difficulties in obtaining seeds from varieties prevent the spread of the product in Turkey [17]. For this reason, *I. batatas* production areas in our country are limited to Iskenderun, Kahramanmaraş, Gaziantep Adana and, especially in Hatay. Cultivars with different skin and inner color are grown in the Hatay region with names such as Hatay Native, Hatay Red, Carrot, Pencil and Yellow sweetpotato (Figure 2.2) [18].

In the studies conducted in our country, it is seen that there are few studies on *I. batatas*, and most of the studies are only in fields of agriculture. In the field studies, it has been seen that the *I. batatas* local variety Hatay Red has high yield performance and it has been stated that it is suitable for growing in the Aegean site of Turkey in terms of yield and quality. In addition, the Hatay Red variety has high content of sugar, starch, vitamin A and vitamin C. Therefore, Hatay Red can be selected as a sufficient cultivar to be grown in the Aegean Region for highest field performance [18].

Genotypes	Storage-root shape	Skin color	Flesh Color
Hatay Red	Long-eliptic	Dark red	Pale yellow
Hatay Native	Irregular	Dark red	Cream
Carrot	Round-eliptic	Pale yellow	Orange
Yellow	Long-eliptic	Pale yellow	Cream

Figure 2. 2. Local sweetpotato genotypes with different inner and skin color (modified from Aydemir *et al*., 2021) [18].

As an outcome of recent studies, it has been demostrated that *I. batatas* have a great ability for adaptation and yield potential on Mediterranean coasts and semiarid conditions. Thus, it has been shown that new fields such as Diyarbakır, Şanlıurfa, and Adana can be applicable areas for agricultural production besides Hatay, the location for traditional sweetpotato production in our country [19].

2.2. Global Production of Sweetpotato

The *I. batatas,* a tuberous root plant, can be produced in the countries located at both tropical and subtropical areas. Furthermore, in the 16th century, it is first transferred to Europe from South America by the Spanish and Portuguese citizens [20]. According to data provided by Food and Agriculture Organization of United States (FAO), *Ipomoea batatas* is the seventh most significant functional food product in the earth with high production rate. In addition, as stated by FAO, it is grown with an average of 140 million tons per year, which corresponds to an area of 9 million hectares [11]. Looking at world production parameters, China leads for roughly 80 per cent of the total *I. batatas* production and ranks first in production with an annual output of 100 million tons per year. Therefore, today *I. batatas* is ranking as an important food product in many parts of the world (Figure 2.3) [21].

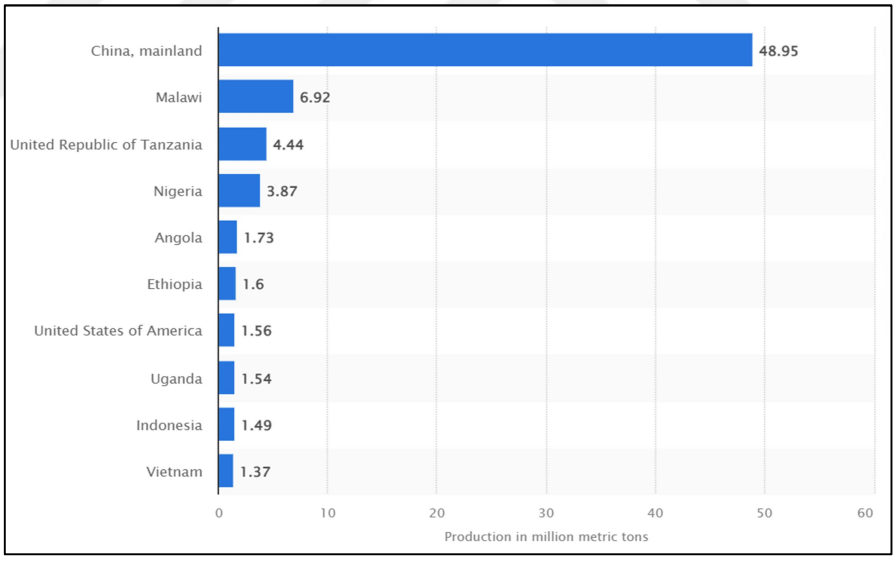

Figure 2. 3. Worldwide production rates of *I. batatas* in 2018 for million metric tons [21].

Sweetpotato is the most vital food product for global consumption after corn, potatoes, wheat, rice and barley (Figure 2.4). This widely consumed product is accepted as a functional food. Functional foods provide both health benefits and basic nutritional needs with their ingredients. The use of such functional foods is aimed at preventing chronic diseases, cancer and diabetes [22].

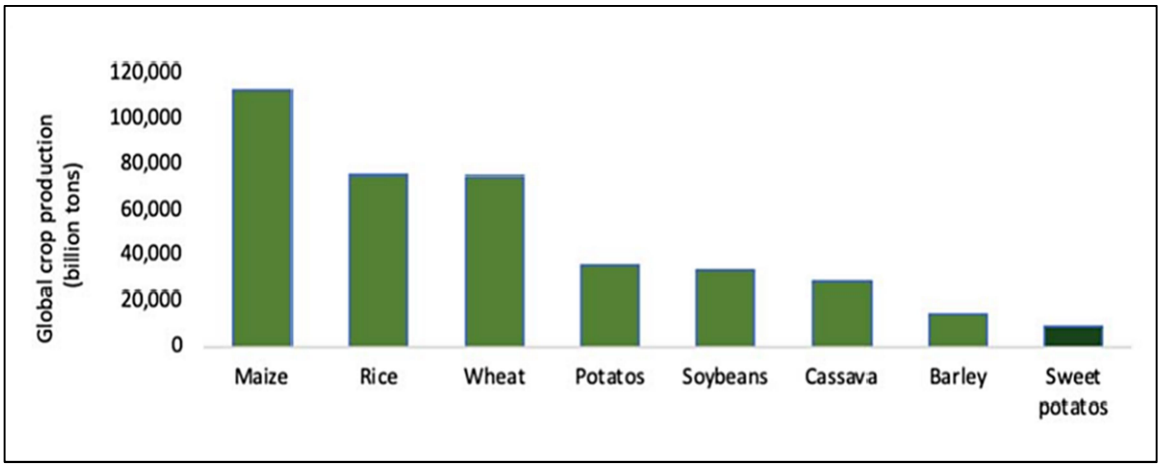

Figure 2. 4. Global production of major crops including *I. batatas* as a functional food. The production numbers were received from (FAO) Statistics, 2019 [23].

It is estimated that *I. batatas* was introduced to Turkey from Cyprus at the beginning of the 20th century. However, in our country the production of *I. batatas* are only at Hatay, İskenderun, Kahramanmaraş, Gaziantep and partially Adana regions. For this reason, there is limited statistical data on *I. batatas* production [24]. According to TÜİK data for 2019, the production of *I. batatas* in our country was 495 tons from an area of 344 decares [3].

It is reported that the production area in the villages of Hatay region where *I. batatas* cultivation is carried out in our country is between 1000-1500 decares. In addition, the total production amount of *I. batatas* is around 1.5-2 thousand tons per year [3].

Looking at the data for each region, it is known that most of the production is made in the Hatay area. Due to the lack of attention to this tuberous plant, it could not find the opportunity to spread to other parts of the country. The reason for this is the lack of agricultural improvement practices. Besides, its taste is unfamiliar to Turkish people and they are not informed about its health benefits.

Also, it is not used as an alternative feed source in animal feeding. In addition, it is not known that it can be utilized as a raw material in the industrial field [24]. For this reason, *I. batatas*, which is very popular in the world with its different taste and nutritious properties, is produced only as a small-scale family business in Turkey [19].

2.3. Application of Biotechnological Tools for Sweetpotato

Due to the difficulty in meeting the food demand for the increasing world population and the inadequacy of agricultural productivity, studies in the field of plant genetic resources and agricultural practices are gaining importance. Plant biotechnology uses different genetic engineering and plant tissue culture techniques to create genetically modified plants that exhibit beneficial property to meet the demands in these fields.

In vegetatively propagated plants, the spread of diseases is easy and the seeds can get harmed in a short period of time. Black rot disease, produced by the fungus *Ceratocystis Fimbriata,* is considered the highest dangerous disease of *I. batatas* in many parts of the world. The disease agent can develop on the plants in the field or on the roots during storage. As a result, plant growth and yield are significantly decreased with reducing agricultural production [25].

In addition, sweetpotato chlorotic dwarf virus (TPKBV) and sweetpotato hairy spot virus (TPTBV) are viruses that cause diseases in plants. The co-infection of these two viruses results in sweetpotato virus disease (TPVH). As a result of these viruses, damaged, dwarf or chlorotic leaves are formed in the *I. batatas* plant [26]. Viral diseases also directly affect the production by reducing the productivity of the plant in the agricultural areas and nutrient content. Studies in the field of plant science are gaining importance in order to prevent plant diseases that are harmful in many areas.

Plant biotechnology provides new contributions to agricultural production by focusing on seed-variety development with molecular-assisted breeding and the production of genetically modified products.

The application of genetic information and sequencing with biotechnological studies is of great importance in increasing agricultural productivity by making advances in plant breeding, reducing the damage of diseases to plant yield and eliminating global famine [27]. In our country, for *I. batatas*, there are few studies in the biotechnology field with local cultivars. By carrying out tissue culture studies against pathogen and virus problems, genetic resources can be preserved and also agricultural productivity will be raised.

In addition, by examining the optimum conditions in the fields for safe production, and the economic and industrial contribution of *I. batatas*, production rates can be increased [28]. Additionally, use of recombinant DNA technology, can overcome mismatch mechanisms by adding desired features to target genomes.

Genetic modification methods are important for crop improvement with an efficient plant formation process using transgenic approaches. Transformation technology gives the ability to regenerate the genetically transformed plant using isolated plant cells or tissues. This method forms the basis of most plant transformation systems. Therefore, tissue culture and plant regeneration are integral parts of most plant transformation strategies [29].

2.3.1. Tissue Culture and Regeneration of Sweetpotato

With the increase in global consumption, the demand for herbal products is increasing and the importance of plant biotechnology in this field is rising over the years. Plant reproduction by plant tissue culture method was initiated by the German scientist Haberlant in the early 20^{th} century. Today, it is currently one of the most important methods used in the fields of agricultural biotechnology, molecular biology and related fields [30].

The tissue culture method, also called micropropagation ensures the reproduction of cells, organs and tissues under sterile conditions to propagate millions of virus and disease-free plants in the world [31]. In Turkey, *I. batatas* is commercially propagated vegetatively by using shoots and sprouting the depot roots. Viral diseases can be seen again in these reproduced tubers and shoots. Therefore, it is important to perform in vitro micropropagation in a suitable laboratory environment for plants to be free from viruses. There are two methods of plant regeneration to obtain new plants that are virus-free and identical to the mother plant using the plant tissue culture method. These are called somatic embryogenesis and organogenesis [32].

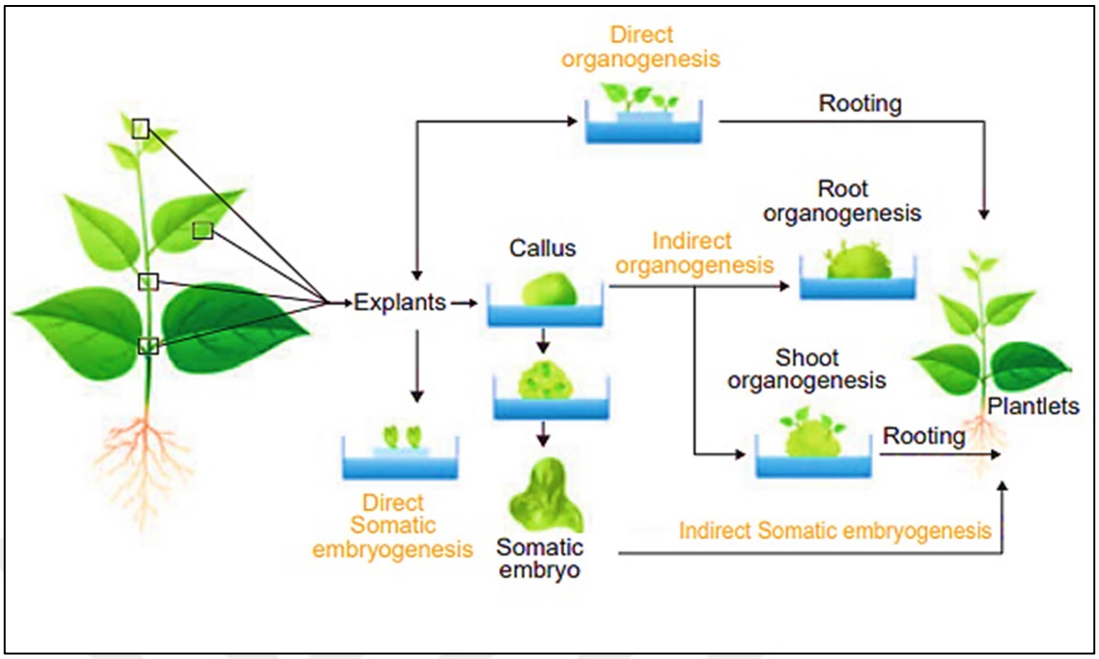

Figure 2. 5. Production of whole plant by different plant regeneration methods using mother plant as an explant in order to obtain virus-free identical plantlets [33].

In the somatic embryogenesis method, embryo-like structures are formed directly or indirectly by using somatic tissues obtained from different parts of the plants to regenerate plantlets. At direct somatic embryogenesis, an embryo is obtained as an explant directly from the plant. Whereas in indirect embryogenesis, callus, which is called an unorganized cell community is used and an embryo is obtained by using this callus tissue or suspension culture method [30]. In the other regeneration method, called the organogenesis, the developing organs of the plant are used directly. Thus, a plant is obtained from the callus culture without the need for embryo germination [31]. Organogenesis or somatic embryogenesis formation in plant tissue culture differs in each plant species (Figure 2.5). In the process in which plants are produced in vitro in a culture medium with aseptic techniques, plantlets growth is supported by plant growth regulators [34].

Tissue culture techniques used to solve problems related to food safety and agricultural production have opened a new page in agricultural science. As a result of this innovation in agricultural science, more importance has been given to the use of biotechnological methods for the genetic improvement of important characteristics in sweetpotato [35].

As in every plant, the regeneration of the plant to be obtained as a result of gene transfer in sweetpotato is of great importance for the control of transformation [34]. Based on this information, it was stated that the transformation success largely depended on the regeneration capacity of the target sweetpotato explant used [36]. The deficiency of an effective system for regeneration in sweetpotato has been a barrier for the application of biotechnological tools. One of the main challenges for gene transfer in sweetpotato is that it is a persistent species for regeneration control [37].

The efficiency of micropropagation depends on different factors these are, including plant genotype, physiology of plant, composition of basal medium with suitable PGRs, and type of light source [38]. Sweetpotato regeneration is mostly restricted to certain genotypes. In addition, when regeneration studies are carried out to expand the genotype variety, it has been determined that it is a stubborn species and resistant to regeneration as each variety reacts differently [39]. Plant growth regulators added to the prepared basal medium contribute to the development of plants with desired conditions. Plant hormones and their synthetic forms are widely used as plant growth regulators. As in every plant species, for *I. batatas*, the use of correct hormone combination ratios is a critical factor in the regeneration and at the same time, it contributes to the high regeneration efficiency in the tissue culture for micropropagation [40].

In a study conducted in our country, micropropagation was obtained in Hatay Red and Hatay Native local sweetpotato varieties when 1.0 BAP mg/L, 0.01 mg/L NAA and 1.0 mg/L GA3 growth regulators were used on node plant segments [19]. Petioles, meristem tissue, leaf disk or callus tissue of *I. batatas* are useful sources for regeneration; however, efficiency is genotype dependent [41]. The micropropagation rate varies according to each sweetpotato variety; therefore, it is important to optimize growth regulators for different varieties. In addition, regenerative capacity of the genotype is crucial in genetic studies for successful genetic transformation [42].

Meristem and shoot tip culture techniques are used in tissue culture to obtain virus-free plants in many plant species [43]. Considering the studies, mostly in vitro meristem culture was used for the propagation of virus-free sweetpotato with tissue culture.

It was stated that the success of sweetpotato tissue culture in China depended on the use of meristem culture [44]. Also, the use of shoot tips from lateral buds as explants also showed successful results [45].

Another study reported that the use of buds as explants was a good tool for sweetpotato regeneration. In addition, different growth regulators and doses were investigated to determine the regeneration rates of genotypes, and as a result, it was determined that plant growth regulators played an significant role in the success of callus formation, organogenesis, or somatic embryogenesis in tissue culture [46]. Thus micropropagation of sweetpotato using nodal explants has become popular in the recent years.

In a study, 'Carmen Rubin', 'White Triumph' and 'Gaozi' node explants have been reported to be used for micropropagation of sweetpotato cultivars [47]. In another study, 'KSP 36' and 'KEMB 36' sweetpotato cultivars were micropropagated using nodal explants in Brazil [48]. When the tissue culture studies on the local *I. batatas* in our country were examined, it was found that the sweetpotato varieties (Carrot and Hatay Native) had higher plant formation with the supply of cytokinin and gibberellin as plant growth regulators compared to those grown in auxin containing media [49]. As a result of another study conducted in our country, it was stated that there were significant differences among different sweet potato genotypes in terms of regeneration rate and subsequent growth rates in meristem culture [19].

2.3.2. Transformation Studies for Sweetpotato

Sweetpotato breeding is common in our country however, there is insufficient molecular knowledge on sweetpotato. In addition to the traditional breeding program, it is important to use genetic engineering technology to improve plants with different traits. Especially in recent years, it has been shown that gene engineering has great potential in obtaining improved plants in terms of resistance to abiotic and biotic stress conditions. For that reason, today genetic transformation is an alternative or complementary to conventional breeding [50]. Genetic transformation is a way to create a transgenic plant with required traits by giving the desired genetic material to selected plants by using different tissues of plants called explants. Transgenic roots, calli, somatic embryos or plants with desired properties can be created with the transformation methods [42].

Considering the studies of the last 30 years, there is a continuous development in gene transformation approaches. As a result of the transformation methods adopted in genetic engineering, the way of crop improvement is changing and important developments are taking place in agricultural production, crop protection from diseases and crop improvement for different environmental conditions [51].

The methods developed for genetic transformation are divided into two groups as direct and indirect according to their effects and application conditions. Different methods such as biolistics, microinjection, electroporation, liposome-mediated gene transfer and PEG-mediated gene transfer have been developed as a direct transfer method. The chosen method depends on the characteristics of the plant and DNA is directly transferred [52]. In the vector-mediated transfer method, known as indirect methods, the desired DNA is transferred to the target genome indirectly with the help of biological vectors. Therefore *Agrobacterium*-mediated gene transfer and plant virus vectors are used as indirect methods. Compared to protoplast or biolistic methods as plant transformation methods, *Agrobacterium* transformation is a more effective method due to the stable expression of gene, possible to transfer of large size of DNA and low copy number of the transgene [53].

Until today, agronomically important crops have been genetically modified by using *Agrobacterium*. To overcome nutritional quality, abiotic and biotic stress conditions of *I. batatas,* there have been studies about success of *Agrobacterium*-mediated transformation. Today as a result of the progress of studies with *Agrobacterium*, transgenic *I. batatas* with broad virus resistance were developed [54]. However, for effective transformation of *I. batatas*, protocols have been developed only for few cultivars in the world. Genetic transformation is limited by the unsuccessful response of several *I. batatas* cultivars to in vitro regeneration. Thus, transformation methods of sweetpotato are still genotype dependent [55].

When transformation studies are optimized for the local varieties in our country, pathogen-free varieties with the desired traits can be obtained and this will have a positive impact on the economy. Many studies carried out to develop efficient transformation in *I. batatas* variety in the world. Particle bombardment and electroporation of sweetpotato have been tried hoewever, only transformed callus, transient gene expression, or a several transgenic sweetpotato plants have been obtained [56]. In addition to these studies, an *Agrobacterium*-mediated transformation system has been researched for various *I. batatas* genotypes.

Studies carried out for several *I. batatas* types to identify tolerance for salinity and drought [57], resistance to diseases and pests [58], starch, anthocyanins and carotenoids biosynthesis [8]. In *A. tumefaciens*-mediated transformation studies, a different section of sweetpotato plants was used to measure transformation efficiency, and stable transgenic plants were also obtained. However, the studies mostly showed low transformation efficiency in sweetpotato using leaves, petioles, stems and storage roots as an explant sources [59]. The delivery of the target gene to the sweetpotato protoplast and its expression in the callus tissue was also reported [59].

In the following years, high transformation success was achieved in embryogenic suspension cultures using *A. tumefaciens* EHA105 strain involving binary vector pCAMBIA1301. Therefore, binary vector system cultures containing EHA105 strain and suspension of embryogenic calli are strongly suggested for transformation of sweetpotato varieties [60].

Several transgenic plants have been reported to be produced with embryogenic suspension cultures using *A. tumefaciens* strains A208SE and LBA4404. Additionally, an efficient *A. tumefaciens*-mediated transformation system has been also reported for sweetpotato using the electrocompetent *A. tumefaciens* strain EHA105 [61]. It has been seen that the introduction of agriculturally important genes into commercial sweetpotato varieties can be carried out on a large scale with the transformation studies carried out around the world. In addition, traditional cultivation techniques and molecular methods should be studied together to develop efficient sweetpotato varieties with adapted to conditional stress with increased nutritional quality [62].

2.3.3. The mechanism of *Agrobacterium tumefaciens*-mediated transformation

A. tumefaciens generally infects dicotyledonous plants in nature, can genetically transform a wide variety different plant species under sterile conditions in vitro by using developing technological methods [63]. Therefore, it has become the most preferred genetic transformation method for manipulation of different plant species. Since 1853, the gram-negative *Agrobacterium* known to cause crown gall diseases in the roots of plants by transferring its own bacterial DNA (Figure 2.6). Due to its flagella structure, *A. tumefaciens* progress towards the photo-assimilates in the roots and forms tumor [64].

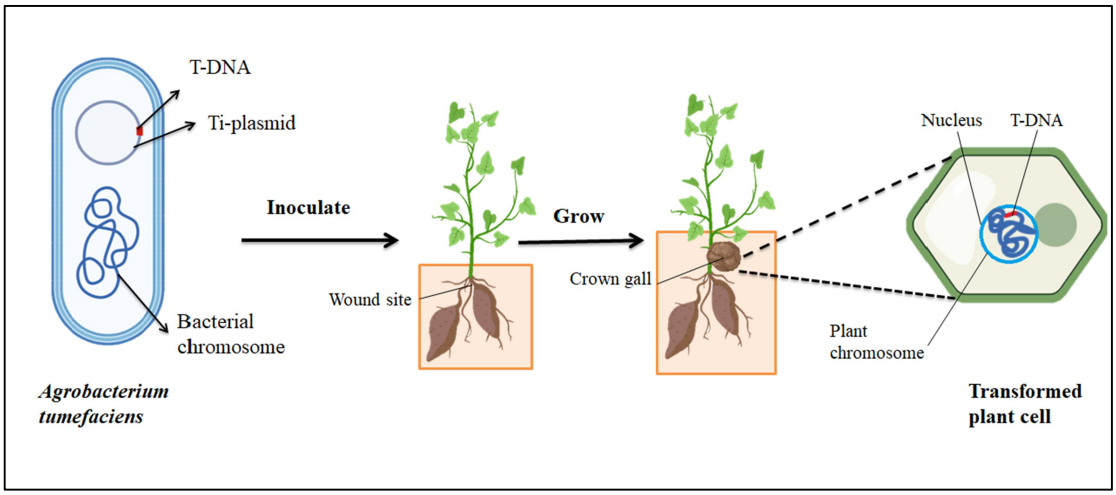

Figure 2. 6. The process for crown gall disease in nature with tumor formation in plant roots by the infection from *A. tumefaciens* (modified from Clark *et al*., 2019) [65].

This virulent bacterium is now used by plant biotechnologist for examining cellular and basic biological mechanisms. Besides, they are widely used in genomic experimental fields to regulate cellular expression. In literature, expression of transgene, chromatin targeting, DNA repair and proteasomal degradation can be given as examples for these studies that used *Agrobacterium*-mediated transformation [66]. In addition, subcellular localization of scalable and rapid protein, production and interactions of recombinant proteins can be performed using *Agrobacterium* [67]. As a result, in *Agrobacterium*-mediated transformation using gram-negative *A. tumefaciens,* it is possible to transfer the multiple or relevant genes into target plant cells [68].

Agrobacterium drives the opine biosynthesis since it requires energy and nitrogen for its metabolism [67]. It is stated that opine biosynthesis is already present in hairy roots or crown galls produced by *Agrobacterium*, but synthesis does not occur in the lack of infection. Therefore, with the infection of the plant cells, *Agrobacterium* drives the opine synthesis by specific enzymes encoded by genes found in the Ti-plasmid [69]. According to the type of opine synthesized by the tumor tissue it is called octopine, nopaline or mannopine type depending on the *Agrobacterium* strain [70]. Generally, all virulent *Agrobacterium* strains carry a ring-shaped plasmid called tumor-inducing (Ti) plasmid, which enables tumor formation and cell proliferation. Ti plasmids in plants are in double stranded DNA structure with approximately 200-250 kb in size.

The target gene to be transferred, transfer DNA (T-DNA), and virulence (*vir*) regions on tumor-inducing plasmid play a role together for tumor formation. Therefore, the T-DNA region restricted to 25 bp is transmitted and integrated into the target plant genome for genetic studies [71].

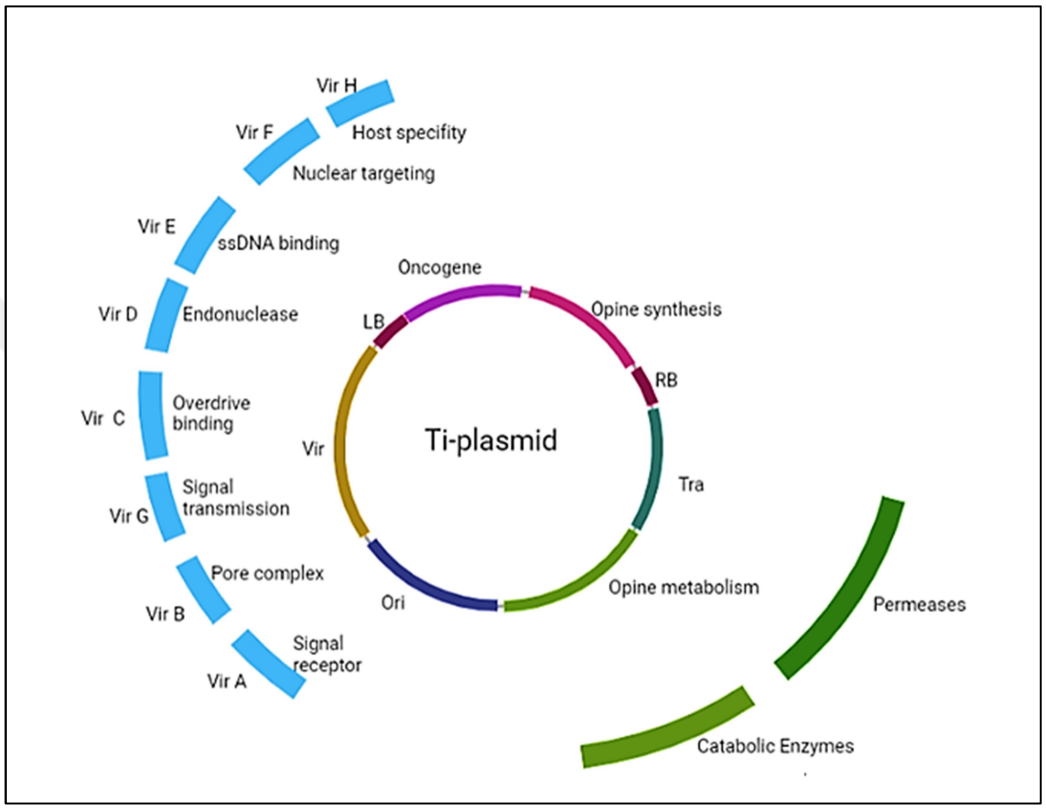

Figure 2. 7. Typical Ti- plasmid cloning for plants and its integration to the plant genome by *Agrobacterium* using binary vector strategy (modified from Aditya *et al*., 2018) [3].

Generally, in typical Ti-plasmid, there are four crucial regions. These are including the T-DNA region, *vir* region, origin of replication and opine breakdown (Figure 2.7). *Vir* region encodes virulence genes to initiate T-DNA integration into plant genome [72]. T-DNA region is a portion of the Ti-plasmid and is transferred to the target host plant cells by conjugation. Besides in Ti- plasmid, T-DNA contains two group of genes called as Onco-genes and opine synthesizing genes. With the integration to the plant genome, these genes are expressed. Oncogenes synthesize plant growth hormones (phytohormones) such as auxin and cytokinin to stimulate cell division [73].

In the literature, the transfer mechanism of *Agrobacterium* to target plant is named under 4 headings. These are; 1) plant cell recognition by bacteria with the activation of the virulence system, 2) contact of *Agrobacterium* with plant cells, 3) transfer of T-DNA to the plant cell and integration into the plant genome, and 4) expression of T-DNA in the target genome respectively (Figure 2.8).

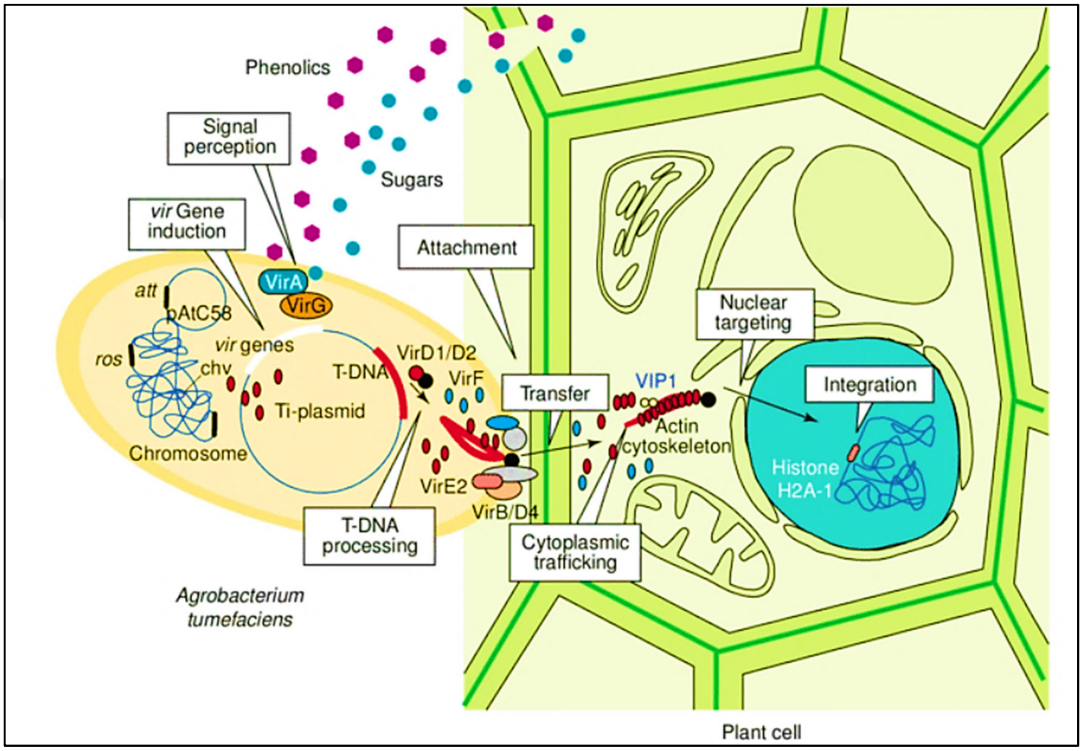

Figure 2. 8. General process about transformation of specified gene to target plant genome. The image shows basic steps for the transport of T-DNA molecule in the *Agrobacterium* Ti plasmid into the host nucleus and its integration process into host genome [74].

Normally undamaged, healthy plants do not produce or produce at low levels of phenolic compounds without injury; however, when plants become wounded, the production of these compounds is dramatically increased. Phenolic compounds such as acetosyringone, syringaldehyde or acetovanillone leak from wounded plant tissues (Figure 2.9). As a result of injury and stimulation, phenolics are released from the injured plant. By the presence of phenolic compounds, in the *Agrobacterium vir* genes are activated. In addition to phenolic compounds, it has been observed that different factors such as low pH and monosaccharide ingredients of the plant cell wall can activate virulence genes [10].

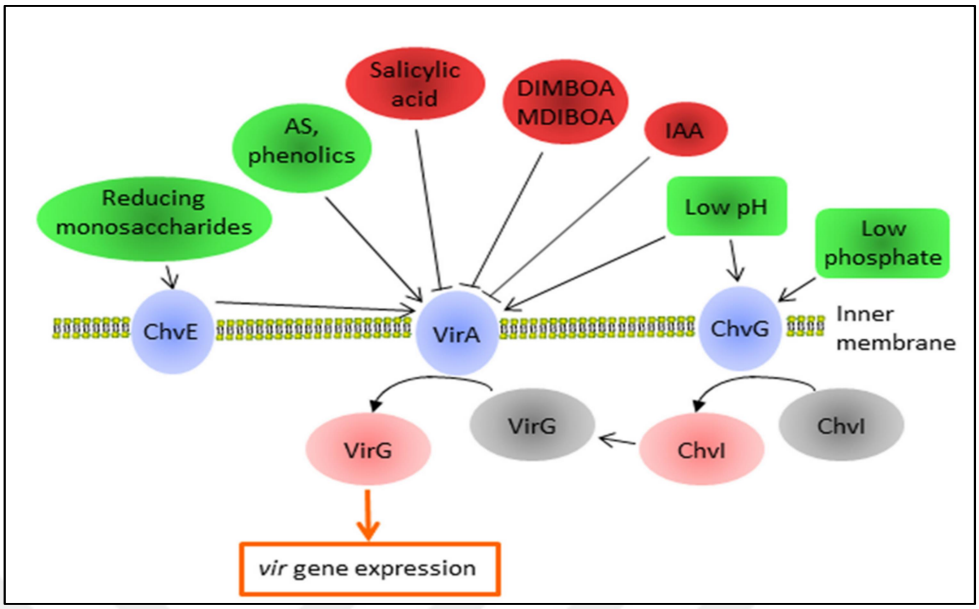

Figure 2. 9. Different plant and environmental signal effecting expression of *vir* genes with the interaction to *Agrobacterium* VirA/VirG two-component regulatory system [10].

In the literature, the *vir* genes *VirA* and *VirG* are referred to as a two-component regulatory system. When the phenolic signal molecules secreted by the plant, this signal binds and activates the transmembrane protein called as VirA. VirG gene is activated after autophosphorylation of histidine residue (His474) and acts as a transcription factor. It provides activation of *vir* genes by binding to the 12 bp sequence element in the promoters of all *vir* operons. Thereby, transcription of *vir* genes in the promoter of the Ti-plasmid become induced [66].

In addition, for the activation of VirA, the periplasmic sugar-binding protein called as the Chromosomal virulence gene E (*chvE*) collaborate with the VirA/VirG two-component regulatory process. *chvE* which is homologous to the galactose-binding protein of *E. coli*, stimulates *vir* genes by binding of sugars [75].

In bacterial cells T-DNA exists as a single stranded (ss) DNA-protein complex called as immature T-complex. Generally in immature complex, VirD2 molecule is covalently bound to the 5 'end of the T-helix [76]. VirD1 and VirD2 proteins act together during transformation and contribute to the formation of (ss) T-DNA [77].

Therefore, VirD2 endonuclease works to separate left and right boundaries of T-DNA. Based on this information, it is stated that the nucleosome complex probably also fuses at the left T-DNA border of the Ti-plasmid [78]. The single stranded T-DNA molecule formed after activation of VirD1&D2 proteins. (ss) T-DNA is covered with a large number of VirE2 molecules along its entire length in order to transport T-DNA to the target plant cell nucleus. The VirB / D4 type IV secretion system is used to transfer T-DNA to the host cell (Figure 2.7). T-DNA is thought to exist as a mature T-DNA complex after its entry into the host cell cytoplasm [79].

Extracellular polysaccharide called as unipolar polysaccharide (UPP) is a polar type binding that supports of *Agrobacterium* cells. Recent *Agrobacterium* studies have reported that binding of *Agrobacterium* and type of UPP synthesis depends on concentration of calcium ions. Also, the presence of calcium ions in high concentrations eliminates UPP synthesis and the number of *Agrobacterium* cells attached to plant surfaces decrease [80].

Plant cytoskeleton is important for *Agrobacterium* in intracellular movement to the nucleus. As a result of a study, it was stated that *Agrobacterium* have a purpose of dynein-like plant engine to transmit its T-complex to the nuclear pores. The final stages of the transformation process is travelling of the T-DNA to nucleus through to the cytoplasm of the plant cell. As a final step of movement into the nucleus, T-DNA integration occurs [81].Due to its dense structure, cytoplasm of the plant cell limits Brownian diffusion of large macromolecules consisting of microtubule, actin and intermediate filament networks [82]. The T-complex protein component known as VirD2 and VirE2 have been found to interact with host proteins for nuclear import into host cells [83].

VirE2-interacting protein 1 (VIP1) and its functional homologue, the bacterial VirE3 protein act as molecular adapters. They provide the connection between VirE2 and the host cell karyopherin a [84]. It is also known that for nuclear import VirD2 interacts with a member of the *Arabidopsis* karyopherin family AtKAPa [83].H2A histone is a plant chromatin protein essential for T-DNA integration. *Agrobacterium* interacts with H2A histone for T-DNA integration using affinity of VIP1 (Figure 2.8.) [85]. Therefore, VirD2 and VirE2 are both required for nuclear import of (ss) DNA. As a result, host bacteria and host proteins must organize together for the target T complex to integrate into the host cell nucleus.

2.3.4. Plant Transformation Vector Construction and Gene Constructs

For genetic engineering of plants cells, Ti-plasmid preffered since it is a natural vector. These vectors known as wild type, that are not suitable for gene transfer to plants due to the presence of tumor-forming oncogenes in the T-DNA region on the Ti-plasmid causing unorganized cell growth. Therefore, the use of vectors that do not carry oncogenes has become mandatory for genetic studies. Disarmed plasmids are the vectors whose oncogenes have been removed [86]. The absence of oncogenes prevents crown gall formation and ensures the transfer of target T-DNA. As a result, it is possible to achieve transgenic plants by shoot regeneration from transformed plant cells by using disarmed Ti-plasmids.

In the plant sciences, the binary vectors are the most prevalent tools. The term binary vector generally refers to the unarmed Ti plasmid with the T-DNA region removed. Binary vector is usually small and easily manageable plasmid without the T-DNA region of *Agrobacterium* [87]. Thus, binary vector system consists of the combination of two plasmids. One of them is called *Agrobacterium* Ti-helper plasmid.

The helper plasmid contains only the *vir* genes needed for T-DNA transfer. Activated *vir* genes assist transfer of T-DNA in the host genome. On the other hand, the other plasmid, known as the binary plasmid, is obtained from *E. coli* cloning vectors. This plasmid contains 25 bp of terminal repeat within the right and left borders for T-DNA [87]. In the literature, the first binary vectors created were known as pBin19 [88]. Afterwards, pPZP vectors and pCAMBIA vectors were modified from pPZP vectors for binary vector system [89]. It was stated that in binary vectors with recent modifications, it was possible to have wide selection of cloning sites and high copy numbers in *E. coli*. Thus, high cloning capacity, improved compatibility with preferred *Agrobacterium* strains, a large pool of selectable markers for plants and high frequency cloning capacity of plant transformation will be available in the upcoming years.

The presence of genes called markers in the T-DNA of the vectors is of great importance for the selection of transformed plants. To demostrate the success of the transformation efficiency, selectable marker and a reporter gene are used in the recombinant molecule.

A selectable marker is a gene to decide transformed cells from non-transformed cells as an outcome of transformation. At the end of the transformation, tissues that contain selectable markers can survive on selective media containing specified selection agents such as antibiotics. However, non-transformed cells which are indurable to antibiotic with lack of antibiotic resistance gene, cannot survive on the same media. Generally, in transformation studies, kanamycin (neomycin phosphotransferase II) and hygromycin (hygromycin phosphotransferase) resistance genes have been widely used as selectable markers [80].

Likewise, reporter gene is another gene that is used to differentiate the transformed cells and to determine the function of the inserted gene in the host [87]. Transformed tissue can be distinguished by reporter gene expression. In the transformed tissue, to visualize the expression of reporter genes, staining or auto-fluorescence methods are in use. *β-glucuronidase* is a gene that isolated from *E. coli* and has been commonly used in molecular study of plants. Luciferin, and GFP are also commonly used reporters after GUS for observation of transgene expression histochemically and fluorometrically in transgenic plants [90].

Promoter is the region of DNA where the transcription of gene is initiated. Promoter region controls location and time where the gene of interest is expressed in the organism. Since it is the DNA segment that initiates the transcription of promoter genes, it is used in in transgenic studies to direct the expression of the selectable marker to determine the transformed callus and shoots after transformation. Thus, it detects the segregation of T-DNA with the target gene in consequent generations to determine in the progeny for the expression and specificity of gene of interest [42]. These promoters are used in basic research in plant biotechnology to study the functions of plants and to overproduce related proteins to produce superior seeds or plants for agricultural purposes.

The 35S CaMV (Cauliflower Mosaic Virus) promoter is widely used in plant biology since it is a constitutive type of promoter, provides a high transgenic expression and is diversely well characterized in both monocotyledonous and dicotyledonous plants. Consequently, it contributes to the development of commercial product varieties with new agricultural properties such as insect resistance and herbicide tolerance [91].

2.5. AIM OF THE STUDY

The aim of the study is to optimize; 1) *Agrobacterium*-mediated gene transfer method with pCAMBIA1301 vector implementing to *Agrobacterium* strain LBA4404, using GUS (*β*-glucuronidase) reporter gene for Turkey originated Hatay Red *I. batatas* variety, and 2) Micropropagation of (C-1, K-1, K-2, K-3, K6, K7, K8) different *I. batatas* genotypes provided by Korea Research Institute of Bioscience and Biotechnology (KRIBB), South Korea.

To our knowledge, this is the first study in our country on the transformation of the Turkish *I. batatas* variety. Thus, it will form the basis for future agricultural practices and advanced biotechnological research studies.

With the outcomes of this study, viral disease problems in Turkish sweetpotato cultivars will be overcome by using optimized plant tissue culture protocols. Besides, preserved germplasm collection can be used in plant breeding practices for the development of new varieties in the future.

3. MATERIALS

3.1. PLANT MATERIAL

Ipomoea batatas L. (Lam.) seeds (K-1, K-2, C-1, K-3, K4, K6, K7, K8) were kindly provided by Korea Research Institute of Bioscience and Biotechnology (KRIBB), South Korea. Turkey originated Hatay Red [*Ipomoea batatas* (L.) Lam.] sweetpotato variety was supplied from Mustafa Kemal University, Turkey.

3.2 BACTERIAL STRAIN AND PLASMID

Agrobacterium tumefaciens LBA4404 was used for transformation of *I. batatas*. The strain was choosen according to its transformation efficiency [92]. LBA4404 strain carried pAL 4404 octopine-type Ti plasmid resistant to streptomycin. The *A. tumefaciens* LBA4404 gene was carried the plasmid pCAMBIA1301 (CAMBIA, Canberra, Australia) that contained *β*-glucuronidase uidA (GUS) gene under the control of CaMV35S promoter. For bacterial selection, neomycin phosphotransferase (*nptII*) gene, providing resistance to kanamycin was used. For plant selection, hygromycin phosphotransferase (*hptII*) gene providing resistance to hygromycin was utilized.

3.3. CHEMICALS

Thiamine hydrochloride (Sigma Aldrich, CAS#T4625), MS basal salt (Duchefa, CAS#M0221), Gelrite (Duchefa, CAS# 71010-52-1), Ethanol (Sigma Aldrich, CAS#64-17-5), Sodium chloride (Sigma Aldrich, CAS#7647-14-5), Preservative for Plant Tissue Culture Media (Phytotech, CAS#P6820), Sucrose (Duchefa, CAS#57-50-1), Giberellic Acid-3 (Caisson, CAS#77-05-5), Magnesium sulphate heptahydrate (Sigma Aldrich, CAS#2773), Potassium dihydrogen phosphate (Sigma Aldrich, CAS#P5655), Plant agar (Phytotech, CAS#9002-18-0), 1-Naphtalenacetic acid (Sigma Aldrich, CAS#86-87-3), 6-Benzylaminopurine (Duchefa, CAS#1214-39-7), Mannitol (Sigma Aldrich, CAS#69-65-8), 2-(N-morpholino) ethanesulfonic acid (Sigma Aldrich, CAS#4432-31-9), Yeast extract (Sigma Aldrich, CAS#801301-2), Myo-inositol (Sigma Aldrich, CAS#87-89-8),

MS media including vitamins (Duchefa, CAS#M0222), 2,4 Dichlorophenoxyacetic acid (Duchefa, CAS#94-75-7), Nutrient broth (Himedia,CAS#M002), Acetosyringone (Phytotech,CAS#2478-38-8),Generuler DNA ladder mix(ready to use) (Thermo Fisher, CAS#SM0333), 6X DNA loading dye (Thermo Fisher, CAS#R0611) , EDTA (Sigma Aldrich, CAS# 6381-92-6), Potassium ferricyanide (Bioshop, CAS# 13746-66-2), Potassium ferrocyanide (Bioshop, CAS# 144539-95-1), Tris-HCI (Genemark, CAS#1185-53-1), Triton X (Sigma Aldrich, CAS# 9036-19-5), β-mercaptoethanol (Sigma Aldrich, CAS# 60-24-2), Xgluc (Phytotech, CAS#114162-64-0), Sodium phosphate monobasic (Sigma Aldrich, CAS# 10049-21-5), Sodium phosphate dibasic (Isolab, CAS#10028-24-7), TE buffer (Invitrogen, CAS#12-090-015), RNaseA (Thermo Fisher, CAS# EN0531), Cefotaxime (Merck, CAS# 64485-93-4), Streptomycin (Bioshop, CAS# 3810-74-0), Hygromycin (Merck, CAS#3810-74-0), Agar (Sigma Aldrich, CAS#05039), Agarose (Sigma Aldrich, CAS#9012-36-6), Ethidium bromide (Biorad, CAS#161-0433), TAE buffer (Thermofisher, CAS#B49), Sodium acetate (Bioshop, CAS# 6131-90-4), Potassium acetate (Merck, CAS# 127-08-2), dNTP mix (Thermo Fisher, CAS#R0191) Glycerol (Sigma, CAS# 56-81-5), Kanamycin (Thermo Fisher, CAS#11815024), LB Agar (Sigma Aldrich, CAS# L2897), LB Broth (Sigma Aldrich, CAS# L3022), Magnesium Chloride (Sigma Aldrich, CAS# 7786-30-3), Taq Buffer (Thermo Fisher, CAS# B38), Taq Buffer (Thermo Fisher, CAS# B38), Taq DNA polymerase (Thermo Fisher, CAS# 9012-90-2), X-gal (Sigma Aldrich, CAS#7240-90-6)

3.4. GLASSWARE AND CONSUMABLES

Petri dishes 90 mm, Magenta GA-7, Pasteur Pipettes, Weighing dishes, Micropipettes (0.1-10 µl, 20-200 µl, 100-1000 µl), Filter paper Plastic Viols, Plastic Pots, Glass graduated cylinder (500 ml and 1000 ml), Metal Test Tube Racks, Micropipette tips (100 µl, 200 µl, 1000µl), Porcelain mortar and pestle, Aluminium Foil Roll, Glass bottles borosilicate (500 ml and 1000 ml), Liquid nitrogen tank, Falcon tubes (15ml and 50ml), Polystyrene Spectrophotometer cuvette, Centrifuge Tubes (15 ml and 50 ml), Scapel, Scapel Blade (No:11), Forceps, Microtube racks for 1.5 ml and 2 ml, Parafilm

3.5. EQUIPMENTS

Analytical Balance with 0.0001 and 0.001 precision (Shimadzu), Agarose Gel Electrophoresis System (Bio-Rad, USA), Gel Electrophoresis Tank (Cleaver Scientific, UK), Climatic Chambers (Aralab, S#1799, 1877, 1778), Inverted Phase Contrast Light Microscope (Nikon Eclipse TS-100, USA), Laminar Flow Cabinet (TEZ-SAN Class II Biohazard Safety Cabinet Type 2A), Stereo Microscope (Zeiss Stemi Dv4), Incubator (Memmert TUN55), Water bath (Grant subaqua 12 Plus S#QS1126010), Water Bath (Memmert, Germany), Microwave MD809 (Arçelik, Turkey), Nanodrop2000 Spectrophotometer (Thermofisher, USA), Spectrophotometer (Genesys 10S UV-Vis), pH meter (Mettler Toledo, Sevencompact), Spectrophotometer (Thermo Scientific,LUX multimode microplate reader), Laboratory Fume Hood, Vortex Mixer (WiseMix, Wisd, VM-10), Magnetic Stirrer With Hot Plate (SciLogex MSH280-Pro), Shaking Incubator (Inova, USA), Thermal Cycler (Bio-Rad, USA), Autoclave (Wisd, MeXterile 60), Centrifuge (Eppendorf, S#5811AK563617), Shaker (Sartorius Stedim Biotech Cermomatis Orbital Shaker), Centrifuge (Eppendorf, 5424, S#5424ZR734628) , -20 °C Freezer (Arçelik, Turkey), -80 °C Freezer (Thermo Scientific, USA), UV Transilluminator (Fisher Scientific, USA) +4 °C Refrigerator (Arçelik, Turkey).

4. METHODS

4.1. OPTIMIZATION OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF TURKISH SWEETPOTATO CULTIVAR BY USING GUS (*β*-GLUCURONIDASE) AS A REPORTER FOR EMBRYOGENIC CALLUS

4.1.1. Growth conditions of Hatay Red Sweetpotato variety

The Hatay Red *I. batatas* seedlings provided by Mustafa Kemal University were propagated under greenhouse conditions (16/8 h light/dark photoperiod at 25/22 °C, relative humidity between 60-70 per cent, photosynthetic photon flux of 320 μmol m^{-2} s^{-1} at canopy height) for callus formation.

4.1.2. Surface Sterilization and Callus Formation

Callus induction was obtained from the internodes of the Hatay Red plants grown in the greenhouse. Sterilization protocol for the Hatay Red variety was optimized according to Yang *et al*. (2011) [6]. In this study, totally 80 explants were used. Two trials were set at different time periods. In the first trial, 6 replicates (5 explants/petri dish) and in the second trial 10 replicates (5 explants/petri dish) were used. To surface sterilize plant material nodal explants were sterilized with 70 per cent ethanol for 5 minutes. After continuous shaking, each explant was kept with 10 per cent sodium hypochlorite for 10 minutes. The sterilized explants were rinsed with autoclaved water 3 times for 1 minute at each wash. For transformation studies, callus induction was carried out according to Kwon *et al*. (2004) (Figure 4.1) [92]. Callus tissue was sub-cultured in each 4 weeks interval. Callus formation (CIM) media (pH 5.8) of Hatay Red [*Ipomoea batatas* (L.) Lam.] variety was listed in Table 4.1.

Table 4. 1. Media composition for callus induction of Hatay Red variety.

	MS (including vitamins) (g/L)	**Sucrose (g/L)**	**Thiamine - HCL (mg/L)**	**Myo-Inositol (mg/L)**	**2,4 D (mg/L)**	**Gelrite (g/L)**
CIM (Callus Induction Media)	4.43	30	4.0	0.1	1.0	4.0

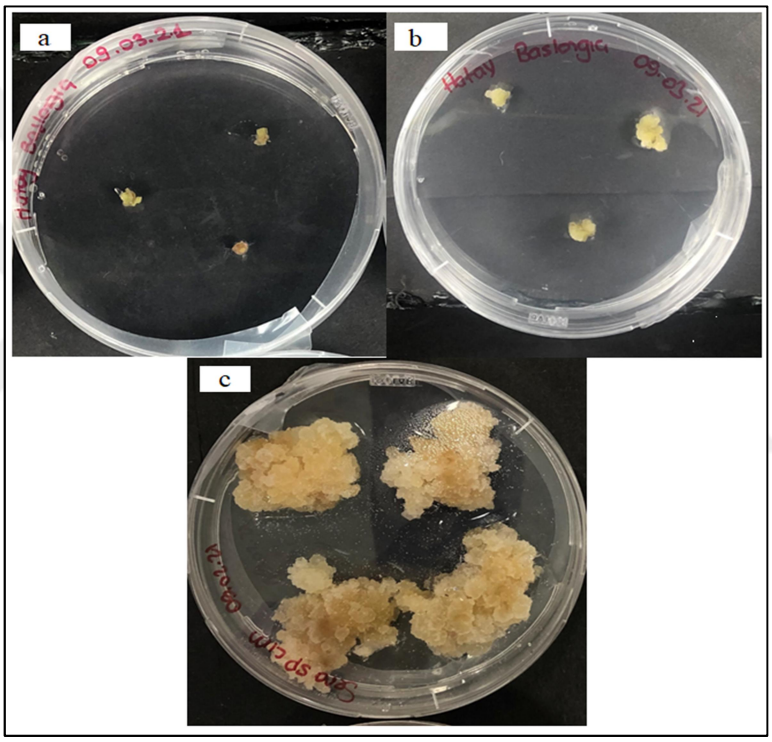

Figure 4. 1. Callus induction of Hatay Red variety of *I. batatas* for transformation. (a) Callus initiation from internode part of the explants (b) Callus formation at 1st sub-culture (c) The 5th subculture of the callus tissue, whose subculture is still continued.

4.1.3. Plasmid Transformation of *Agrobacterium* cells

In this study, the *β*-glucuronidase (GUS) reporter gene was used with the pCAMBIA1301 binary vector under the control of CaMV 35S promoter. The binary vector includes hygromycin B for plant selection and kanamycin for bacterial selection (Figure 4.2).

For the study, electrocompetent LBA4404 (Invitrogen, USA) *A. tumefaciens* strain was used. LBA4404 strains carried the Ti-plasmid pAL 4404, which has only the *vir* and *ori* region of the Ti-plasmid. The pCAMBIA1301 vector was previously grown using chemically competent DH5α competent *E. coli* [92]. According to the manufacturer's instructions, the extracted pCAMBIA1301 plasmid was introduced into electrocompetent *A. tumefaciens* strain LBA4404 (Invitrogen, USA).

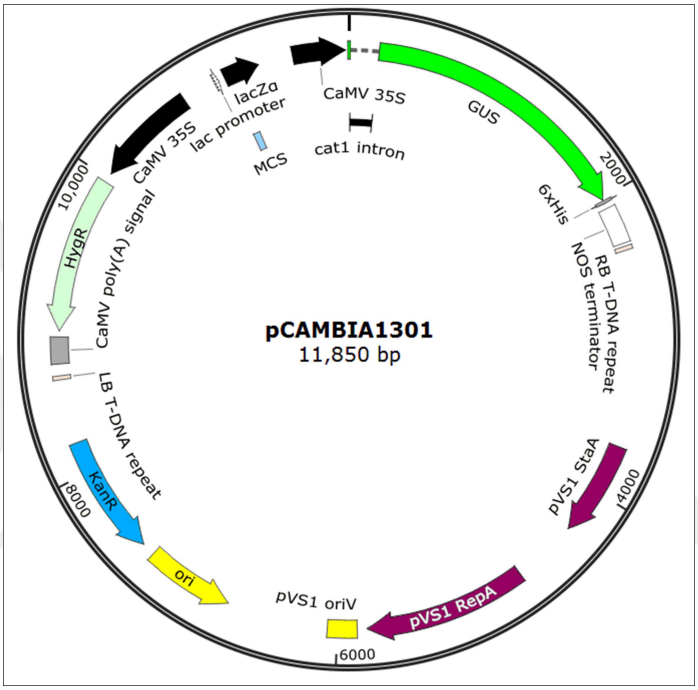

Figure 4. 2. pCAMBIA1301 *Agrobacterium* binary vector gene map, containing hygromycin-kanamycin resistance and GUS gene [94].

The pCAMBIA1301 plasmid was added into a 0.1 cm electroporation cuvette containing LBA4404 cells and 200 Ω, 2-2.5 kV, 25 µF electrical pulse was applied. After electroporation, cells were mixed with 1 ml YM medium [0.4 g yeast extract, 10.0 g mannitol, 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g $K_2HPO_4.3H_20$ (pH 7.0)] and transferred to 15 ml falcon tubes to incubate at 30 °C at 225 rpm. After 3 hours of incubation, cells were plated on YM agar plates with kanamycin (50 mg/L) and streptomycin (100 mg/L) for 2 days at 28 °C. Glycerol stocks were stored at -80 °C.

4.1.4. Growth of *Agrobacterium* strain

The growth of *Agrobacterium* strain and induction of *vir* genes was studied according to Özdemir *et* al. (2018) [92]. In order to prepare LBA4404 competent cells, the tip of the loop was touched to the glycerol stock of LBA4404 cells from -80 °C and spread on YEB agar plate [1 g/l yeast extract, 13.5 g/l nutrient broth, 2 mM $MgSO_4 \cdot 7H_2O$, 5 g/l sucrose, 15g/L bacto-agar (pH 7.2)] with kanamycin (50 µg/ml) and streptomycin (50 µg/ml) and incubated at 30°C for 2 days.

A single colony observed in the plates was taken and transferred to a 5 ml YEB medium supplemented with kanamycin (50 µg/ml) and streptomycin (50 µg/ml). Then it was agitated in the dark at 30 °C, 150 rpm. The next day 1 ml of all bacterial cultures were transferred in 50 ml YEB media supplemented with specified antibiotics (100 mg/ml streptomycin and 50 mg/ml kanamycin) and the OD_{600} was measured several hours apart (Figure 4.3). When bacterial concentration (OD_{600}) is between 0.3 and 0.8, the cells were collected by centrifugation at 1400 g for 15 min at 4 °C.

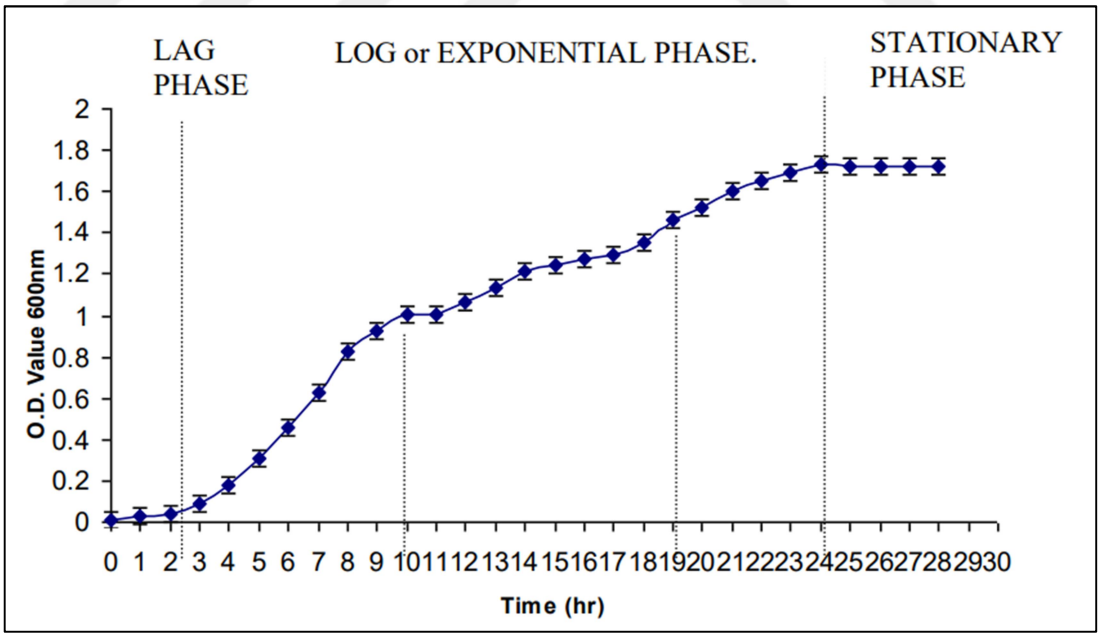

Figure 4. 3. The growth curve of *A. tumefaciens strain* LBA4404 [95].

4.1.5. Inoculation and Co-cultivation of Sweetpotato Embryogenic callus with *Agrobacterium* and Induction of *vir* genes

In order to create, wounded tissue, using a needle and scalpel callus tissues were damaged. The callus tissues were resuspended in a CIM medium containing 20 µM acetosyringone for induction of the virulence genes. Then, they were transferred to 50 ml falcon tubes with centrifugated cells and inoculated with shaking at dark in RT for 30 min. After incubation, calli were transferred to CIM agar with acetosyringone (20 µg/ml) and incubated at 25°C in dark between 2-6 days. Then the callus tissues were washed with distilled water and transferred to the CIM agar media containing cefotaxime (250 mg/L) to remove excess of *Agrobacterium*, and hygromycin for antibiotic resistance (15 mg/L).

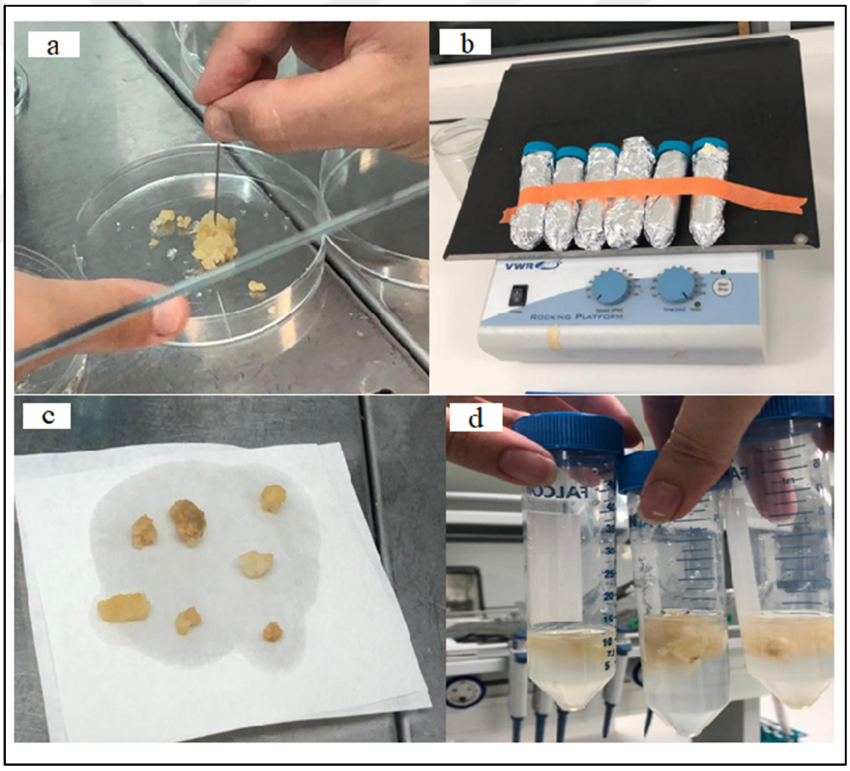

Figure 4. 4. The steps of electrocompetent LBA4404 *Agrobacterium* strain transfer to callus cells by *Agrobacterium* mediated method. (a) wound tissue formation in callus cells, (b) inoculation of *Agrobacterium* and callus tissue, (c) calli recovered on filter paper after inoculation (d) activation of virulence of callus tissues with CIM medium containing acetosyringone.

4.1.6. Regeneration and Selection of the Transformed Calli

After 2 weeks, for regeneration, necrotic tissues were eliminated, and surviving calli were transferred to an RM medium [4,43g/L MS, 30g/L sucrose, 1mg/L BAP, 0.01 mg/L NAA, 8g/L plant agar (pH 5.7)] containing only hygromycin (30 mg/L). Plantlets were kept in magenta boxes under the same environmental conditions until the putative transgenic plants were fully developed. Transformed calli in the regeneration medium were continued to sub-culture at 3 weeks interval [54].

4.1.7. Confirmation of Transient Gene Expression

In the transformation study, the T-DNA region of plasmid pCAMBIA1301 contains the GUS gene. To reveal the success of transformation, expression of the GUS gene was detected by using the histochemical GUS Staining method [92].

Table 4. 2. Chemicals and quantities used for the preparation of GUS selection.

Component	**Amount**
EDTA (0.1M)	1ml
Potassium ferricyanide (5Mm)	1ml
Potassium ferrocyanide (5mM)	1ml
Sodium phosphate buffer	5ml
Triton X-100 (0.1 %)	100 µl
X-gluc (0.3 mg/ml)	150 µl
Total	**10ml**

The prepared GUS staining solution [5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1 M EDTA, 5.0 ml sodium phosphate buffer (61 per cent (0.1 M) Na_2HPO_4 and 39 per cent (0.1 M) NaH_2PO_4, pH 7.0), 0.1 per cent Triton X-100, 0.3 mg/ml X-gluc] with autoclaved distilled water add up the volume to 10 ml was added to cover the explants and these explants were incubated for 48 hours at 37°C in the dark (Table 4.2). After 48 hours of incubation, GUS expression was recorded by counting the blue spots (putatively transformed cells or cell aggregates) under the microscope. Explants were kept in GUS fixative solution (70 per cent ethanol).

4.1.8. Genomic DNA Isolation from Sweetpotato

Isolation of genomic DNA was optimized [96]. 500 mg wild type and transgenic calli were freeze-thaw using liquid nitrogen to obtain fine powder. Each sample received 800 µl of cetyltriethyl ammonium bromide (CTAB) extraction buffer [1.4 M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), 2 per cent w/v of CTAB]. To lyse the cells, each tube were incubated at 65 °C for 15 min. 2M, 225 µl sodium acetate was added and mixed gently. Each sample tube was incubated for 15 minutes on ice. Then tubes were centrifuged at 12,000 g for 10 min, RT. At the end of the centrifuge, supernatant was transferred to a new tube followed by an equal volume of isopropanol.

To form a pellet, precipitated DNA was centrifuged at 12,000 g for 5 minutes at room temperature. Each tube that formed a pellet was washed with 0.5 ml of 70 per cent ethanol for 10 minutes. Then air-died and dissolved in 50 µl of ddH2O. The isolated genomic DNA was analyzed for concentration and purity (A260/280) by using Nanodrop2000.

RNase treatment and ethanol precipitation was carried out with isolated DNA of both wild-type and transgenic samples. The top of the DNA volume was completed to 200 µl with TE buffer (pH 8.0). 10 µl RNase A was added and each tube was incubated at 37°C for 60 minutes. 2M sodium acetate was added 20 µl to RNase treated DNA samples. Then 550 µl of 100 per cent ethanol was added and spin down the sample. Each tube were placed to -80°C for 30 minutes. After incubation, samples were centrifuged at 12000 g for 2 minutes at 4 °C. Supernatants were poured after centrifugation. Each pellet was washed with 500 µl, 70 per cent ethanol. Samples were spin again at 4°C for 10 minutes.

Ethanol inside of the tubes was removed out and tubes were air-dried. Pellets were re-suspended with 100 µl of TE buffer and concentration and purity results were taken from Nanodrop2000.

4.1.9. PCR (Polymerase Chain Reaction) Amplification of GUS gene from putative transgenic cells

PCR analysis was performed to identify putative transgenic sweetpotato using DNA samples isolated from *I. batatas* calli. PCR amplification was carried out to detect ~1700 bp region to prove the presence of the GUS gene. The GUS gene sequence belonging to binary vector pCAMBIA1301 was obtained from the NCBI database (AF234297) and the GUS gene-specific primers with 20 bp extensions homologous to vector ends were designed by using the Benchling primer designing tool. Primers used in the thesis study for amplification of GUS gene are shown in Table 4.3.

The PCR protocol for amplification of the GUS gene was as follows; 5 minutes at 94 °C for initial denaturation, then 35 cycles of 94 °C for 1 min, 55 °C for 45 sec, and 72 °C for 1 min for annealing, and the final extension at 72 °C for 10 minutes.

The final concentrations were adjusted as, 2.0 mM MgCl2, 1X Taq buffer, 0.15 mM $dNTP_{mix}$, 0.4 mM of each primer, 0.05 units/µl Taq enzyme and 100 ng wild type and putative genomic DNA in a total reaction volume of 25 µl. In addition, negative controls were included (PCR without any DNA and wild-type DNA).

Table 4. 3. Primer base sequences, annealing temperatures and product size of the GUS gene for confirmation of putative transgenic plants in Hatay Red cultivar of *I. batatas* in PCR studies.

Primer Name	Primer Sequence	Annealing Temperature	Product Size (bp)
GUS-F	5'TCGTCCGTCCTGTAGAAACC3'	55 °C	1700
GUS-R	5'TTTCACCGAAGTTCATGCCA3'		

The detected PCR results were confirmed by the gel electrophoresis method with 1 percent agarose gel. For this, 1 gram agarose was placed in 100 ml 1X TAE (Tris-acetate EDTA) solution. The solution melted in a microwave oven for about 5 minutes, and 1.75 µl ethidium bromide was added so that DNA could be visualized under UV. Afterwards, both sides of the tank were taped with casting dams and the buffer solution was poured in a gel tray. After waiting for about 20 minutes, the gel tray and casting dams were removed from the hardened the gel and the gel transferred to buffer tank containing 1X TAE buffer. 10 µl of sample was mixed with 2 µl 6X loading dye (Thermofisher, R0611). As a DNA ladder, Generuler DNA ladder mix ready to use (SM0333) was used and loaded only to the first well as 5 µl. For each well, 12 µl samples were loaded and run at 150 V/cm voltages for 45 minutes. Then, the running gel was placed on the UV transilluminator, checked, photographed and the results were determined.

4.2. MICROPROPAGATION OF SOUTH KOREAN SWEETPOTATO CULTIVARS FROM NODE EXPLANTS AND ACCLIMATIZATION TO THE GREENHOUSE

4.2.1. Surface Sterilization of South Korean Sweetpotato Node Explants

Node explants of seven (C-1, K-1, K-2, K-3, K6, K7, K8) different South Korean *Ipomoea batatas* genotypes were treated with ethanol and sodium hypochlorite [6].

Two nodes per explants were excised with a scalpel from the propagated *I. batatas* nodes at the greenhouse and were transferred to sterile tissue culture vessels containing distilled water. For surface sterilization, the explants were rinsed under running tap water for 30 minutes. Then, explants were surface-sterilized by immersing in 70 per cent ethanol for 3 minutes with continuous shaking followed by one washing with 10 per cent sodium hypochlorite for 10 minutes. The each explants were rinsed three times with autoclaved water for 1 minute. After the explants were rinsed with distilled water, they were placed in magenta boxes containing micropropagation media (MS1D) with 4 seeds in each box.

4.2.2. Basal Medium Preparation and Micropropagation of Sweetpotato Cultivars

Media compositions for the micropropagation of South Korean sweetpotato [*Ipomoea batatas* (L.) Lam.] varieties are listed in Table 4.4.

Table 4. 4. Media composition for *Ipomoea batatas* micropropagation [97].

	MS (including vitamins) (g/L)	**Sucrose (g/L)**	**BAP (mg/L)**	**NAA (mg/L)**	**GA3 (mg/L)**	**Gelrite (g/L)**	**PPM (ml)**
MS1D (Micropropagation Media)	4.43	30	0.25	0.1	0.2	4.0	2.0

Nodal cuttings of *I. batatas* were used as explants and routinely sub-cultured at 21 days intervals into a new micropropagation media for shoot initiation. Each sub-cultured *I. batatas* genotypes were maintained in plant growth rooms (photoperiod 16h light/8h dark, 25°C, 65 per cent humidity).

4.2.3. Acclimatization of Micropropagated Sweetpotato Plantlets

The developed plantlets were transferred to a greenhouse for acclimization. Plants at the greenhouse were transferred into planting pots including mixed soil, peat with perlite at a 1:1 ratio for adaptation. To enhance the survival rate, a gradual reduction of the relative humidity from 90 per cent to 70 per cent in the humidity chamber for 3-week intervals was applied. Afterwards, adapted plantlets of different genotypes were grown under a optimal environment (relative humidity 60-70 per cent, 16/8 h light/dark photoperiod at 22/24 °C, and a photosynthetic photon flux of 320 µmol m^{-2} s^{-1} at canopy height provided by fluorescent lamps) for plant growth in the greenhouse [98].

4.3 Statistical Analysis

Statistical analysis of the data was carried out with Student t-test and one-way ANOVA by using MS Excel 2010.

5. RESULTS

5.1. INDUCTION AND MAINTANENCE OF CALLUS

For *Agrobacterium*-mediated transformation study, *I. batatas* explants were taken from the internode parts of the Hatay Red cultivar and sub-cultured in callus induction medium (CIM) at 4 weeks intervals. Internodal explants were excised from shoot tips under a stereomicroscope and cut to 1 mm length from the in vitro plants to obtain callus from Hatay Red *I. batatas* cultivar. Five developing buds were placed in each petri-dish in a circular arrangement and sealed using parafilm (Figure 5.1). At the end of 4 weeks, the calli that had reached the appropriate size were used in the transformation study.

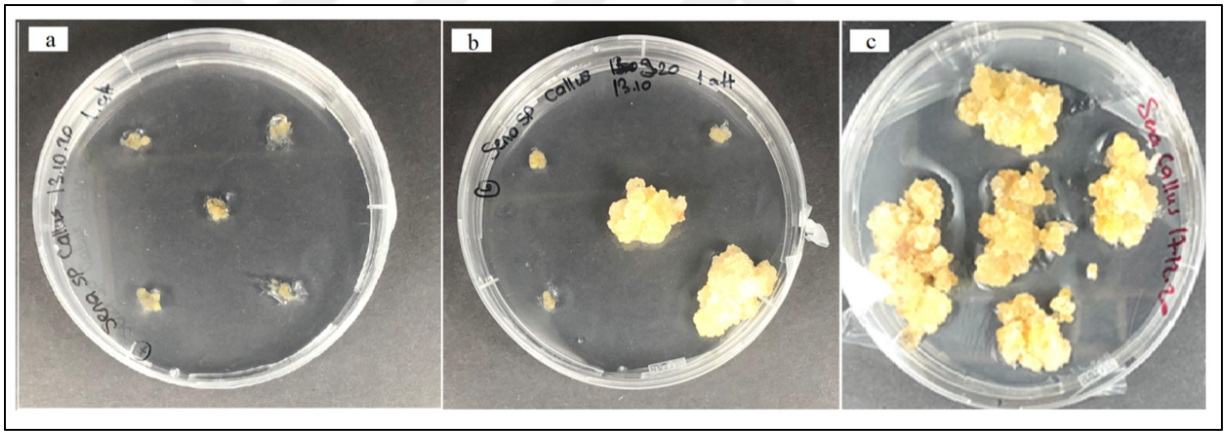

Figure 5. 1. Callus formation from internodal explants. (a) Initiation of callus formation (b) Start of callus formation at the end of the 2 weeks (c) Pale yellow embryogenic calli formed at the end of the 28th day.

Callus induction was performed in 2 repetitions and the average calli formation is shown in the Table 5.1. The callus induction frequency was found as 79 per cent on MS medium containing 0.1 g/L myo-inositol, 4 ml/L thiamine-HCL, 1 mg/l 2,4-D within 28 days of culture (Table 5.1). There were not significantly differences ($P \leq 0.05$) at each trial for callus formation.

Using CIM medium, callus induction was first observed 13.5 days after culture initiated from internodal segments is given in the Table 5.1. After 30 days of culture, callus proliferation was obtained throughout the explant. At the end of 3 month the induced callus color was observed as pale-yellow.

Table 5. 1. Callus formation from internodal explants of Hatay Red I. *batatas* cultured in CIM medium with 1 mg/l 2,4-D as auxin.

Trial Number	**No. of Explants**	**Days to first calli formation**	**No. of calli formed**	**Callus Formation (%)**
Trial 1	30	15	23	76
Trial 2	50	12	41	82
Mean	40	13.5	32	79

These results were in agreement for *I. batatas* varieties BARI 6 that induced with 1 mg/l 2, 4-D for callus induction with 50 per cent efficiency. It is revealed those days for callus induction is more than 7 days and the callus weight taken between 15-35 days is approximately between 4.23-5.29 grams [93]. In this study, the values of the calli whose weights were taken are given in the Table 5.2. At the end of 4 weeks, the average of four callus weight in a petri dish was found as 5.50 gram. For this reason, at the end of 4 weeks, the calli growing between 4.0-6.0 grams on average were divided into two and transferred to the CIM medium and their proliferation was continued (Figure 5.2).

Table 5. 2. Weights of calli (in grams) in a petri dish before subculture.

Callus Number	**Callus Weight (gram)**
1	4.4
2	5.4
3	6.6
4	5.6
Average	5.50 ± 0.90

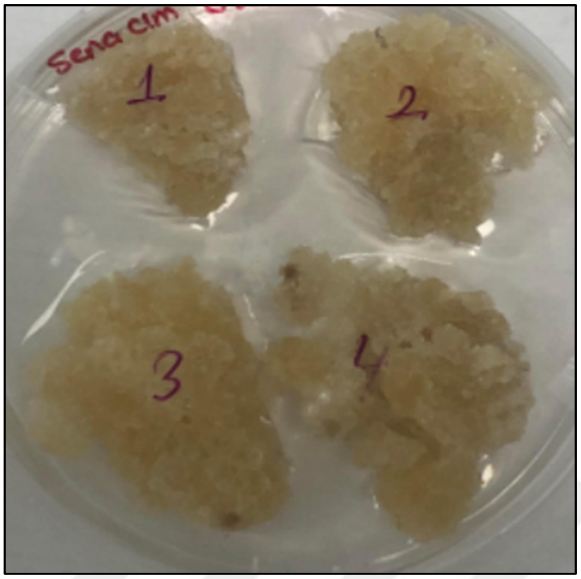

Figure 5. 2. Calli that reached a sufficient size before sub-culture.

5.2. EFFECT OF TEMPERATURE AND MEDIUM ON GROWTH OF *AGROBACTERUM* STRAIN

Observation on the effect of temperature and growth medium for single colony formation of LBA4404 strain was carried out and the result was shown in Figure 5.3. To optimize suitable growth medium, YEB [3] and YM medium [99] were applied.

In addition, the effects of temperatures (28 °C and 30 °C) were observed on *Agrobacterium* growth period for LBA4404 strain. Electrocompetent LBA4404 strain was grown on YEB [1 g/l yeast extract, 2 mM MgSO4·7H2O, 5 g/l sucrose, 13.5 g/l nutrient broth, 15g/L bacto-agar (pH 7.2)] and YM agar [10.0g mannitol, 0.4 g yeast extract, 0.1g NaCl, 0.2g MgSO4•7H2O, 0.5g K2HPO4.3H20 (pH 7.0)] medium at both 28 °C and 30 °C for 2 days at dark. For growing LBA4404 strain, appropriate antibiotics 50 mg/L kanamycin and 100 mg/L streptomycin were added to each media for selection. When both YM and YEB media were compared, it was observed that single colony formation was more in YEB media at the end of the 3rd day. In addition, when the temperatures were compared, it was determined that single colonies formed faster at 30 °C at dark (Figure 5.3). Thus, efficient single colony formation using electrocompetent LBA4404 was optimized with YEB medium at 30 °C for 2 days incubation at dark.

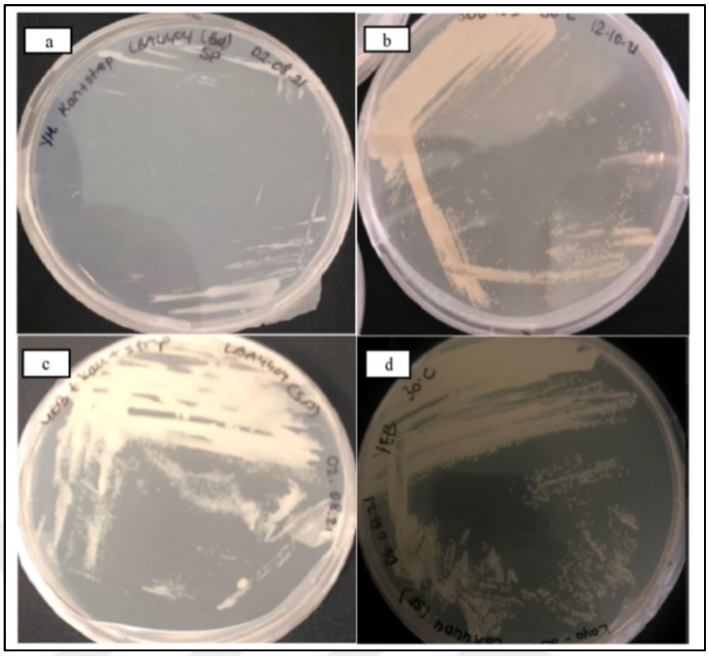

Figure 5. 3. Single colony formation of LBA4404 strain (a) YM medium at 28°C, (b) YM medium at 30 °C, (c) YEB medium at 28 °C, (d) YEB medium at 30 °C.

5. 3. EFFECT OF BACTERIAL CONCENTRATIONS ON TRANSFORMATION

The 4 weeks old calli of Hatay Red variety was immersed in the bacterial suspension at bacterial concentrations (OD_{600}= 0.3, 0.4, 0.6, 0.8) and inoculated for 30 minutes using rotary shaker at 150 rpm. The effect of bacterial concentrations (OD_{600}= 0.3, 0.4, 0.6, 0.8) on Hygromycin resistant calli was optimized and the result can be seen in Figure 5.4.

In this study, there was a significant difference ($p \leq 0.05$) among optical densities (OD_{600}= 0.3 with 0.4) and (OD_{600}= 0.3 with 0.8) of bacterial strain. However, significant difference was not observed between (OD_{600}= 0.4 with 0.8) and (OD_{600}= 0.3 with 0.6). As given in the Figure 5.4., the optical density (OD_{600}= 0.6) of LBA4404 strain gave the highest transformation efficiency as 60 per cent for *I. batatas* callus. The optical density (OD_{600}= 0.3) was also recorded with 50 per cent efficiency for transformation of Hatay Red variety of sweetpotato. Although there was no statistically significant difference between these two, (OD_{600}= 0.6) was preferred for co-cultivation due to the highest percentage of hygromycin resistant calli.

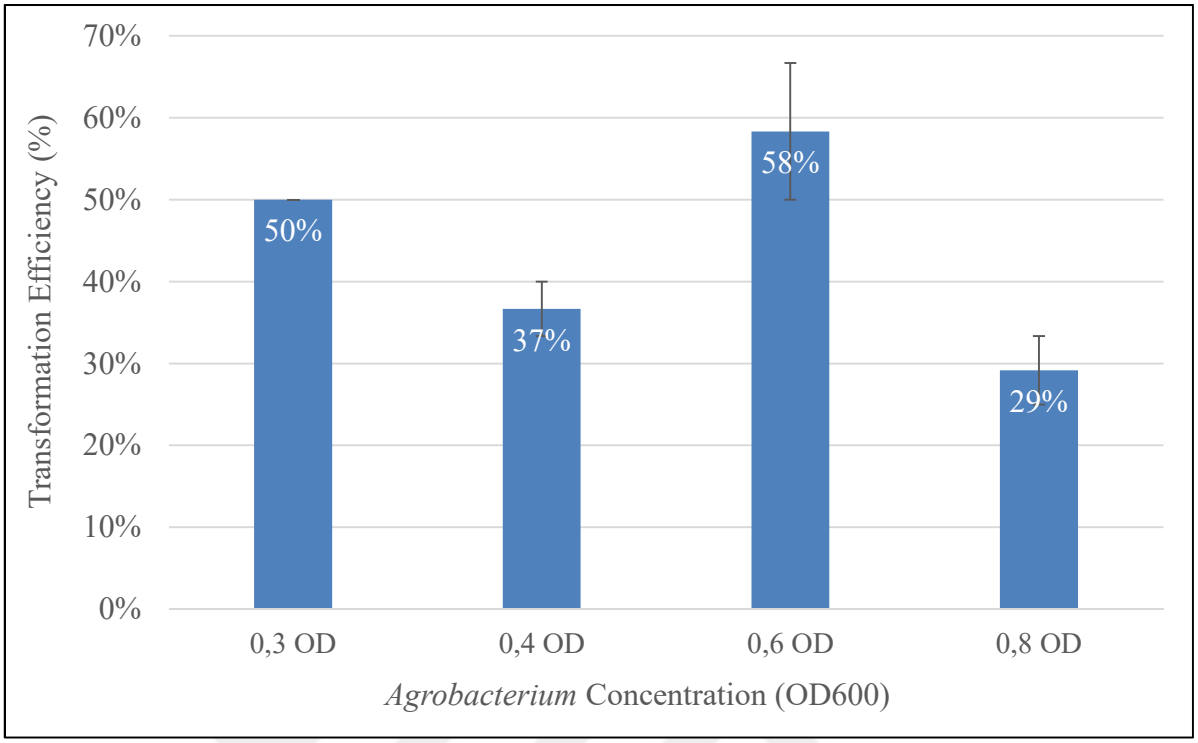

Figure 5. 4. Hygromycin resistant calli (%) depending on different bacteria concentration (OD_{600}= 0.3, 0.4, 0.6 and 0.8) to optimize *Agrobacterium*-mediated transformation of Turkish *I. batatas* cultivar.

5.4. OPTIMIZATION OF INOCULATION AND CO-CULTIVATION PERIODS

4 weeks old calli of Hatay Red variety were subjected to inoculation with *Agrobacterium* suspension for 30 minutes. Calli were transferred to CIM including Acetosyringone (AS) after being immersed with LBA4404 *Agrobacterium* strain. Addition of AS to co-cultivation medium is important to activate bacterial *vir* genes for the success of transformation. Based on this information for LBA4404 strain 20µM AS was supplemented to CIM for co-cultivation.

For the following process after inoculation, calli were co-cultivated with bacteria on CIM medium (50 ml) containing AS (20 µM) at 25 ºC in dark. Since the efficiency of Hygromycin-resistant calli at optical density (OD_{600}= 0.6) is higher than bacterial concentrations (OD_{600}= 0.3, 0.4, and 0.8), optimization for co-cultivation periods were carried out using *Agrobacterium* grown at (OD_{600}= 0.6) optical density.

It is suggested that when callus tissue is co-cultivated with AS for more than 2 days, bacteria formation was observed. For this reason, the waiting period in the dark after inoculation with AS for 30 minute was detected as 2, 3 and 5 days. Extending the co-cultivation period up to three days increased the formation of *Agrobacterium* on the callus (Figure 5.5). However, as the co-culture time increased, the growth efficiency of the bacteria decreased.

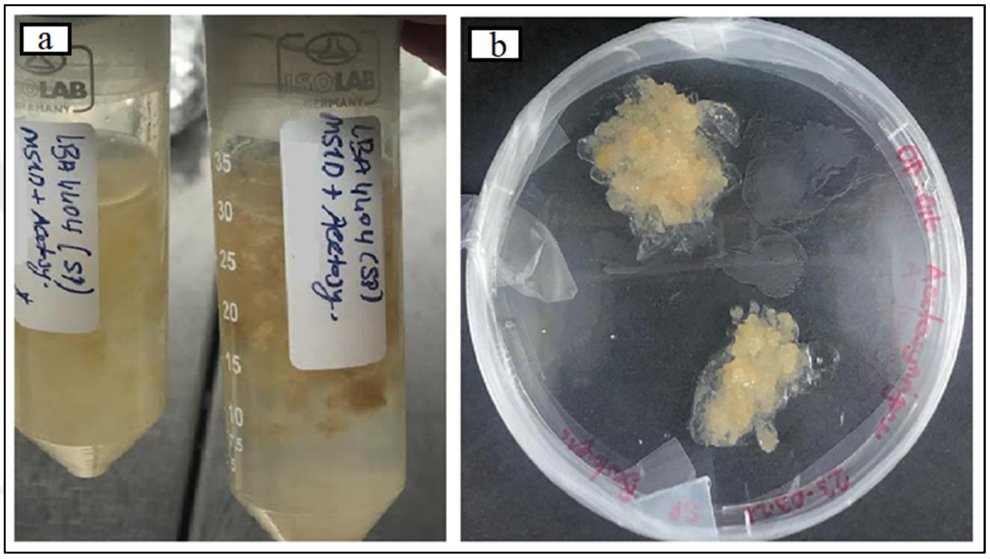

Figure 5. 5. Inoculation and co-cultivation of *I. batatas* callus (a) Inoculation of calli with *Agrobacterium* (LBA4404) suspension for 30 minutes at dark, (b) Co-cultivation of inoculated calli with CIM agar medium containing Acetosyringone at 25 °C in dark.

The results for the effect of co-cultivation time on *I. batatas* calli are shown in Figure 5.6. There is no statistically significant difference ($p \leq 0.05$) between 2, 3 and 5 days of co-cultivation period for *I. batatas* callus. However, the highest percentage of hygromycin resistance transformants was obtained at 3 days of co-cultivation with AS. As the number of co-cultivation days increased, necrosis was observed in the calli that were treated with AS at dark.

The explants co-cultured for 3 days reached a higher percentage of transformed calli than explants co-cultured for 2 and 5 days. Using optical density (OD_{600}= 0.6), 40, 60 and 50 per cent of calli with resistant to hygromycin was obtained in 2, 3 and 5 days of co-cultivation respectively (Figure 5.6). Thus, in *Agrobacterium* studies for *I. batatas* the co-cultivation period was optimized as 3 days and the transformation was continued.

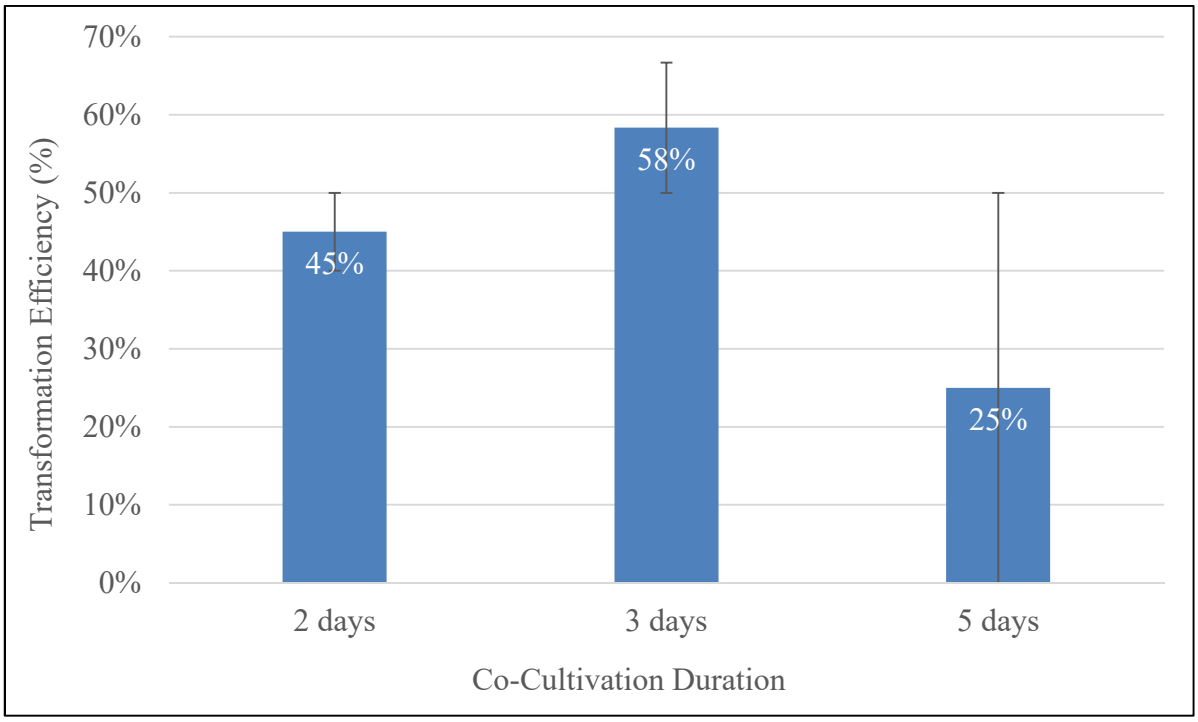

Figure 5. 6. Effect of Co-cultivation days (2, 3 and 5) on efficiency of calli that were infected with LBA4404 strain at bacterial concentration (OD_{600}= 0.6).

5.5. HISTOCHEMICAL GUS ASSAY

Identification of putative transgenic plants and tissues is easy due to the presence of the GUS gene in the T-DNA region of pCAMBIA1301 plasmid. Thus in this study, histochemical GUS analysis was applied to examine putative transgenic callus for Hatay Red *I. batatas* variety. For this purpose, putative transgenic callus tissues were placed in X-Gluc solution and incubated in the dark at 37 °C for 3 and 6 days (Figure 5.7).

Wild-type callus was used as a negative control for the reliability of the GUS assay. When putative transgenic callus react with 4-Brom-3-Chlor-5-glucuronide substrate, the GUS gene product *β*-glucuronidase enzyme being synthesized and with its substrate give blue color for transgenic plant tissues.

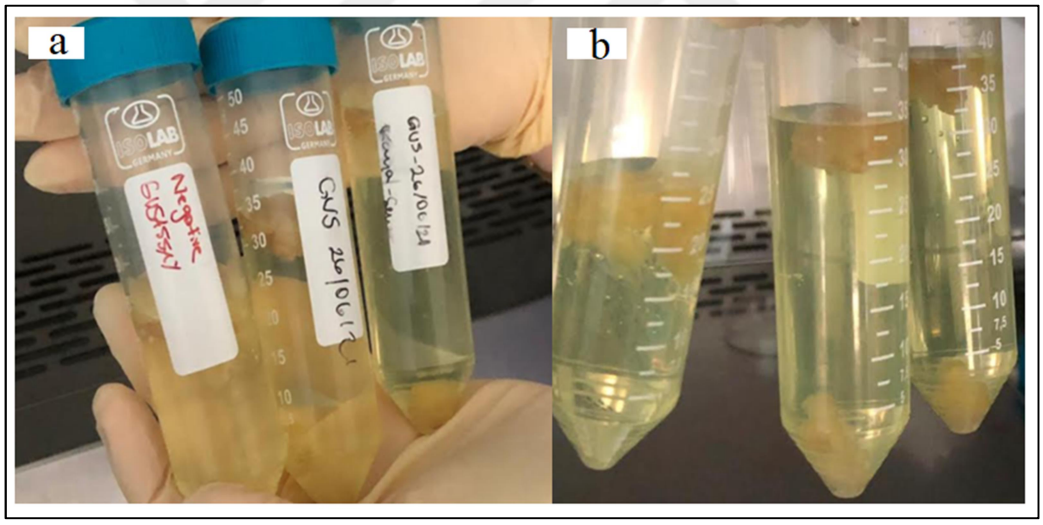

Figure 5. 7. Calli transferred to X-Gluc solution for GUS assay. (a) Wild type callus was used as a negative control, (b) there are at least 2 calli for each falcon.

Subsequently in this study, GUS expression with different bacterial concentrations (0.3, 0.4, 0.6 and 0.8) at optical density (OD_{600nm}) was observed in candidate transgenic Hatay Red variety by blue staining of resistant calli.The GUS expression of cells incubated for 3 and 6 days was examined. There was an increase in the blue dots as the holding time in X-Gluc solution increased for both bacterial concentrations (OD_{600} = 0.3 and 0.6) (Figure 5.8). In addition, GUS activity was not detectable for optical density (OD_{600} = 0.4 and 0.8).

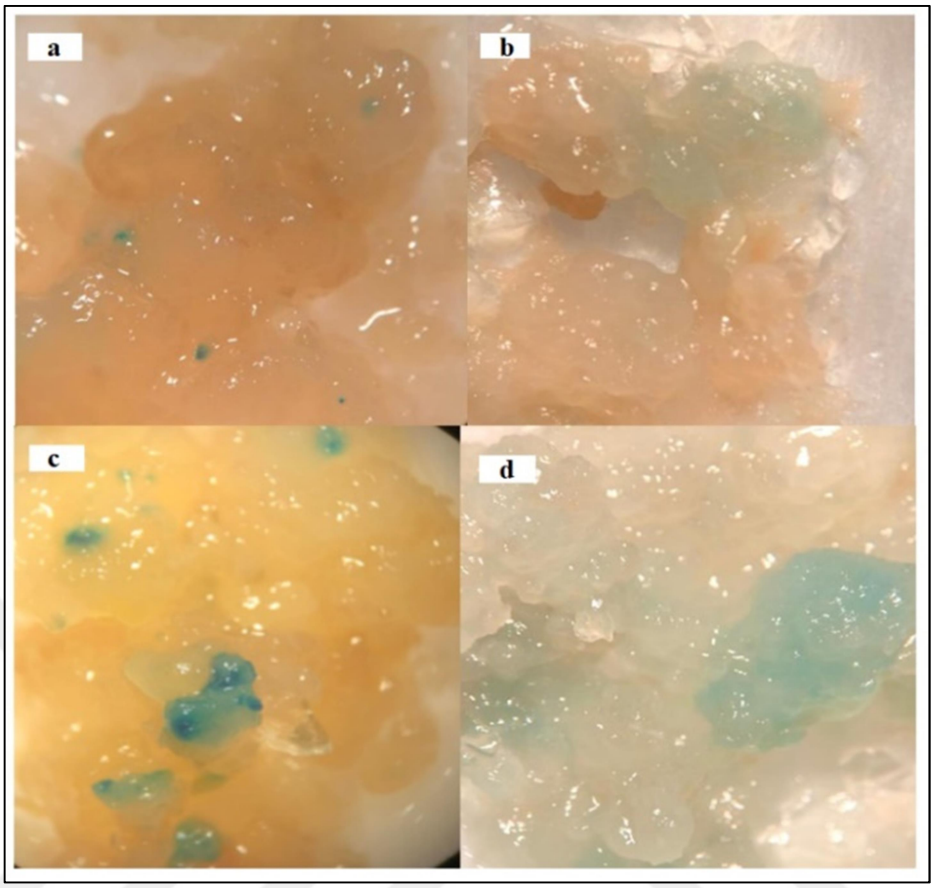

Figure 5. 8. Effect of bacterial concentration (OD_{600nm}) and co-cultivation time for callus of Hatay Red variety. Blue spots showing transient GUS activity after co-cultivation with *Agrobacterium.* (a) (OD_{600nm}=0.3) with 3 days co-cultivation, (b) (OD_{600nm}=0.6) with 3 days co-cultivation, (c) (OD_{600nm}=0.3) with 6 days co-cultivation, (d) (OD_{600nm}=0.6) with 6 days co-cultivation.

GUS activity increased gradually for optical density (OD_{600} = 0.3 and 0.6) from 3 to 6 days on X-Gluc. An increase of GUS activity was detected when transgenic callus tissue at optical density (OD_{600} = 0.6) was treated with X-Gluc after 30 days transferred to RM medium can be seen in the Figure 5.9.

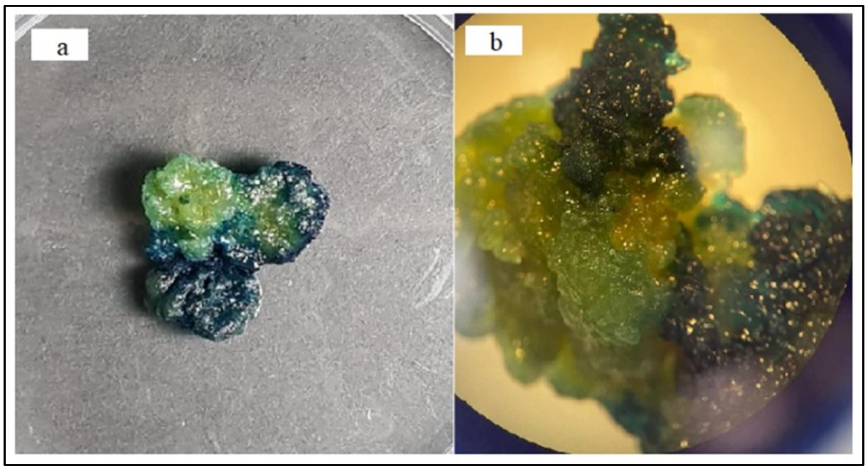

Figure 5. 9. Putative transgenic calli showed GUS activity, indicating that GUS gene expression was relatively stable after transformation. (a) Blue spot visible to the naked eye, (b) Blue spots under the light microscope.

When the percentages of GUS expression at different bacterial concentrations (OD_{600}= 0.3, 0.4, 0.6, 0.8) was examined, the highest GUS expression percentage was seen as 69.2 per cent at optical density at (OD_{600}= 0.6) given in the Table 5.3. However, GUS formation was not observed with optical density (OD_{600}= 0.4 and 0.8) with 3 days incubation at X-Gluc solution.

Table 5. 3. GUS expression efficiency rate (%) depending on different *Agrobacterium* concentrations (OD_{600}= 0.3, 0.4, 0.6, 0.8).

Bacterial OD	**No. of callus for GUS staining**	**No. of GUS positive callus**	**GUS Rate (%)**
0.3	12	8	66.6
0.4	4	0	0
0.6	13	9	69.2
0.8	4	0	0

5. 6. SELECTION AND RENEGERATION

Selection and regeneration are the significant factors for the efficiency of transformation. Selection can be applied for the putative plants at the end of transformation. For this reason, antibiotic selection is one of the significant steps to confirm the success of transformation. In this study, hygromycin resistance (coded by phosphotransferase (*hptII*) gene) was used. After co-cultivation, calli were transferred to CIM media containing hygromycin (15 mg/L). After 2 weeks, necrotic tissues were eliminated, and surviving calli were transferred to RM medium [1 mg/L BAP, 0.01 mg/L NAA, 4,43 g/L MS, 30 g/L sucrose, 8 g/L plant agar (pH 5.7)] containing 30mg/L hygromycin. Selection and regeneration with hygromycin was optimized (Figure 5.10) [93].

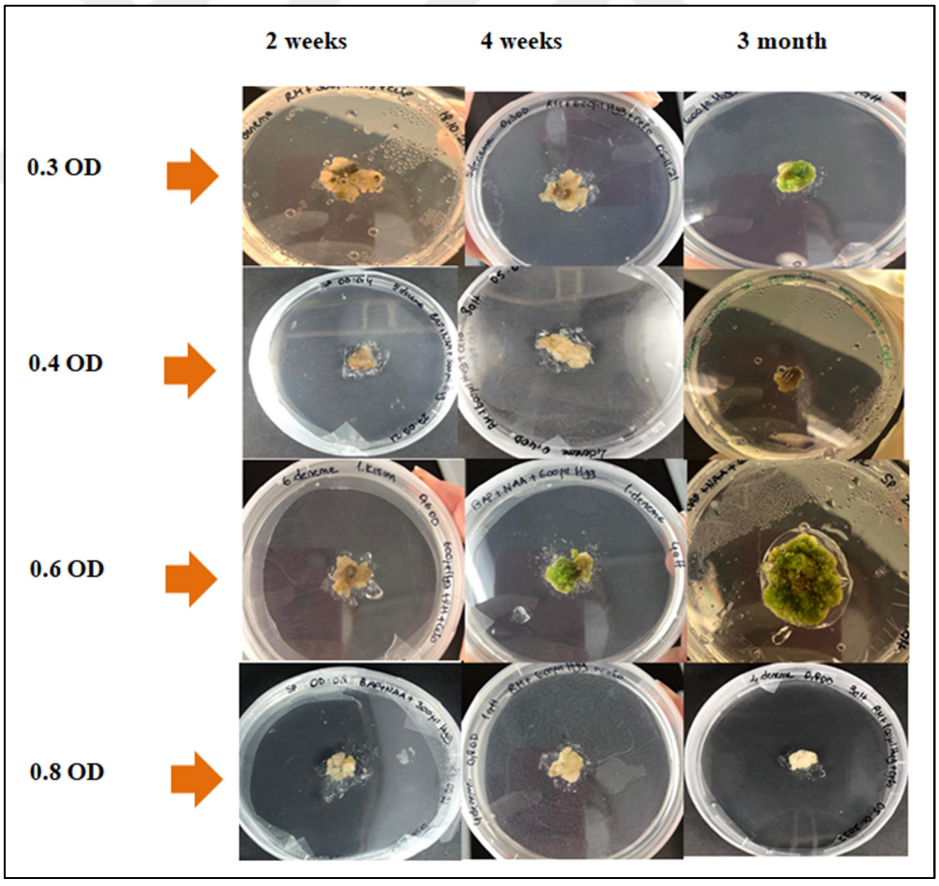

Figure 5. 10. Selection and regeneration of Hatay Red calli at different time intervals for each optical density.

Transformed calli in the RM medium sub-cultured at 3-week intervals. Calli transformed with different optical densities showed resistance to hygromycin and necrosis was not observed. Although the color of the calli transferred to the regeneration medium turned green after 2 weeks of selection, shoot or root formation was not observed and regeneration could not be achieved (Figure 5.11).

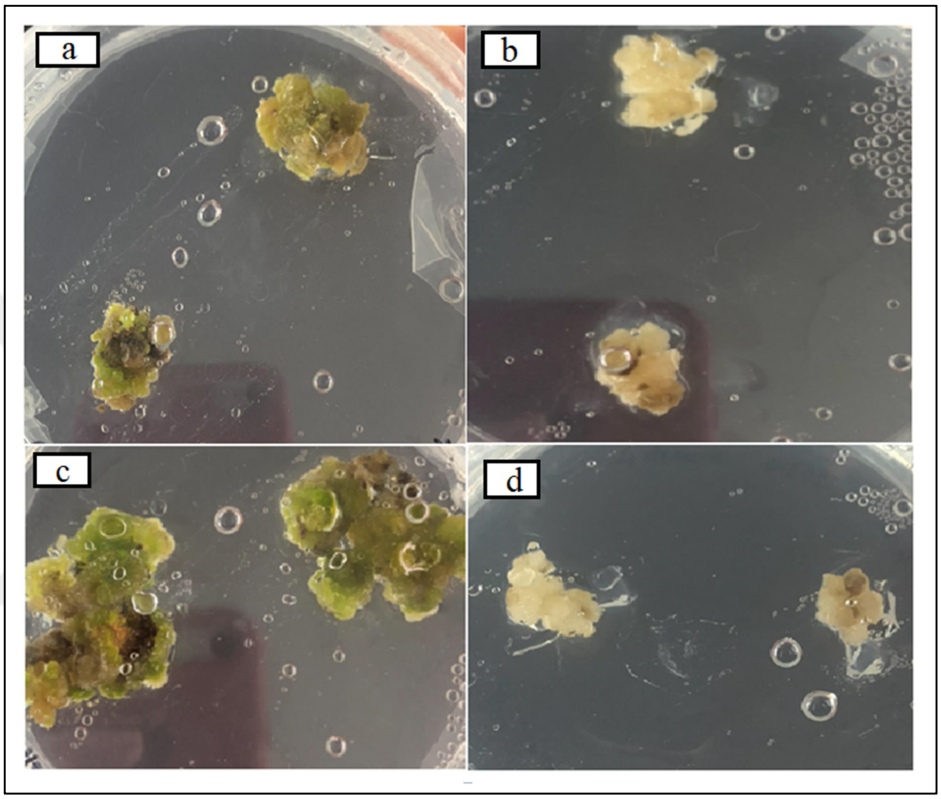

Figure 5. 11. Antibiotic resistant calli at RM medium supplemented with 30 mg/L hygromycin for each optical density. (a) OD_{600nm}=0.3 (b) OD_{600nm}=0.4 (c) OD_{600nm}=0.6, (d) OD_{600nm}=0.8.

Sensitivity of non-transformed explants to antibiotic was also examined. Although the calli turned green in the regeneration medium, no shoot or root formation was observed. However, when wild-type calli was transferred to RM medium supplemented with hygromycin, necrosis was observed at the end of 2 weeks and it was understood that it was sensitive to antibiotic (Figure 5.12). These results also showed the difference between untransformed and transformed callus under antibiotic selection confirming the transformation event in the puatative transgenic tissues.

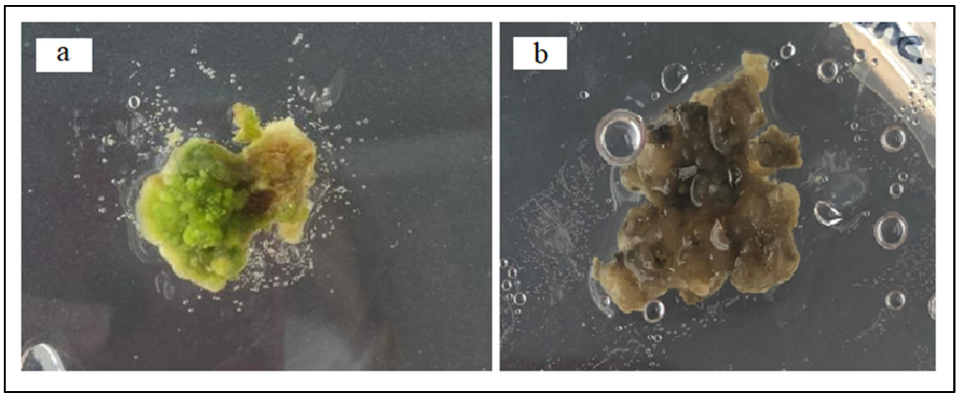

Figure 5. 12. Effect of hygromycin (30 mg/L) on plant necrosis to. *I. batatas* callus that infected with bacteria at optical density ($OD_{600nm=}$ 0.6) were incubated 2 weeks at 25ºC on RM media containing plant growth regulators (1 mg/L BAP, 0.01 mg/L NAA). (a) Hygromycin resistant transformed calli (b) Wild-type calli formed necrosis.

5. 7. DNA ISOLATION FROM HATAY RED CALLUS

Both wild-type and transgenic genomic DNA of *I. batatas* were isolated by CTAB protocol [96]. For DNA isolation, candidate transgenic and wild-type callus tissues were used. To isolate transgenic DNA, optical density (OD_{600nm}= 0.6) was used. DNA concentration of wild-type and putative transgenic samples were determined as 137.77 ng/µL and 217.38 ng/ µL respectively. With RNase treatment, expected DNA purity was achieved for both samples (Table 5.4). Isolated DNA for *I. batatas* callus were used in PCR for amplification of the GUS gene.

Table 5. 4. Nucleic acid concentration (ng/µL) and purity results of wild-type and transgenic DNA after ethanol precipitation and RNase treatment.

	Nucleic acid concentration (ng/µL)	**(A260/A280)**	**(A260/A230)**
Wild-type	137.77	2.18	1.58
Transgenic	217.38	2.15	1.60

5.8. CONFIRMATION OF GUS GENE WITH PCR ANALYSIS

PCR was carried out to prove the presence of the GUS gene in putative transgenic tissues using their genomic DNA. Both transgenic and wild-type DNA was isolated from callus tissue of Hatay Red variety of *I. batatas.* GUS gene-specific primers with 20 bp extensions homologous to vector ends were used to identify the GUS gene sequence belonging to binary vector pCAMBIA1301. Transformed calli for LBA4404 strain for Hatay Red genotype produced a fragment band of the expected size of 1700 bp can be seen in Figure 5.13.

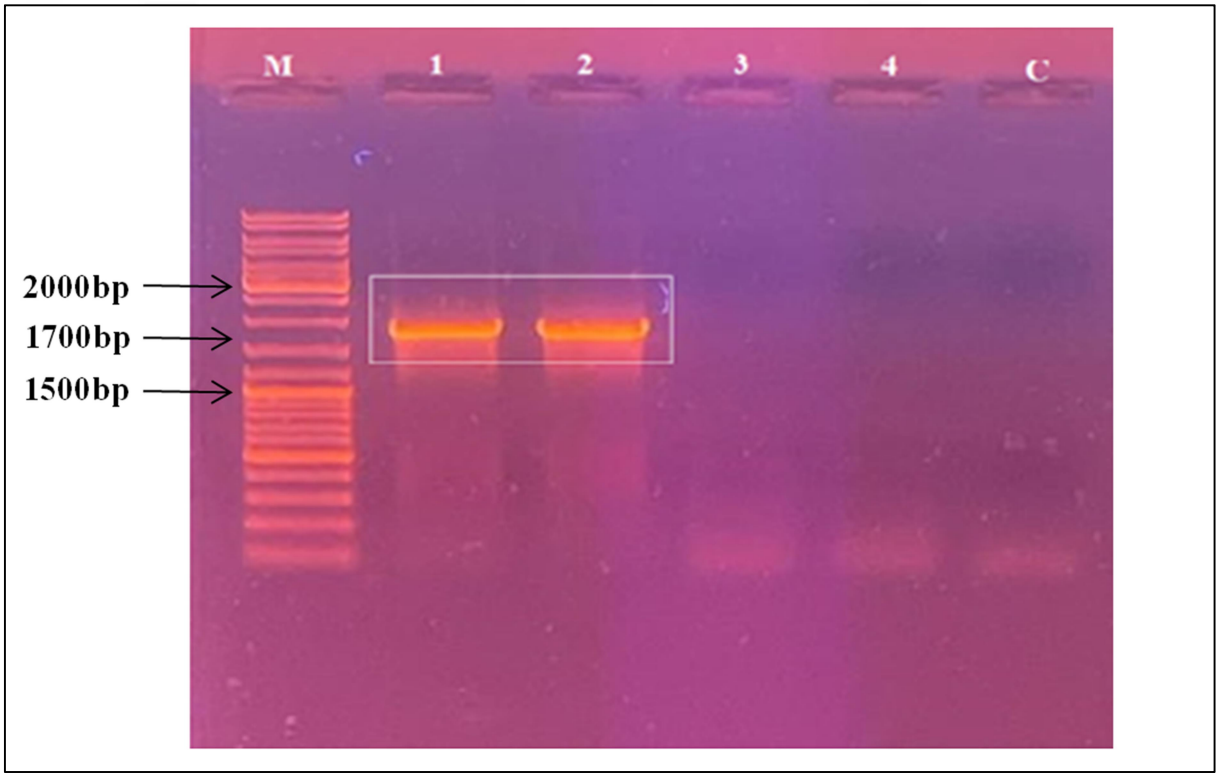

Figure 5. 13. PCR amplification of GUS gene in transformed line of *I. batatas* Hatay Red. Bands visualized with 1% agarose gel stained with ethidium bromide. The bands shown in frames indicate the expected PCR product (1700 bp). Lane (M) 1 kb Generuler DNA ladder mix, Lanes (1) and (2) DNA of putative transgenic tissues, Lanes (3) and (4) wild-type DNA as negative control, (C) ddH20 as template for negative control.

5.9. MICROPROPAGATION OF SOUTH KOREAN VARIETIES

This study was carried out to determine the responses of seven (C-1, K-1, K-2, K-3, K6, K7, K8) South Korean sweet potato varieties to nodal culture and their growth performances at the micropropagation stage under in vitro conditions. Micropropagation of different *I. batatas* genotypes provided by Korea Research Institute of Bioscience and Biotechnology (KRIBB), South Korea were cultured on MS1D media with combined growth regulators. For acclimatization, the developed plantlets were transferred to a greenhouse. Micropropagation capacities of each genotype were investigated under tissue culture conditions. Also, the experience gained from these studies were applied in the tissue culture and *Agrobacterium* studies with local varieties. Total numbers of shoots with respect to sub-cultures for each South Korean variety were shown in Table 5.5. Micropropagation was initiated with 4 explant per variety. Presently, high propagation rates were obtained, and the reason might be the auxin: cytokinin: auxin ratio on MS1D medium. Total numbers of shoots were increased regularly for each genotype. It was determined that there were differences between *I. batatas* genotypes in terms of growth rates in nodal culture and can be seen in Figure 5.14.

Table 5. 5. Total number of shoots at each subculture for sweetpotato varieties.

South Korean sweetpotato varieties							
No. Sub-culture	**K-1**	**K-2**	**K-3**	**K-6**	**K-7**	**K-8**	**C-1**
Subculture 1	5	6	4	7	4	8	9
Subculture 2	5	6	4	8	4	10	10
Subculture 3	5	8	5	8	10	10	13
Subculture 4	6	12	5	9	11	11	18
Subculture 5	9	17	6	24	30	13	29
Subculture 6	12	18	7	31	42	16	53
Subculture 7	16	26	9	43	54	16	65

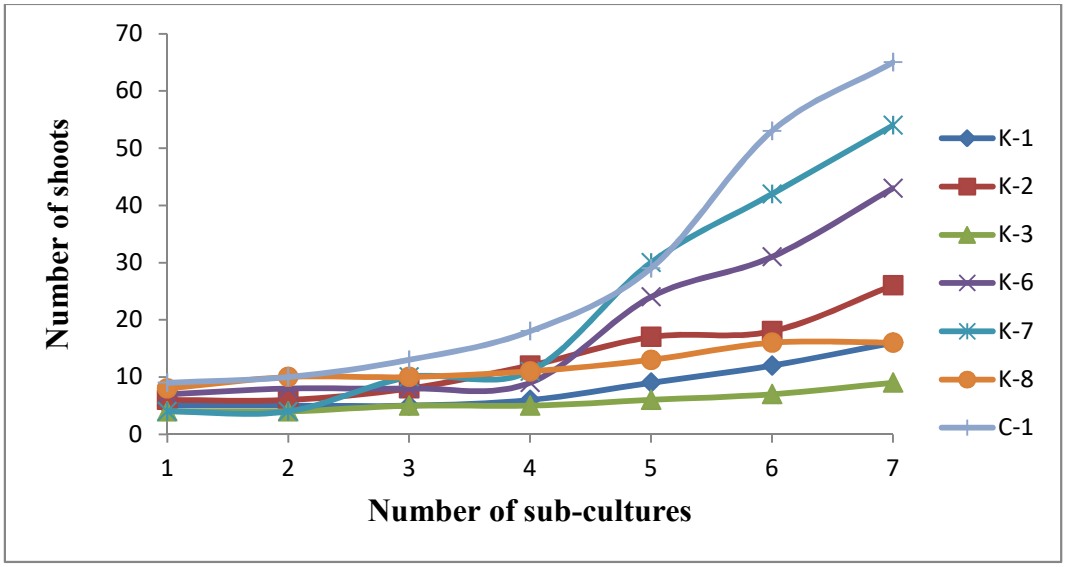

Figure 5. 14. Total number of shoots toward sub-culture for each Korean sweetpotato varieties.

The micropropagation percentage of each Korean sweetpotato cultivar was calculated and their efficiencies were investigated. The growth rate between the 1^{st} and 7^{th} sub-cultures was calculated. For the C-1 and K-7 variety highest propagation percantage was calculated as 96 and 94 per cent respectively as seen in Figure 5.15.

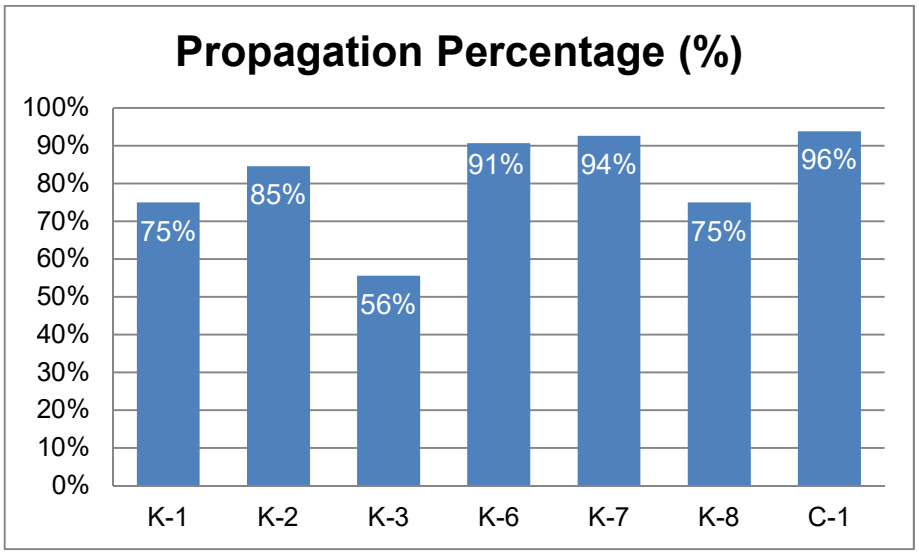

Figure 5. 15. Micropropagation Percentage (%) of plantlets obtained on average from seven subcultures.

Average number of shoots per variety were calculated between the cultivars as seen in Table 5.6. It was observed that highest average shoot number was obtained from C-1 variety (Figure 5.16). With these data, transformation and regeneration successes will be determined by using the successful Korean variety and the native Hatay Red variety in further studies. Finally, after roughly seven months with 3-week interval subculturing, multiplication rates increased. In addition, all of the plantlets for each variety that were subcultured showed healthy morphology without darkening or crystallizing as given in the Figure 5.17.

Table 5. 6. Average number of shoots through seven subcultures for each genotype.

Genotypes	**K-1**	**K-2**	**K-3**	**K-6**	**K-7**	**K-8**	**C-1**
Average number of shoots	8.28	13.28	5.71	18.57	22.14	12.0	28.14

Figure 5. 16. 1st sub-culture of South Korean C-1 variety.

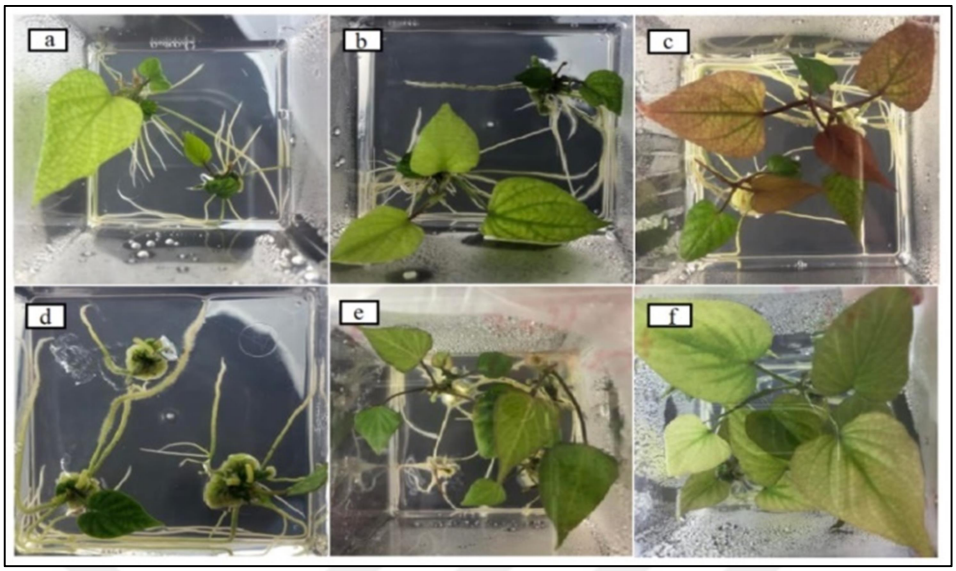

Figure 5. 17. South Korean varieties (a) K-1, (b) K-2, (c) K-3, (d)K-6, (e) K-7, (f) K-8.

To adapt micropropagated plantlets to the new environment, plantlets in the tissue culture vessels were transferred to greenhouse (Figure 5.18). After hardening was finished plantlets were transferred to soil. For acclimatization, humidity was gradually decreased. Well-adapted plants showed tuber formation in the greenhouse and tubers were used for vegetative production in the further greenhouse trails.

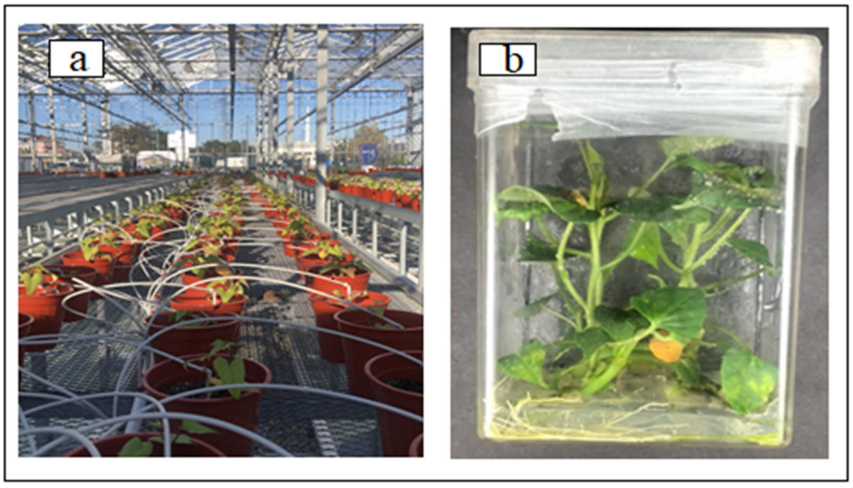

Figure 5. 18 Growth of *Ipomoea batatas* (L.) Lam. (a) Acclimatization of micropropagated plantlets to greenhouse conditions (b) Maintanence of tissue cultured plants in the plant growth room.

6. DISCUSSION

Consumed as a functional food, *I. batatas* is grown in limited regions of our country, especially in Hatay. The production capacity is limited since the work in the field of agriculture is still new and the virus problems for local varieties prevent its production. Studies on the cultivation and consumption of sweetpotato have a great potential in terms of nutritional values and its agricultural production has increased in recent years [101]. For this reason, transgenic studies are also gaining importance besides field studies for agricultural improvement. Transgenic biotechnology is aimed to overcome traditional breeding problems to obtain high nutritional value, virus resistance, and stress-tolerant varieties. Among the difference gene transfer techniques, transformation using *Agrobacterium* preferred since it does not require complex materials [60]. There are studies of *Agrobacterium*-mediated gene transfer in different sweetpotato cultivars [29], [61], [102]. For local sweetpotato varieties in our country there are only field studies [3], [12], [15], [19], [49]. Transformation study is unavailable for local sweetpotato cultivars. Therefore, transformation success for Turkish local varieties using *Agrobacterium* is unknown. To our knowledge, transformation using *Agrobacterium* was performed for the first time in our country with the Hatay Red variety. The present study evaluated the transformation efficiency optimized for the Turkish sweetpotato variety by *Agrobacterium* using the GUS gene as a marker. In addition, it was aimed to produce it safely, free from harmful pathogens and viruses using plant tissue culture methods.

Studies revealed that the success of the transformation depends on several factors such as the type of explant, time for inoculation, the type of crop, type of *Agrobacterium* strain, bacterial density, co-cultivation time, and selection medium for regeneration [55]. Callus cells obtained from plant tissues, called unorganized cell populations, are used in transformation studies. The most common plant growth regulator as auxin suitable for sweetpotato cultures is 2,4-D at callus formation. It has been stated that the optimal concentration of 2,4-D used in the media is critical to form cell differentiation with totipotency among cultivars. This is because the cultivars are extremely sensitive to different auxin levels supported in the growth media [103].

The results obtained reported that the long-term culture using the same media had negative effects on callus formation, proliferation, plant regeneration, shoot and root formation among the cultivars. Another important point is that calli should not be cultured for long time period as the cells lose the capacity to differentiate and develop abnormalities with long culture period [30]. Studies have shown that the use of 2,4-D between 2 mg/l to 4 mg/l is suitable for callus formation in *I. batatas* varieties [104], and 1 mg/l has been accepted as optimal in Korean sweetpotato transformation studies [105], [93]. For this reason, in this study 1 mg/l 2,4-D was used for callus formation from internode explants. At CIM medium with using 1 mg/l 2,4-D as auxin, the average calli formation day was determined as 13.5, and the callus formation percentage was found as 79 per cent. At the end of the 4 weeks, the suitable calli obtained and were used in the transformation studies. These results corroborate with Kwon *et al*. (2002) whom revealed that after 4 weeks of culture period on callus induction medium including 1 mg/l 2,4-D, callus induction rate of *I. batata*s varieties Yulmi, Zami and White Star were 78, 86 and 80 per cent respectively [93].

The growth of *Agrobacterium* strains to be used in transformation studies is one of the initial steps. The medium used and the temperature is a critical step in the colony formation of the *Agrobacterium* strains. In this study, YEB [92] and YM [106] mediums were used at 28 and 30 degrees individually. To optimize the growth of *Agrobacterium* LBA4404 strain, YEB and YM agar plate supplemented with kanamycin (50 µg/ml) and streptomycin (50 µg/ml) incubated at 28°C and 30°C until 2 days at dark. At the end of 2 days, efficient single colony formation was observed using YEB medium at 30 °C for LBA4404 strain.

In nature with the injury of the plant tissue, phenolics are secreted in the damaged plant. It is stated that the presence of phenolic compounds takes part in the transformation study by activating virulence genes in *Agrobacterium* to transfer target gene to the plant genome. In our study, the activation of *vir* genes in the Ti-plasmid for LBA4404 strain was mimicked *in vitro* by adding the phenolic compound known as AS. The concentration of AS used in studies for sweetpotato varieties between 10 and 40 µM [61].A research reveals that the presence of 20 µM AS increases GUS expression for *I. batatas* embryonic callus transformation [61].

In this study, AS concentration was optimized as 20 µM for 30 minute inoculation with *Agrobacterium*. 20 µM AS has been used in both inoculation and co-cultivation. The co-cultivation time has been optimized. In research for embryogenic suspension callus of sweetpotato, the highest rate of GUS positive calli was determined at 4 days of co-cultivation [61]. In addition, it was revealed that more than 5 days of co-cultivation limits bacterial growth and decreased the efficiency of transformation [107]. It was also stated that between 2 to 3 days of co-cultivation were the standard time intervals for general transformation protocols [108]. Thus, longer time period than these days may cause necrosis and cell death [56]. The same idea was supported by another study for sweetpotato variety Yulmi. It is stated that 2, 3 and 5 days of co-cultivation were examined and 2 days were found suitable for transformation efficiency. Based on this information, in this study, the effect of co-culture periods of 2, 3 and 5 days at 25 °C on the formation of antibiotic resistant calli were examined. No statistically significantly difference ($p \leq 0.05$) between 2, 3 and 5 days of co-cultivation period for *I. batatas* callus were observed. However, the highest percentage of hygromycin resistance transformants was evaluated by 3 days of co-cultivation with AS. Based on the number of resistant calli, the optimal time was determined as 3 days with a 60 per cent efficiency rate. These results corroborate with Song *et al*., (2006) whom reported that the growth period for *I. batatas* variety Beniazuma was 3 days [95].

Bacterial concentration is another important factor affecting the transformation success of LBA4404 strain, whose co-culture duration was optimized as 3 days. For sweetpotato, it is revealed that higher optical density (OD_{600}) significantly decreased the transformation efficiency and for sweetpotato suspension cell culture, optimal density was (OD_{600}) found as 0.8 [61]. In another study, *I. batatas* transformation was applied when OD_{600} reached between 0.6-0.8 [61]. As a result of researches in this study, the effect of bacterial concentration ($OD_{600=}$ 0.3, 0.4, 0.6, 0.8) on transformation efficiency was examined. Statistically significant difference ($p \leq 0.05$) among optical densities (OD_{600}= 0.3 with 0.4) and (OD_{600}= 0.3 with 0.8) was determined. However, there is no significant difference was observed between (OD_{600}= 0.4 with 0.8) and (OD_{600}= 0.3 with 0.6.).

In addition, highest hygromycin resistance calli was obtained when optical density (OD_{600}) reached 0.6 with 60 per cent efficiency. Although there was no statistically significant difference between (OD_{600}= 0.3 with 0.6.), (OD_{600}= 0.6) was preferred for co-cultivation due to the high percentage of hygromycin resistant calli.

The *uidA* gene in the pCAMBIA1301 vector is enabling to identify candidate transgenic plants at a short time after transformation. The functional uidA gene produced a blue color when treated with the X-Gluc solution. Thus, in our study histochemical GUS analysis was applied to Hatay Red variety after co-cultivated with AS for 3 days. GUS activity increased gradually with each optical density ($OD_{600=}$ 0.3 and 0.6) from three to six days on X-Gluc solution. When each bacterial concentrations ($OD_{600=}$ 0.3, 0.4, 0.6, 0.8) were analyzed, the highest GUS efficiency was seen as 69.2 per cent and 66.0 per cent for optical density ($OD_{600=}$ 0.3 and 0.6) respectively. Whereas, blue color formation was not observed for optical density ($OD_{600=}$ 0.4 and 0.8) with 3 and 6 days incubation at X-Gluc solution.

Since GUS activity was observed at each optical density ($OD_{600=}$ 0.3 and 0.6), and the number of hygromycin resistant calli was higher at optical density ($OD_{600=}$ 0.6) at 3 day co-culture time, PCR analysis was performed using optical density ($OD_{600=}$ 0.6) of transformed calli. Both wild-type and transgenic genomic DNA of sweetpotato callus were isolated by using CTAB protocol. 137.77 ng/ µL and 217.38 ng/ µL DNA concentration were obtained for wild-type and putative transgenic callus respectively, and used in PCR analysis. In the PCR analysis, in order to amplify GUS gene, specific primers were designed. As a result, a 1700 bp long amplicon was determined in callus co-cultured at 3 days with selected optical density (OD_{600}= 0.6).

Selection and regeneration are the last stage of transformation studies and is important for the determination of the obtained antibiotic resistant plants. In the study, two-stage selection methods were used. At first, transformed calli were placed to the media including hygromycin (15 mg/L) and cefotaxime (250 mg/L). At the end of the 2 weeks, hygromycin resistant putative calli were transferred to RM media containing only hygromycin (30 mg/L).

As a result of two-stage selection for Hatay red variety, the number of hygromycin resistant calli was observed with 60 percenatge efficiency at 3 days co-cultivation AS with optical density (OD_{600}= 0.6). These results suggested that *Agrobacterium* efficiency for transformation was strain and variety dependent. Regulators and their ratios are important to obtain plantlets from hygromycin resistant calli as a result of selection. After selection of resistant calli, research suggested that an acceptable percentage of shoot formation was obtained when apical shoot tips were cultured on media supplemented with 0.01 mg/L NAA and 1 mg/L BAP [61]. A research revealed that sweetpotato regeneration was dependent on the cultivar [30]. In addition, it was stated that while adventitious roots develop easily from callus, for sweetpotato differentiation of adventitious buds was not rapid with respect to roots. [109].

In our study, this information was confirmed and limitations have been observed in regeneration of Hatay Red variety. It has been understood that the regeneration problem experienced in our study is due to the limitations of inducing morphogenesis and the manipulations of the media components and the external environment. For further investigation, regeneration studies should be carried out in local varieties using different plant growth regulators. In a study, successful regeneration of *I. batatas* in a medium including BAP, NAA and GA3 growth regulators was reported by Dasgupta *et al.* (2016) [109]. In addition, it was found that using each PGRs with specified concentrations of some *I. batatas* cultivars in Ethiopia became successful for regeneration. It was reported by the study of López *et al*. (2004) [110] that the highest shoot induction rate for *I. batatas* varieties was obtained using BAP, GA3 and NAA as PGRs. In another study, the best results were obtained when culture medium was supplemented with BA, GA3 and NAA growth regulators with concentrations (0.05, 0.5 and 2.0 mg/l) for Gandera *I. batatas* variety for micropropagation [3]. According to each reference percentage of shoot regeneration was obtained for Korean type sweetpotato varieties when apical shoot tips were cultured on medium containing the combined growth regulators (0.1 mg/L NAA, 0.25 mg/L BAP and 0.1 mg/L GA3) [104]. The micropropagation coefficients of seven Korean sweetpotato cultivars were calculated and their efficiencies were investigated. Differences for average number of shoots were determined between the cultivars, with 97 per cent and 94 per cent efficiency, for each variety (C-1 and K-7) respectively.

7. CONCLUSION AND FUTURE PROSPECT

- *Agrobacterium*-mediated transformation was successfully optimized in *I. batatas* local variety Hatay Red with using LBA4404 strain carried the plasmid pCAMBIA1301 using GUS (*β*-glucuronidase) marker gene.
- Successful calli was created using nodal segment as an explant. The callus induction frequency for Hatay Red variety was found as 79 per cent and first callus induction was observed at 13.5 days after culture at the internodal segments.
- Efficient single colony formation using LBA4404 *Agrobacterium* strain was optimized with YEB medium at 30 °C for 2 days at dark.
- AS concentration was optimized as 20 µM with a 30 minute immersion time for the Hatay Red variety. 20 µM AS has been used in both inoculation and co-cultivation.
- The duration of co-culture affected the transformation success. The highest hygromycin resistance transformants percentage was optimized by 3 days of co-cultivation.
- The highest GUS efficiency was increased at optical density ($OD_{600=}$ 0.3 and 0.6) respectively. Whereas, GUS formation was not observed for optical density ($OD_{600=}$ 0.4 and 0.8) with 3 and 6 days incubation at X-Gluc.
- To amplify GUS gene using specific primers, PCR analysis was done. As a result, a 1700 bp long amplicon was determined in callus co-cultured at 3 days with optical density (OD_{600}= 0.6).
- The number of hygromycin resistant calli was increased at 3 days co-cultivation at AS with optical density (OD_{600}= 0.6).
- Regeneration was not successful in Hatay Red variety with the growth regulators used.
- For micropropagation, Korean type of *I. batatas* cultivars were used. For average number of shoot formation, differences were determined between the cultivars and the highest efficiency was observed in C-1 variety.

- This study showed that efficiency of *Agrobacterium*- mediated transformation for *I. batatas* have many factors to investigate such as the plant genotype, the type of *Agrobacterium* strain, the age of explant, bacterial density, selective medium for regeneration, inoculation and co-cultivation time.
- In conclusion, to the best of our knowledge, *Agrobacterium*-mediated transformation for local *I. batatas* variety Hatay Red for the first time. The output of this study has a great potential to provide new insights for transformation studies in local cultivars of *I. batatas*.

In future studies;

- Callus formation should be ensured in South Korean cultivars and compared with the local cultivar, Hatay Red variety.
- Micropropagation should be started in Hatay Red cultivar in addition to South Korean cultivars and the multiplication coefficient between cultivars should be examined.
- In addition to the optimized Hatay Red variety, transformation study will be carried out on the South Korean variety with selected high micropropagation success variety. The transformation success of the local variety Hatay Red, should be compared with other varieties.
- Regeneration studies should be first performed using untransformed calli. Thus, transgenic calli with success in transformation will be regenerated from the callus using appropriate plant growth regulators.

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