ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEVELOPMENT OF AN *IN VITRO* REGENERATION PROTOCOL, SYNTHETIC SEED PRODUCTION AND DETERMINATION OF ANTIOXIDANT ACTIVITY OF GOLDENBERRY (*PHYSALIS PERUVIANA* L.)

MASTER OF SCIENCE

ALIYU MOHAMMED

BOLU, APRIL - 2016

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APPROVAL OF THE THESIS

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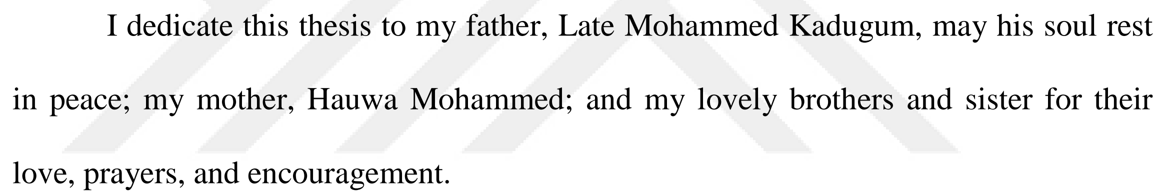

I dedicate this thesis to my father, Late Mohammed Kadugum, may his soul rest in peace; my mother, Hauwa Mohammed; and my lovely brothers and sister for their love, prayers, and encouragement.

DECLARATION

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

Aliyu Mohammed

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ABSTRACT

DEVELOPMENT OF AN *IN VITRO* REGENERATION PROTOCOL, SYNTHETIC SEED PRODUCTION AND DETERMINATION OF ANTIOXIDANT ACTIVITY OF GOLDENBERRY (*PHYSALIS PERUVIANA* L.)

M.Sc. THESIS

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BOLU, APRIL - 2016

Physalis peruviana L., commonly known as goldenberry or cape gooseberry contains polyphenols and carotenoids with anti-inflammatory and antioxidant activities used against diabetes. A highly efficient *in vitro* culture protocol of this plant was developed via direct and indirect organogenesis. To establish a rapid regeneration system, nodal explants excised from 4-week-old germinated seedlings were cultured on different plant growth regulators (KIN, BAP, Zeatin, TDZ, GA3, IAA, IBA, NAA) at various concentrations (0.1, 0.5, 1.0 or 2.0 mg/l) and combinations. The highest mean number of shoots was obtained on MS medium containing 0.5 mg/l TDZ, producing 6.5 shoots per explant with 100% explants developing shoots within four weeks of incubation. The highest root formation was achieved on MS containing 2.0 mg/l IAA, producing 9.0 roots per explant, with 100% rooting responses. Due to the high regeneration capacity of nodal segments, synthetic seed was developed using sodium alginate (NaAlg) encapsulation techniques. Four different matrix compositions, including NaAlg, NaAlg together with MS medium with or without 3% (w/v) sucrose or in combination with 0.5 mg/l abscisic acid (ABA) as a growth retardant, were tested for the regrowth performance of synthetic seeds after storage at +4 °C up to 70 days. Of the tested matrix compositions, the highest re-germination rate was observed from nodal segments encapsulated in NaAlg bead containing MS medium and sucrose, with 60%

conversion after 70 days of storage. In addition, the effects of soil and MS as a sowing medium for synthetic seeds were also compared, and MS medium was found more productive than soil. Antioxidant activity of fruit, leaf, stem, calyx and callus extracts obtained from *in vitro*-derived regenerants as well as fruit, leaf, stem and calyx extracts obtained from seedlings germinated under *ex vitro* were also compared. Regardless of plant resource, the highest antioxidant capacity, total phenolics and flavonoids were obtained from fruit extracts followed by stem and calyx extracts. Leaf and stem extracts obtained from regenerants show more antioxidant activity than those obtained from seedlings. This study not only demonstrates the interest in alternative sources of natural antioxidants but also provides a new insight into protocol development for micropropagation and synthetic seed production of many solanaceous species with economic relevance.

KEYWORDS: *Physalis peruviana* L., *In vitro* regeneration, Goldenberry, Encapsulation, Synthetic seed, Antioxidant activity, Thidiazuron.

ÖZET

ALTIN ÇİLEKTE (*PHYSALİS PERUVİANA* L.) İN VİTRO REJENERASYON PROTOKOLÜ GELİŞTİRİLMESİ, SENTETİK TOHUM ÜRETİMİ VE ANTİOKSİDAN AKTİVİTESİNİN BELİRLENMESİ

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BOLU, NİSAN - 2016

Altın çilek olarak bilinen *Physalis peruviana* L., içeriğindeki polifenol ve karotenoidler sebebiyle anti enflametuar ve antioksidan aktiviteleri ile diyabete karşı kullanılmaktadır. Bu bitki, yüksek verimli in vitro rejenerasyon protokolü oluşturularak boğum eksplantlardan çoklu sürgün yoluyla üretilmiştir. Hızlı rejenerasyon sistemi oluşturmak için, boğum eksplantları 4 haftalık çimlenmiş fidelerden kesilip, farklı bitki büyüme düzenleyicilerinin (KIN, BAP, Zeatin, TDZ, GA3, IAA, IBA, NAA) farklı konsantrasyonlarında (0.1, 0.5, 1.0 veya 2.0 mg/l) kültüre alınmıştır. Dört haftalık inkübasyon süresince 0.5 mg/1 TDZ içeren MS ortamında, eksplant başına 6,5 sürgün üretimiyle en yüksek ortalama değer gözlenmiştir. En yüksek kök oluşumu, %100 köklenme yanıtları ile sürgün başına 9.0 kök üreten, MS 2.0 mg/l IAA içeren besi ortamından elde edilmiştir. Boğum eksplantlarını yüksek rejenerasyon kapasitesi ile sentetik tohum üretimi, aynı zamanda sodyum aljinat (NaAlg) kaplama teknikleri kullanılarak incelenmiştir. Dört günden 70 güne uzayan depolanma sürecinden sonra 3% (w/v) sukroz içeren MS ortamında bulunan ya da bulunmayan sodyum aljinat veya çimlenme geciktirici etkisi olan 0.5 mg/l absisik asit (ABA) kombinasyonuyla sentetik tohumları yeniden çimlendirme performanslarını test etmek için 4 farklı matriks bileşeni kullanılmıştır. Test edilen matriks bileşinleri arasında en yüksek çimlenme hızı 70. günün sonunda %60'lık başarıyla, MS ortamı ve sukroz içeren NaAlg içinde kapsüllenmiş boğumlarda elde edilmiştir. Buna ek olarak, dikim ortamı olarak MS ve

toprak ortamının etkileri sentetik tohum için karşılaştırılmıştır. Test edilen dikim ortamı arasında MS ortamının toprağa göre daha verimli olduğu saptanmıştır. İn vitro koşullarda üretilmiş olan rejenerantların meyve, yaprak, gövde, çanak ve kallus dokularının yanı sıra ex vitro koşullar altında çimlenen fidelerin de meyve, yaprak, gövde ve çanak yaprak özütlerinin antioksidant aktiviteleri karşılaştırılmıştır. En yüksek antioksidan kapasitesi, toplam fenolik ve flavonoid miktarı, gövde ve çanak yaprak özütlerini takiben meyve özütlerinden elde edilmiştir. Rejenerasyonlardan elde edilen yaprak ve gövde özütleri, fidelerden elde edilenlere göre daha fazla antioksidan özelliği ihtiva ettiği gözlemlenmiştir. Bu çalışma sadece doğal antioksidanların alternatif kaynaklarına ışık tutmanın yanında, aynı zamanda mikro çoğaltım ve birçok patlıcangiller familyasından farklı türlerin sentetik tohum üretimi için protokol geliştirmesine de yeni bakış açısı sağlamaktadır.

ANAHTAR KELIMELER: *Physalis peruviana* L., İn vitro rejenerasyon, Altın çilek, Enkapsülasyon, Sentetik tohum, Antioksidan aktivitesi, Thidiazuron.

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LIST OF ABBREVIATIONS

$^1O_2$	Singlet oxygen
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
B5	Gamborg's B5 vitamins medium
BAP	6-benzylaminopurine
$CaCl_2$	Calcium chloride
CE	Catechol equivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
GA_3	Gibberellic acid
GAE	Gallic acid equivalent
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HCl	hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC_{50}	50% inhibition concentration
KIN	Kinetin
MeOH	Methanol
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthalene acetic acid
Na_2CO_3	Sodium carbonate
NaAlg	Sodium alginate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
O_2^-	Superoxide anion radicals
OH•	Hydroxyl radicals

ORAC	Oxygen radical absorbing capacity
PGR	Plant growth regulator
RAMs	Random amplified microsatellites
ROS	Reactive oxygen species
SD	Standard deviation
SE	Standard error
TDZ	Thidiazuron
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TPC	Total phenolic content
TRAP	Total radical-tapping antioxidant parameter
UV	Ultraviolet

ACKNOWLEDGEMENTS

I wish to acknowledge the indispensable assistance afforded to me by so many people throughout my life and this experience. It is an honor to have many people around me who have made great sacrifices and aids to make this thesis possible.

First of all, I would like to express my deepest appreciation and gratitude to my supervisor Prof. Dr. Ekrem Gürel for his guidance, supports, efforts and enthusiasm throughout the research. I also want to thank him for believing in me. It is an honor to have a thesis supervisor like him who was motivated and patient. In addition, his incredible knowledge and encouraging comments on the subject are some other qualities that make him a great mentor.

I would also like to express my heartfelt thanks to my co-supervisor Assist. Prof. Dr. Bahtiyar Buhara Yücesan for his academic supports and suggestions over the course of my studies. His flexibility, genuine caring and concern, during the dissertation process, enabled me to achieve this goal. He has been motivating, encouraging, and enlightening all the time.

I would like to emphasize my delightfulness to my lab colleagues Assist. Prof. Dr. Fatma Pehlivan Karakaş, Özge Kaya, Dr. Günce Şahin, Merve Arslan, Cansu Cihangir, Barış Kılıçsaymaz, Assist. Prof. Dr. Sameeullah Muhammad and Tuğçe Ayşe İlal for everything they have done for me. Without them, this thesis would be impossible. They all provided me with not only experimental support and inspiration but also created a working atmosphere with full of genuine friendship, enjoy and helpful.

I would like to thank my friends here in Bolu for sharing the stressful moments with me and for helping me whenever I needed. I want to also send my special thanks to my friends in Nigeria for their prayers, well-wishes, and encouragement all the time.

Lastly but most importantly, by dedicating this dissertation to them, I wanted to express my sincere appreciations to my family members for all their unconditional love and support both during my research and throughout my life. I want to especially mention the support and inspiration of my mother, Hauwa Mohammed; my brothers,

Sani Mohammed Kadugum, Saleh Mohammed and my lovely sister, Maryam Mohammed Kadugum. I could not have been able to complete this long journey without their love, support and prayers. They have always given me confidence and motivation in all what I have done. Finally, I apologize for those not mentioned here; they are, however, not forgotten.

CHAPTER I

1 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Physalis peruviana L. is one of the most important medicinal plant species belonging to Solanaceae family and genus *Physalis*. It is an herbaceous, semi-shrub and perennial plant native to South America and other warm temperate and subtropical regions throughout the world. This species comprises more than 80 varieties that can be found in the wilderness (Fang et al., 2012). The genus is characterized by its small orange fruit similar in size, shape and structure to a small tomato. *P. peruviana* has been known by different names such as uchuva (in Columbia), uvilla (in Ecuador), aguaymanto (in Peru), topotopo (in Venezuela), altın çilek (in Turkey) and in English-speaking countries it is known as “goldenberry” or “cape gooseberry” (Salazar et al., 2008; Puente et al., 2011; Demir et al., 2014).

This plant has an increasing popularity due to its nutritional and medicinal values. In 2005, more than 1.8 million acres of berry crops worldwide were reported including 966 acres of goldenberries (Strik, 2007). Goldenberry is a source of health-related compounds found in the fruits and other parts of the plant including leaves and stems. It has been widely used in folk medicine for treating diabetes, hepatitis, ulcer, malaria, asthma, dermatitis, leukemia, rheumatism and several other diseases (Mayorga et al., 2002; Wu et al., 2004a, b; Arun and Asha, 2007; Franco et al., 2007; Ramadan, 2011). Goldenberry’s fruit also contains high levels of vitamin A, C, and B-complex, as well as compounds with anti-inflammatory, antioxidant, anticancer, antibacterial, antipyretic, diuretic and immunomodulatory properties (Strik, 2007; Zhao, 2007).

Besides using this plant for medicinal purpose, its fruits are often eaten fresh in fruit salads, preserved as jam or sometimes are stewed with honey and eaten as dessert. In Turkey, fresh or dried fruits and preparations containing this fruit are frequently use

for losing weight and treating diabetes, and many health practitioners are recommending eating of at least five fruits a day (Demir et al., 2014). However, some studies reported that taking too much of these fruits might cause side effects such as hypertension, ventricular tachycardia, and manic episode (Perk et al., 2013).

Despite growing interest in this species, little is known about its genetic diversity and population structure. Although it has been reported that goldenberry is a diploid species with 2n = 48 (Menzel, 1951); different chromosome numbers might exist among genotypes given that 2n = 24 has been reported for wild ecotypes, 2n = 32 for the cultivated Colombia ecotype and 2n = 48 for the cultivated Kenya ecotype (Nohra et al., 2006). However, recent studies by Missouri Botanic Garden (2012) refers mostly to tetraploids (2n = 48) with one reference to a hexaploid (2n = 72). In addition, Rodriguez and Bueno (2006) consider the basic chromosome number to be n = 8 and referred the wild ecotypes as triploid (2n=24) and tetraploid (2n=32) for the cultivated Colombia ecotype, while cultivated forms in Kenya as hexaploid (2n=48). The genetic diversity of this species at the molecular level has been poorly studied, only one report applying dominant markers RAMs (Random Amplified Microsatellites) in 43 individuals from five geographical regions in Colombia, suggesting high heterozygosity and genetic diversity in this species (Munoz-Florez et al., 2008).

1.2 LITERATURE REVIEW

1.2.1 History of Origin

Physalis peruviana L. is a plant native to the Andes region, transcending the history of the pre-Incan and Incan periods throughout South America (Cailes, 1952; Legge, 1974). The center of origin according to Legge (1974) was the Peruvian Andes, but according to a study made by the countries belonging to the Andres Bello Convention in 1983, a larger area was identified as the origin of this species, ranging from Ecuadorian Andes to Chile, Colombia and Venezuela. From South America, this plant has been widely distributed across Africa, Asia and then finally to Europe, as a fruit and ornamental crop. Cultivation in Europe started during the 1700s in

the United Kingdom (Legge, 1974) and later extended to South Africa, Australia, and New Zealand with the first immigrants (Watt, 1948; Cailes, 1952; Anon, 1953; Cann and Sproule, 1963). In Turkey, *Physalis* has long been grown as gardens plant in Kutahya, Antalya, Bitlis, Istanbul, Sakarya, Samsun, and Karaman regions, especially *P. pubescens* L. and *P. alkekengi* L., but commercial production has been on a very small scale (Demir et al., 2014).

Interest in commercial development has fluctuated, stimulated at times of interest in export potential. Many attempts at large-scale production have failed because of problems associated with cultivation, ripening, and harvesting over a sustained period. Apart from an early report on production (Watt, 1948), very little information is available on goldenberry cultivation. However, in Colombia, the fruit has become promissory with high demand in European markets, mainly due to its unique taste and its potential health value.

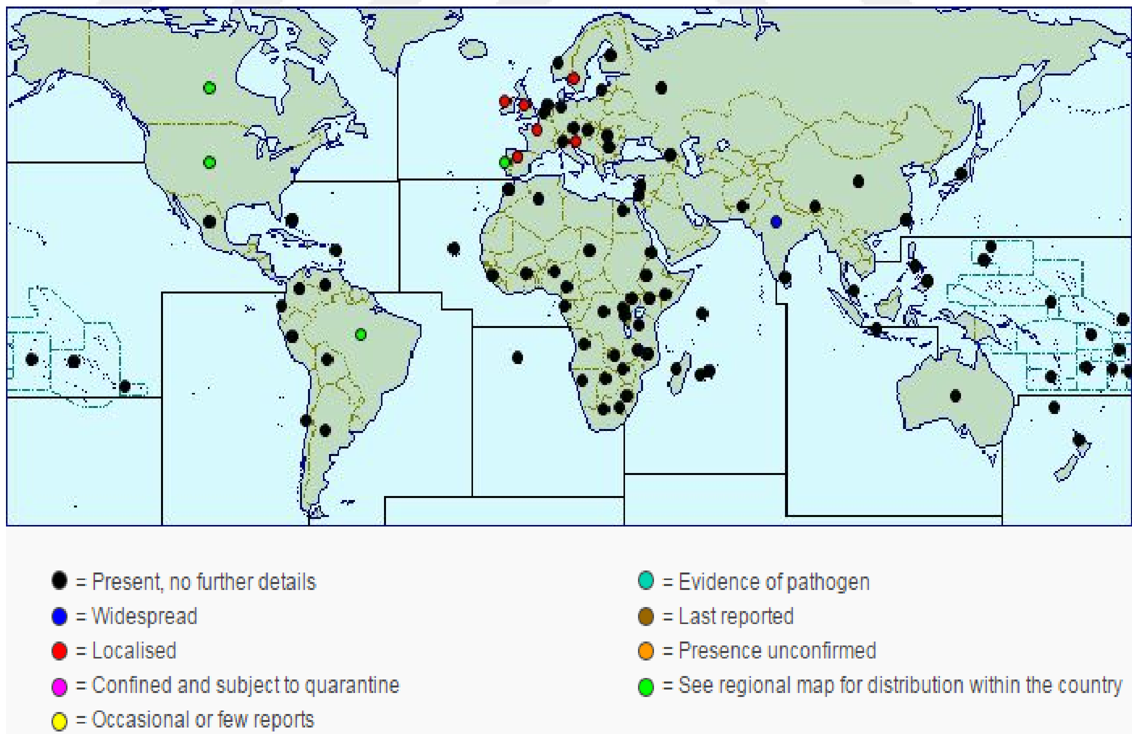

Figure 1.1. Sketch map showing the distribution of *P. peruviana* worldwide (www.agreengarden.com).

1.2.2 Taxonomical Studies

The first description of genus *Physalis* was made by Linnaeus in 1753. However, identification of various species within this genus has been subject to much confusion in the literature (Menzel, 1951). The specific boundaries of this genus were poorly defined with some duplication of names and many changes in the nomenclature during the last 50 years (Hudson, 1986). The complexity of the genus may be due to the wide range of genetic variability present that presumably resulting from interspecific hybridization and also by the ambiguity of the earlier taxonomic descriptions (Waterfall, 1958; Raja-Rao, 1979).

To clarify the taxonomic classification of *Physalis*, Menzel (1957) and Waterfall (1967) made extensive cytologic and taxonomic studies of the genus and try to modify some of the misconceptions, and they classified *P. peruviana* as subspecies under *P. ixocarpa*. Later, Fernandes (1974) made a thorough investigation of this nomenclatural problem and concluded that *P. peruviana* is a distinct species, different from *P. ixocarpa* based on previous cytological studies. Nevertheless, the taxonomic complexity of the genus is not yet clarified. The recent taxonomic classification of genus *Physalis* according to "Plants Database" from United States Department of Agriculture (USDA, 2012), classified goldenberry as: Kingdom Plantae; Subkingdom, Tracheobionta; Superdivision, Spermatophyta; Division, Magnoliophyta; Class, Magnoliopsida; Subclass, Asteridae; Order, Solanales; Family, Solanaceae; Genus, *Physalis* L.; Species, *peruviana*.

1.2.3 Botany

Physalis peruviana L. main stem is herbaceous or soft-wooded, capable of growing up to 0.6 to 0.9 m and in some cases up to 1.3 m, with main root ranging from 30 to 60 cm long. It has sparingly branches (10 to 15 centimeters), which are densely pubescent and ribbed, often purplish in color (Kindscher, 2012). Leaves are simple, alternated, 6 to 15 cm long and 4 to 10 cm wide (Fischer et al., 2000). The leaf lamina is broadly ovate to cordate, on 2–5 cm long petioles, densely pubescent, base cordate,

margin entire or with a few irregular indistinct teeth, apex shortly acuminate. Flowers are unique, pedunculate and hermaphrodite, derived from the axillary bud, with five yellow petals, stamens with blue-purple filaments and anthers. The flower can be easily pollinated by insects, wind or self-pollinated (Lagos, 2008). The fruit is a juicy berry with ovoid shape, which turns from green to golden-yellow upon ripening, with a diameter around 1.25 to 2.50 cm and weight from 4 to 10g, containing inside 100 to 200 tiny (2 mm diameter), yellowish, flat seeds (Fischer et al., 2011). The fruit is also protected by the calyx or fruit basket (formed by five sepals around 4 cm long) which completely covers the fruit along its development and protecting it against insects, birds, diseases and adverse climatic conditions (Muniz et al., 2014).

Ganapathi et al. (1991) provide a key that clarifies the morphological differences between *Physalis* species. They indicated that *P. peruviana* can be distinguished by its larger flowers (corolla over 1.3 cm diameter) from *P. pubescens*, *P. acutifolia, P. minima* and *P. angulata* whose corollas are less than 1 cm diameter. Other species, including *P. Philadelphia, P. angulata* and *P. minima*, are distinguished from *P. peruviana* by being annual, not perennial, and having anthers less than 3 mm long (Flora of China, 2012).

Figure 1.2. Pictures showing morphological characteristics of natural populations of *P. peruviana* (www.agreengarden.com).

1.2.4 Agroecological Studies

Most studies reported that this plant can grow in a wide range of altitude from 3300 m above sea level (Fischer et al., 2011). However, some authors suggested that altitude has a strong influence on *Physalis* plants and their fruits because an increase in ultraviolet radiation and decrease in the air temperature affect the growth and development of this plant. According to Miranda (2004) the favorable temperature for growing and development of plants is 18 °C. Similarly, Fischer (2000) stated that *Physalis* shows better growth and development in regions with annual temperatures between 13 to 18 °C. Moreover, Kindscher (2012) also proposed the optimal temperature for *Physalis* to range between 12 to 20 °C. In contrast, Salazar et al. (2006) explained that 6.3°C is the physiologic-base temperature for *Physalis* growing. High temperatures (higher than 30 ºC) damage flowering and fruiting stages, promoting early aging while low temperatures (nocturnal lower than 10 ºC) can also obstruct the growth of this plant (Muniz et al., 2014). The same report further indicated that temperature and light have an important role in relation to size, color, nutritional content, taste and fruit ripening.

Physalis peruviana can also grow in a huge range of soil and climatic conditions and it is classified as a very tolerant species due to its adaptability to Mediterranean climates and to several soil types (Fischer, 2000). This plant thrives best in slightly acid soil though it can tolerate pH values between 5.5 to 7.3 with good organic matter content and rainfall between 1000 and 2000 mm (Muniz et al., 2014). The average relative humidity of this plant ranges from 35 to 60% during growing season (Popova et al., 2010).

1.2.5 Biological Activity Studies on *P. peruviana*

Physalis peruviana extracts have demonstrated important biological activities, as: antioxidant (Wu et al., 2005; Franco et al., 2007; Vasco et al., 2008; Quispe-Mauricio et al., 2009; Puente et al., 2011; Fang et al., 2012; Valdenegro et al., 2013; López et al., 2013; Izli et al., 2014; Demir et al., 2014), anti-inflammatory (Wu et al., 2006; Pinto et

al., 2010; Martínez et al., 2010; Ramadan, 2011; Demir et al., 2014), hepatoprotective (Chang et al., 2008; Pinto et al., 2009), antihepatotoxic (Arun and Asha, 2007), anticancer (Wu et al., 2004b; Zavala et al., 2006; Franco et al., 2007; Lan et al., 2009; Yen et al., 2010; Gautam et al., 2011; Demir et al., 2014), antibacterial (Veleiro et al., 2005; Wu et al., 2006; Lan et al., 2009; Jaca and Kambizi, 2011; Demir et al., 2014), cytotoxic (Lan et al., 2009) and antitumor (Chiang et al., 1992; Zavala et al., 2006).

Mayorga et al. (2002) reported that goldenberry has an anti-ulcer activity and it is effective in reducing blood cholesterol level. In addition, Wu et al (2009) also reported that supercritical carbon dioxide extracts of *P. peruviana* leaves induce cell cycle arrest and apoptosis in human lung cancer cells, and 4-β-hydroxywithanolide (4βHWE) isolated from aerial parts (stems and leaves) has also confirmed to be a potential DNA-damaging and chemotherapeutic agent against lung cancer. Extracts from leaves and stems of *P. peruviana* L. were also notified to inhibit tumor cell growth (Zavala et al., 2006).

1.2.6 Tissue Culture Studies on *P. peruviana*

Because *P. peruviana* was reported as recalcitrant towards shoot organogenesis in terms of adventitious regeneration (Torres, 1991), there are limited number of *in vitro* regeneration studies concerning its micropropagation (Rodrigues et al., 2013a). Several studies on *in vitro* propagation of other *Physalis* species including *P. minima*, *P. ixocarpa* and *P. pruinosa* were reported (Bapat and Rao, 1977; George and Rao, 1979; Gupta, 1986; Ramirez-Malagon and Ochoa-Alejo, 1991; Rao et al., 2004). One of these studies reported high number of shoot regeneration in *P. minima* on full-strength MS medium fortified with 5 mg/l BAP and 0.25 mg/l IAA (Sheeba et al., 2010). Similarly, Sipahimalani et al. (1981) also reported regeneration from stem explants of *P. minima*.

Ramirez-Malagon and Ochoa-Alejo (1991) reported a failure of adventitious regeneration in *P. peruviana* using hypocotyl explants when cultured on MS medium containing cytokinins or auxins alone. However, Rodrigues et al. (2013b) reported successful shoot regeneration on half-strength MS medium supplemented with BAP. Otroshy et al. (2013) also reported high rhizogenesis and organogenesis from leaf and

nodal explants of *P. peruviana* on MS containing high concentrations of BAP and KIN, with best *in vitro* rooting on full-strength MS with 1.0 mg/l IBA. Ramar et al. (2014) reported a study using node and internode explants of *P. peruviana* and observed maximum shoot multiplication on MS medium with B5 vitamins and 2.0 mg/l BAP + 1.0 mg/l GA3 + 1.0 mg/l 2, 4-D.

Callus culture was also reported using leaf explants on MS medium supplemented with different concentrations of PGRs and found that leaf explants raised on MS medium containing 2% sucrose with 4.0 mg/l NAA and 1.0 mg/l BA induced more callus, with high amount of ascorbic acid accumulation, total protein content and peroxidase activity (Gautam et al., 2011). Callus induction in *P. peruviana* was also previously observed by Torres et al. (1991). Furthermore, BAP and NAA were also reported to increase the accumulation of active metabolites in *P. peruviana* (Gautam et al., 2011). This indicates the possibility of using PGRs to induced further production of secondary metabolites. However, review of the literature shows that there has been limited efforts in the application of tissue culture techniques for regeneration of *P. peruviana*.

Although plants are generated conventionally through seeds, generation from seed is usually season-dependent. Hence, there has been extensive efforts in production of artificial (synthetic) seeds in several plant species (Rai et al., 2008a,b, 2009; Ara et al., 2000; Sharma et al., 2009; Verma et al., 2010; Ozudogru et al., 2011; Yucesan et al., 2014). This technology provides an easy handling of living materials, as well as new understanding towards the storability, uniformity of pathogen-free plant materials (Patel et al., 2000; Winkelmann et al., 2004). Despite the growing interest in synthetic seed production, no report was found regarding encapsulation in *P. peruviana*.

1.2.7 Active Constituents and Nutritional Composition of *P. peruviana*

Recent studies have reported physicochemical parameters of fresh fruit of *P. peruviana* L. The benefits associated with the fruits are mainly due to their nutritional

composition because, besides having good nutritional characteristics, it contains biologically active components that provide health benefits; thus reduces the risks of certain diseases. Among its major components are high amounts of polyunsaturated fatty acids, vitamins A, B and C and phytosterols, as well as the presence of essential minerals, withanolides and physalins, which together give the medicinal properties of this crop (Salazar et al., 2008).

El-sheikha et al. (2009) studied the protein content of the fruit of *P. pubescens* L., showing 31.8% of essential amino acids, mainly leucine, lysine and isoleucine. Similarly, Puente et al. (2011) indicated that the fruit of *P. peruviana* L. has a good protein content, but there is no detailed record of the amino acids and how many of them are essential. In addition, Fischer (2000) also evaluated three sugars in the fruit, with sucrose (disaccharide) being the most abundant sugar after glucose (monosaccharide) and finally fructose (monosaccharide) with limited presence.

According to studies by Ramadan and Morsel (2003), the fruit also contains 2% oil, of which 1.8% is extracted from the seeds and 0.2% from the fruit pulp and skin. The oil consists of 15 fatty acids, among which are linoleic, oleic, palmitic and stearic acids, which constitute 95% of total fatty acids. The oil extracted from the skin and pulp has high levels of plant sterols with campesterol being the most abundant phytosterol, moreover contains β-sitosterol and stigmasterol, which could be responsible for the fruit's ability to reduce cholesterol levels (Ramadan, 2011). Mayorga et al. (2001) reported that the fruit has been also used as a source of minerals, especially iron and potassium. In line with that, Puente et al. (2011) proved the presence of phosphorus, iron, potassium and zinc in the fruit, which together have important metabolic functions related to muscle function, hormonal and nerve stimulation (Rodrigues et al., 2009).

A series of pseudo-steroids known as physalins were isolated from *Physalis* sp. and characterized (Soares et al., 2006), with physalins A, B, D, F and glycosides being the most predominate active constituents of leaf extract, which show anticancer activity (Wu et al., 2004a, 2009). For the phytochemical spectra, Lan et al. (2009) noticed 17 withanolides in this species, out of which seven were newly discovered: phyperunolid A, B, C, D, E, F and peruvianoxid, as well as ten correspond to withanolides previously

known: withanolid C, withanolid E, withanolid S, 4-β hidroxiwithanolid E, physalolactone, physalactona, withaphysanolid, withaperuvin, withaperuvin D and loliolid.

Furthermore, several phenolics are of great interest owing to their important pharmacological properties (Meyer, 1999; Narváez-Cuenca et al., 2014). In goldenberry, quercetin is the main phenolic compound, followed by garlic acid, myricetin and kaempferol (Ramadan, 2011). High content of flavonoids was also indicated in leaf and fruit of goldenberry (Licodiedoff et al., 2013; Al-Olayan et al., 2014). The bioactive components present in this plant makes it an important dietary antioxidant since they reduce the adverse effects of reactive oxygen and reactive nitrogen that can cause damage to macromolecules such as lipids, DNA and proteins, which are also related to cardiovascular, cancer and neurodegenerative diseases (Naidu, 2003).

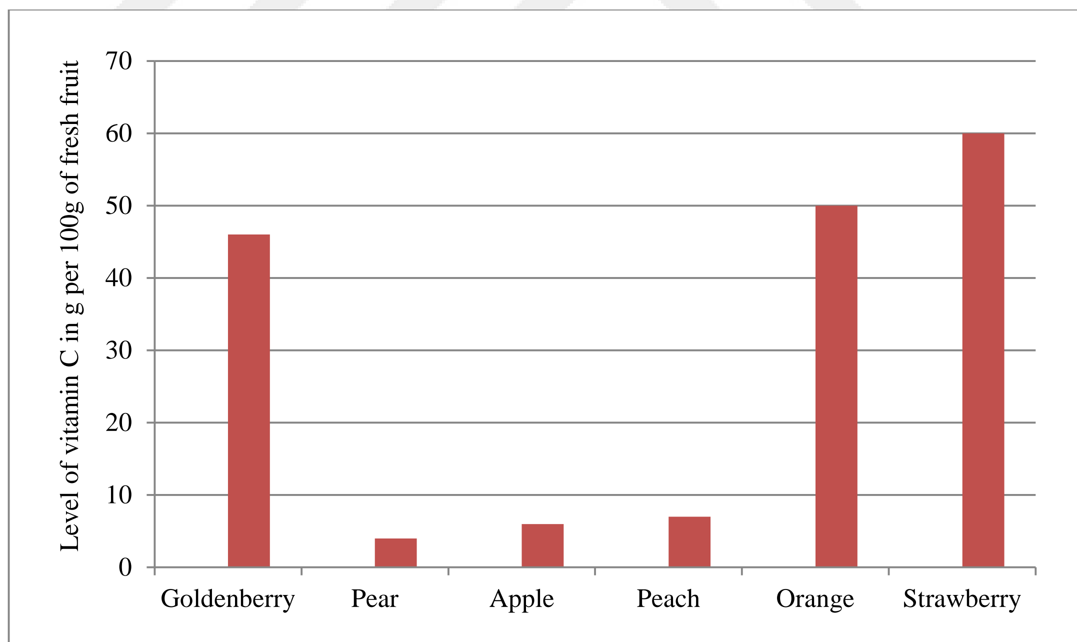

Figure 1.3. Comparison of vitamin C content in *P. peruviana* fresh fruit and other fruits (Ramadan, 2011).

2 AIM AND SCOPE OF THE STUDY

1. To develop an efficient *in vitro* plant regeneration protocol for *P. peruviana* via adventitious shoot regeneration from nodal and leaf explants on MS medium supplemented with different concentrations and combinations of various plant growth regulators. This will provide a new insight for micropropagation of this plant via tissue culture techniques for large-scale production as well as the effects of plant growth regulators in relation to certain physiological and environmental factors under both *in vitro* and *ex vitro* conditions.

2. To understand the viability of nodal explants encapsulated in different sodium alginate compositions and their regeneration capacity on different sowing medium. This protocol provides, for the first time, synthetic seed production in this species.

3. To evaluate and compare the antioxidant activities of leaves, stems, fruits, calyces and callus of *in vitro*-derived regenerants as well as those of *ex vitro*-derived *P. peruviana*.

CHAPTER II

3 *IN VITRO* REGENERATION OF *PHYSALIS PERUVIANA* L. (GOLDENBERRY)

3.1 Introduction

Plant tissue culture is an alternative method of commercial propagation and it has been used widely for the propagation of a large number of plant species including many medicinal plants (Rout et al., 2000). Although medicinal plants can easily be found in the wild, they are generally subjected to some herbicides and attacked by some insect and pathogens. *In vitro* micropropagation not only allows rapid propagation or mass production of identical clones of plant species but also has the following advantages: the elimination of viruses and other pathogens; the storage of essential germplasm instead of conventional seeds; the embryo rescue; and the production of haploids by anther and ovary culture (gametoclonal variants) (Smith, 2004). *In vitro* micropropagation of medicinal plants provides pesticide-free plants and produces large numbers of vegetative planting stock easily. In addition, with an *in vitro* propagation method, unlimited plant material can consistently be obtained throughout the whole year and plant materials with less genetic diversity (genetically uniform) can be produced easily, which will be higher with seed germination (Turker et al., 2008). *In vitro* micropropagation can also provide a rapid multiplication method for producing plantlets exhibiting desirable characteristics. These desirable characteristics may be in terms of disease resistance, high yield, increased secondary product accumulation, superior genotype characteristics, or beneficial morphological characteristics; all of which result in superior planting stock. Moreover, more uniform plantlets can be obtained using an *in vitro* protocol for production, thus ensuring a more consistent crop. An *in vitro* propagation method would

also eliminate seasonal constraints with seedling by providing unlimited planting material on a consistent year-round basis (McCoy, 1998).

All normal living cells within the plant body possess the potential capacity to regenerate an entire plant. This potentiality has been exploited through the culture of protoplasts, cells, tissues and organs *in vitro*. Cells and tissues, which are mitotically quiescent or already committed to some function or pathway of development, can be (re)directed into organ or embryo formation. Organogenesis is the process by which cells or tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissues (Thorpe, 1994). The earliest report of controlled shoot formation *in vitro* was by White (1939). In the same year, the first observation of root formation from callus was reported by Nobecourt (1939), using carrot callus. White's observation was confirmed and extended by Skoog (1944), who showed that auxins could stimulate root formation and inhibit shoot formation. However, Skoog (1944) further found that the inhibitory effect of auxin on shoot formation could be partially overcome by increasing the concentration of sucrose and inorganic phosphate in the medium. Skoog and Tsui (1948) demonstrated that adenine sulfate was active in promoting shoot formation and counteracted the inhibitory action of auxin. They further reported (Skoog and Tsui, 1951) that the extent of organ formation was dependent on both the concentration and proportion of these additives.

The discovery of kinetin by Miller et al. (1956) led to the now classic finding of Skoog and Miller (1957) that the basic regulatory mechanism underlying organogenesis involved a balance between auxin and cytokinin. Both of these substances are required for cell division and enlargement in tobacco callus, but a relatively high level of auxin to cytokinin favored root formation and the reverse favored shoot formation. These observations led them to suggest that quantitative interactions between diverse growth regulators rather than specific morphogenetic substances provided a common mechanism for the regulation of all types of morphogenetic phenomena in plants, that is the basic concept of the regulation of organogenesis *in vitro* (Thorpe, 1990).

Christianson and Warnick (1988) established three phases of organogenesis: dedifferentiation, induction and differentiation. Dedifferentiation involves callus production and the process ends when cells become competent. During the induction phase, cells become determined and in the differentiation phase, cells formed roots or shoots. Plant hormones are centrally involved in the control of organogenic processes. In this manner, the destination of meristemoids can be controlled by manipulation of exogenous hormones or by insertion of T-DNA genes specifying synthesis of auxins or cytokinins (Lakshmanan et al., 2006). Furthermore, the exogenous phytohormones can reverse the T-DNA-induced morphogenesis, suggesting that the phytohormones play a direct role in organized development. Consequently, responding cells enter a state of determination fixing them along a particular developmental pathway, competence is lost and organogenesis follows (Coste et al., 2011).

Biotech crops are grown commercially on a large scale and also being increasingly used for the production of vaccines and many pharmaceuticals (Vasil, 2008). The secondary metabolites of plants include such compounds as pigments and aromas which aid pollination by attracting insects to the flowers, poisons which make the plant inedible, compounds which control invasion by pathogens and grazers and compounds whose function are not yet understood. Many of these secondary compounds have uses beneficial to man. A large number of plant secondary metabolites are used in the food, fragrance, flavorings, cosmetics and pharmaceutical industries. Many medicines were initially isolated from natural plant sources, a gene pool that is diminishing (Burbidge, 1993). Therefore, *in vitro* regeneration protocols for plants, especially medicinal plants have high value.

Micropropagation via direct or indirect organogenesis from different plant organ has been used for clonal propagation of many species, with different auxins and cytokinins used to induce organogenesis (Vasil and Thorpe, 1994). Both types of organogenesis (direct and indirect) can occur in the same culture, and callus proliferation in the cut zone is usually unavoidable (Bigot et al., 1977). De novo induction of shoot buds is commonly promoted by phytohormones, and a combination of cytokinins and auxins is usually necessary (George et al., 2008). Cytokinins play an important role in

plant growth and development (Werner et al., 2001), and participate in the maintenance of meristem function and in the modulation of metabolism and morphogenesis in response to environmental stimuli (Sakakibara et al., 2006). However, appropriate plant genotype and physiological status of explants are crucial for an organogenic response (Bianchi et al., 1999; Lin et al., 1998; Magyar-Tabori et al., 2010).

Goldenberry is a valuable medicinal plant, but there are no adequate studies on *in vitro* propagation of this species. Therefore, the main aim of this study was to establish an efficient *in vitro* regeneration protocol of this species via adventitious shoot formation from nodal and leaf explants on medium containing different concentrations and combinations of various plant growth regulators (PGRs).

3.2 Materials and Methods

Seeds of *P. peruviana* were purchased from commercial sellers in October 2013, in Bolu, Turkey. First of all, the seeds were washed under running tap water and surface disinfected by shaking in 70% ethanol for 4 minutes. Afterwards, the seeds were dipped into 50 ml of 20% commercial bleach, [Domestos®; ~5% (v/v) sodium hypochlorite] in a 250 ml beaker and stirred at 300 rpm on a magnetic stirrer plate for 10 min, followed by rinsing with sterile distilled water 5-6 times. After surface sterilization, an average of 15-20 seeds were aseptically germination in disposable Petri plate (90 × 15 mm) containing 25 ml of Murashige and Skoog (1962) medium (MS) supplemented with 3% (w/v) sucrose. The medium was solidified with 0.8% (w/v) plant-agar prior to autoclaving at 121 °C and 1.06 kg/cm^2 pressure for 20 min after adjusting the pH to 5.8. Nodal segments (~5 mm) were isolated from 4-weeks-old seedlings and subcultured on MS medium without growth regulators for the multiplication of stock sources by recycling the regenerated shoots through nodal segments at three weeks intervals.

To attain direct shoot regeneration, two sets of experiments were carried out independently using nodal segments excised from 4-weeks-old stock plants as explants source. In the first set, nodal explants (~5 mm) were cultured in disposable Petri plates containing MS medium supplemented with four different concentrations (0.1, 0.5, 1.0 or

2.0 mg/l) of plant growth regulators (PGRs): kinetin (KIN), 6-benzylaminopurine (BAP), zeatin, thidiazuron (TDZ), gibberellic acid (GA_3), indole-3-acetic acid (IAA), indol-3-butyric acid (IBA) or naphthaleneacetic acid (NAA), whereas in the second set, the nodal explants were cultured on MS medium supplemented with either 0.5 mg/l of KIN, BAP, TDZ or GA_3 together with 0.25 IAA or 0.25 IBA. After four weeks of culture, the shoots in Petri plates were separated individually from the shoot clusters and transferred to glass jars (400 ml volume) containing 40 ml of MS medium for further growth and development for an additional three weeks. Irrespective of the medium composition, no additional rooting stage was required because almost all the regenerants produced a sufficient number of roots on their respective treatments.

For indirect shoot regeneration, leaf explants (~2.5 cm^2) were excised from 4-week-old stock plants and cultured on MS medium supplemented with NAA (1.0 or 2.0 mg/l) or 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0 or 2.0 mg/l) only or each combined with BAP (0.5 or 1.0, mg/l) for eight weeks. For each treatment, the leaf explants were cultured in two different orientations in such a way that either abaxial or adaxial surface is in contact with the agar. After callus production and subsequent shoot formation on the same medium, the regenerated shoots were transferred to 400 ml glass jars containing PGRs-free MS medium for further growth.

All *in vitro* cultures grown in disposable Petri plates and jars were maintained in a growth chamber at 24±1 °C under a 16/8 hours light/dark photoperiod provided by cool-white fluorescent light with an average of 2000 lux and at 60% relative humidity. The explants were checked daily during the first weeks for symptoms of contamination and thereafter weekly for the sign of growth and development.

After 3 weeks' culture initiation in jars, all shoots with a well-developed root system were randomly selected and carefully removed from the culture vessels, followed by washing their roots with sterile distilled water in order to remove the agar. They were then transferred to the plastic pots containing a mixture of soil, manure, moss, and sand at a ratio of 1:2:2:1. The Potted plantlets were covered with a transparent polythene membrane to ensure high humidity, which was opened after 10 days. The plantlets were maintained in a greenhouse at 25 ± 2 °C with low humidity (35–40%) and 16/8 hours

light/dark photoperiod. The survival efficiency, defined as the percentage of plantlets that survived the transfer from *in vitro* to *ex vitro* growth conditions, was determined three weeks after acclimatization. At the fourth week of acclimatization, the plantlets (~20 cm in height) were directly transferred to the field for further growth and fruit setting. Fruit formation having similar taste with mother plants was achieved 4 months after transferring to the field.

For data recording, all in vitro experiments were performed in 6 replicates, with 4 explants per Petri plate (replicate) and all the experiments were repeated three times. The mean shoot length of each treatment was recorded by measuring the distance (cm) between shoot-base and shoot-tip of 25 randomly selected plantlets. The mean number of leaves per regenerated shoot was determined by counting the leaves of 25 randomly selected plantlets from each treatment. Mean leaf area was measured from the photographs taken by FujiFilm camera (FujiFilm, China) and Adobe Photoshop CS4 was used to estimate the leaf area of third leaves collected from 25 randomly selected plantlets from each treatment (Bradshaw et al., 2007). The total biomass of plant regenerated from each treatment was recorded by measuring the fresh and dry weight of 25 randomly selected plantlets. The data concerning the mean number of leaves, shoots, roots, leaf area, and total biomass were recorded after four weeks' culture. In terms of callus formation, the frequency of explants producing callus was recoded after five weeks while the mean number of shoot formation from callus clump was determined after seven weeks of culture. Data obtained from tissue culture experiments were statistically analyzed by SPSS, Version 17.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to calculate the statistical significance of all data presented and mean ± standard error (SE) differing significantly were determined using Duncan's multiple range test at $P < 0.05$ level.

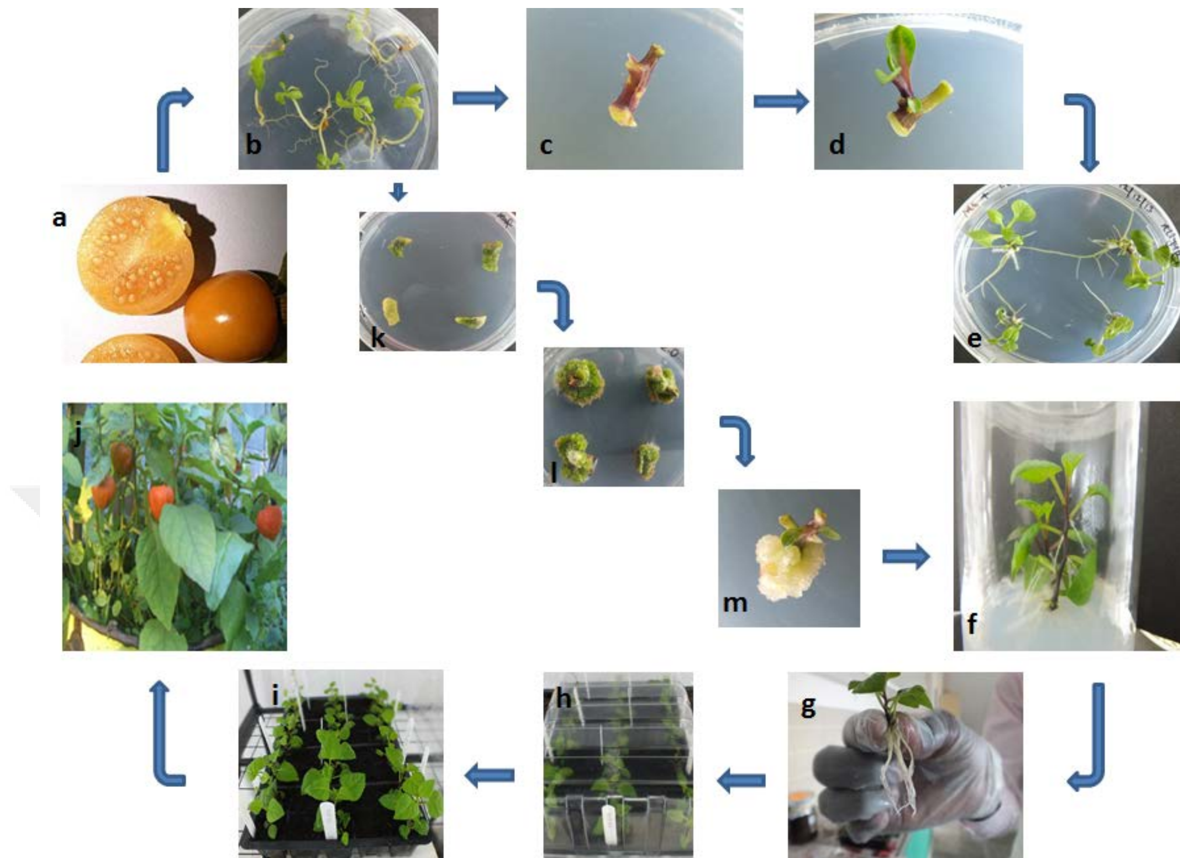

Figure 3.1. Steps of *in vitro* plantlet regeneration in *P. peruviana* via direct and indirect organogenesis: **a**) freshly cut fruits, **b**) seedlings developed from germinating seeds, **c**) nodal segments (5 mm) cultured on growth medium, **d**) shoot development from nodal explant after one week, **e**) more develop shoots after 4 weeks, **f**) rooted plantlets, **g**) well-developed shoot and root system before transferring into the soil, **h**) regenerants covered with transparent plastic cover after transferring to soil for acclimatization, **i**) acclimatized plantlets in greenhouse, **j**) fruit development from *in vitro*-derived regenerants in the field, **k**) leaf explants cultured on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP for callus induction, **l**) callus formation, and **m**) micro-shoot development on the same medium.

3.3 Results

Initial attempt to obtain sterile explants from sterile seedlings was successful due to high seed viability. The seed germination was observed after 10 days of culture and the seedlings obtained were used as explant donor for multiplication of stock plants. The germination rate of *P. peruviana* seeds was high (80%), giving rise to approximately 3 cm seedling with 3 nodes within 4 weeks.

In this study, two different methods of micropropagation were employed (i.e., direct and indirect regeneration) for shoot regeneration. To obtain direct shoot regeneration, two sets of experiments were carried out. In the first set, nodal explants excised from 4-weeks-old stock plants were incubated on MS medium supplemented with four different concentrations (0.1, 0.5, 1.0 or 2.0 mg/l) of PGRs (KIN, BAP, Zeatin, TDZ, GA3, IAA, IBA or NAA; Table 2.1) while in the second set, the nodal explants were incubated on MS medium containing either 0.5 mg/l (KIN, BAP, TDZ or GA_3) together with 0.25 IAA or 0.25 mg/l IBA. Of all the tested concentrations and combinations of PGRs, treatments containing TDZ were found more responsive in terms of shoot proliferation. The highest mean number of shoot formation was obtained on MS medium containing 0.5 mg/l TDZ with a mean of 6.5 shoots per explant within 4 weeks of culture (Table 2.1; Figure 2.2A). TDZ treatments also induced high shooting frequency (ranging from 91.3 to 100%). In addition, treatments containing BAP were also effective, producing shoots within a range of 3.9 to 4.4 shoots per cultured explant (Figure 2.2B). Following TDZ and BAP, zeatin treatments were also successful for shoot formation producing 2.0 to 2.9 shoots per explant with the highest shooting response of 100% at all tested concentrations (Figure 2.2C). However, among cytokinins tested (TDZ, KIN, BAP and zeatin), KIN was the least effective in terms of shoot organogenesis; producing 0.8 to 1.6 shoots per explant (Figure 2.2D). It was observed that all cytokinins tested were more effective at 0.5 mg/l concentration, indicating that, this concentration is the optimum concentration of phytohormones required by this plant. Therefore, any concentration below or above is considered as either insufficient or high for shoot organogenesis in this plant (Table 2.1).

On the other hand, treatments containing auxin (IAA, IBA or NAA) or GA_3 produced almost the same number of shoots, giving rise to 1.0-1.6 shoots per explant (Table 2.1; Figure 2.2E). Among these auxins used, NAA at higher concentration (1.0 and 2.0 mg/l) was observed to produce the least number of shoots (0.7 and 0.5) and with little callus at the lower cut of the explant. In addition, combinations of auxins (in this study IAA or IBA at 0.25 mg/l) with cytokinins (KIN, BAP, or TDZ) or GA_3 caused some inhibitory effect on shoot formation, especially when combined with TDZ, sharply decreasing the number of shoots (from 6.5 to 2.8 or 2.5 shoots). Although shoot formation was noticed on control medium (PGR-free MS), the mean number of shoot production was much lower (0.8 shoot per explant), with the lowest shooting response (46.3%; Figure 2.2F).

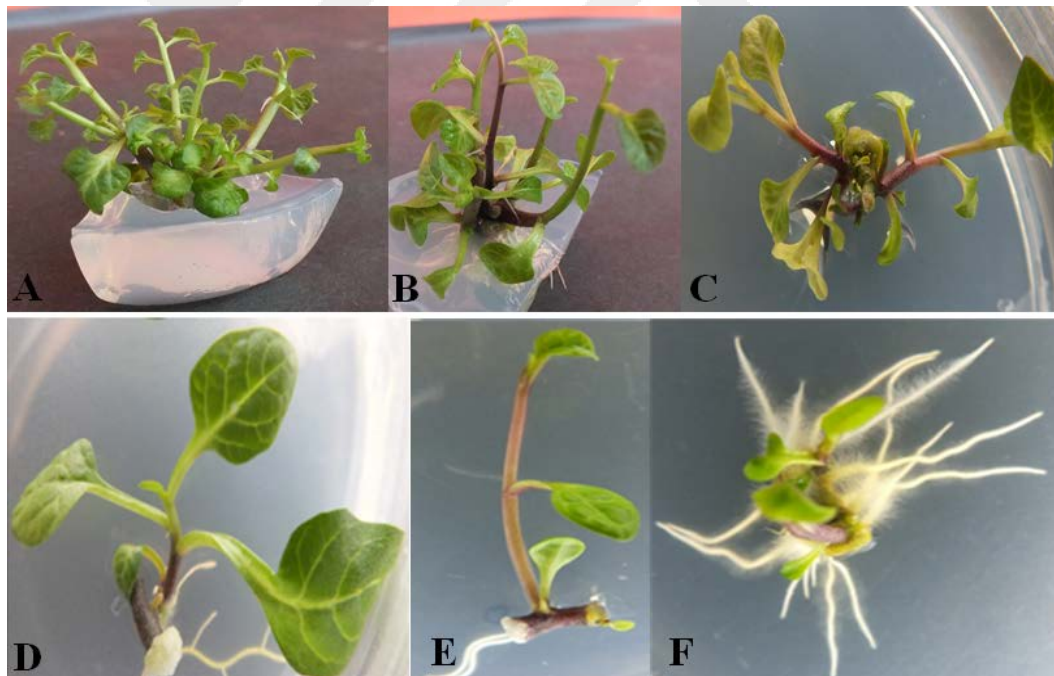

Figure 3.2. Effect of plant growth regulators on organogenesis: multiple shoots development from nodal explant on MS medium containing 0.5 mg/l TDZ (**A**); multiple shoot development on MS medium containing 0.5 mg/l BAP (**B**); double shoots formation on MS medium containing 0.5 mg/l zeatin (**C**); single shoot regeneration with expanded leaves on MS medium containing 0.5 mg/l kinetin (**D**); elongated shoot (4.6 cm) with less and smaller leaves on MS medium containing 0.5 mg/l GA_3 (**E**); and more roots development on PGR-free MS medium (**F**).

Table 3.1. Effects of different concentrations and combinations of plant growth regulators on shoot and root formation in *P. peruviana* after four weeks' culture. Mean numbers with the same letter within the same column are not significantly different at P>0.05.

PGRs	Concentration (mg/l)	Mean number of shoots per explant	Frequency of shoot formation (%)	Mean number of roots per explant	Frequency of root formation (%)
MS (control)	-	0.8 ± 0.1^{k}	46.3	5.0 ± 0.2^{g}	55.2
KIN	0.1	0.8 ± 0.2^{k}	66.7	3.8 ± 0.2^{i}	66.7
	0.5	1.6 ± 0.2^{i}	100.0	3.6 ± 0.3^{i}	86.7
	1.0	1.4 ± 0.1^{ij}	87.5	2.6 ± 0.2^{k}	93.8
	2.0	1.3 ± 0.3^{ij}	75.0	2.3 ± 0.2^{k}	75.0
BAP	0.1	4.2 ± 0.2^{cd}	81.3	1.5 ± 0.1^{l}	62.5
	0.5	4.4 ± 0.1^{c}	93.8	1.4 ± 0.2^{l}	26.7
	1.0	3.9 ± 0.2^{e}	81.3	1.2 ± 0.2^{l}	25.0
	2.0	3.9 ± 0.1^{e}	81.3	1.0 ± 0.1^{l}	16.3
Zeatin	0.1	2.1 ± 0.1^{h}	100.0	3.2 ± 0.2^{j}	93.8
	0.5	2.9 ± 0.1^{f}	100.0	2.9 ± 0.1^{j}	83.3
	1.0	2.4 ± 0.1^{gh}	100.0	2.2 ± 0.2^{k}	87.5
	2.0	2.0 ± 0.2^{h}	100.0	2.3 ± 0.2^{k}	93.8
TDZ	0.1	4.1 ± 0.2^{de}	97.5	3.6 ± 0.2^{i}	62.5
	0.5	6.5 ± 0.1^{a}	100.0	3.5 ± 0.2^{i}	86.7
	1.0	5.4 ± 0.2^{b}	96.3	3.2 ± 0.2^{i}	62.5
	2.0	5.3 ± 0.2^{b}	91.3	2.4 ± 0.1^{k}	60.3
GA_3	0.1	1.6 ± 0.1^{i}	62.5	1.5 ± 0.1^{l}	56.3
	0.5	1.3 ± 0.1^{ij}	68.8	1.4 ± 0.1^{l}	25.0
	1.0	1.4 ± 0.1^{ij}	68.8	1.1 ± 0.1^{l}	18.8
	2.0	1.1 ± 0.2^{j}	77.5	1.1 ± 0.1^{l}	12.5
IAA	0.1	1.2 ± 0.2^{j}	68.8	8.1 ± 0.2^{b}	100.0
	0.5	1.1 ± 0.2^{j}	81.3	8.3 ± 0.3^{b}	100.0
	1.0	1.3 ± 0.1^{j}	56.3	8.9 ± 0.2^{a}	100.0
	2.0	1.5 ± 0.2^{i}	50.0	9.0 ± 0.3^{a}	100.0
IBA	0.1	1.1 ± 0.2^{j}	75.0	6.2 ± 0.4^{f}	95.0
	0.5	1.3 ± 0.2^{ij}	75.1	6.8 ± 0.2^{e}	98.3
	1.0	1.2 ± 0.1^{j}	75.0	6.9 ± 0.2^{e}	97.8
	2.0	1.0 ± 0.1^{j}	74.0	7.4 ± 0.3^{d}	97.5
NAA	0.1	1.0 ± 0.1^{j}	62.5	8.2 ± 0.6^{b}	100.0
	0.5	1.1 ± 0.1^{j}	68.8	7.9 ± 0.3^{bc}	87.5
	1.0	0.7 ± 0.1^{k}	51.3	4.4 ± 0.4^{gh}	41.3
	2.0	0.5 ± 0.1^{k}	50.0	4.2 ± 0.2^{h}	37.5
0.5 KIN + 0.25 IAA		1.2 ± 0.3^{ij}	86.7	4.6 ± 0.4^{gh}	86.7
0.5 BAP + 0.25 IAA		2.4 ± 0.3^{gh}	93.3	3.0 ± 0.2^{j}	86.7
0.5 TDZ + 0.25 IAA		2.8 ± 0.2^{fg}	96.7	4.0 ± 0.8^{hi}	86.7
0.5 GA_3 + 0.25 IAA		1.1 ± 0.1^{ij}	86.7	2.8 ± 0.4^{jk}	86.7
0.5 KIN + 0.25 IBA		1.2 ± 0.3^{ij}	86.7	4.3 ± 0.2^{h}	80.0
0.5 BAP + 0.25 IBA		2.2 ± 0.3^{h}	93.3	2.9 ± 0.2^{jk}	86.7
0.5 TDZ + 0.25 IBA		2.5 ± 0.1^{g}	93.3	4.2 ± 0.6^{h}	73.3
0.5 GA_3 + 0.25 IBA		1.1 ± 0.3^{ijk}	80.0	2.6 ± 0.5^{jk}	73.3

Table 3.2. Effects of different concentrations and combinations plant growth regulators on shoot length, number of leaves and leaf area in *P. peruviana* after four weeks' culture. Mean numbers with the same letter within the same column are not significantly different at P>0.05.

PGRs	Concentration (mg/l)	Mean number of		
		Leaves per shoot	Leaf Area (cm^2)	Shoot Length (cm)
MS (control)	-	4.1 ± 0.3^{ghi}	17.8 ± 1.5	2.0 ± 0.1^{e}
KIN	0.1	7.2 ± 0.4^{cd}	19.4 ± 2.9	2.5 ± 0.3^{cd}
	0.5	7.4 ± 0.1^{c}	16.4 ± 1.1	2.5 ± 0.1^{c}
	1.0	5.7 ± 0.2^{e}	15.0 ± 1.8	2.8 ± 0.2^{c}
	2.0	5.6 ± 0.3^{e}	15.6 ± 1.1	2.4 ± 0.2^{cd}
BAP	0.1	8.9 ± 0.2^{a}	7.50 ± 1.1	2.3 ± 0.2^{cde}
	0.5	8.8 ± 0.2^{a}	6.30 ± 2.4	2.2 ± 0.1^{de}
	1.0	8.1 ± 0.1^{b}	6.30 ± 2.2	2.7 ± 0.2^{c}
	2.0	8.5 ± 0.2^{a}	6.00 ± 0.5	2.6 ± 0.2^{cd}
Zeatin	0.1	4.4 ± 0.2^{gh}	5.00 ± 0.9	2.1 ± 0.2^{de}
	0.5	5.1 ± 0.2^{f}	6.80 ± 2.1	2.5 ± 0.1^{c}
	1.0	4.8 ± 0.3^{fg}	6.50 ± 0.9	2.4 ± 0.1^{cd}
	2.0	5.2 ± 0.2^{f}	5.60 ± 1.1	2.6 ± 0.3^{c}
TDZ	0.1	6.7 ± 0.2^{d}	15.0 ± 1.6	2.6 ± 0.4^{c}
	0.5	6.8 ± 0.2^{cd}	16.2 ± 1.4	2.4 ± 0.1^{cd}
	1.0	7.3 ± 0.3^{c}	13.5 ± 0.7	2.4 ± 0.2^{cd}
	2.0	7.4 ± 0.1^{c}	15.8 ± 0.3	2.6 ± 0.2^{c}
GA_3	0.1	2.8 ± 0.2^{jk}	4.10 ± 1.0	3.4 ± 0.3^{b}
	0.5	3.2 ± 0.2^{j}	4.70 ± 3.2	4.5 ± 0.5^{a}
	1.0	2.8 ± 0.1^{k}	3.30 ± 0.5	4.5 ± 0.3^{a}
	2.0	3.4 ± 0.2^{j}	3.40 ± 0.4	4.6 ± 0.4^{a}
IAA	0.1	5.5 ± 0.1^{e}	16.4 ± 2.5	2.8 ± 0.1^{c}
	0.5	4.6 ± 0.1^{g}	15.7 ± 1.3	2.8 ± 0.2^{c}
	1.0	4.9 ± 0.2^{fg}	16.1 ± 1.4	2.7 ± 0.3^{c}
	2.0	5.4 ± 0.1^{e}	11.4 ± 0.7	2.6 ± 0.2^{c}
IBA	0.1	4.0 ± 0.1^{i}	5.50 ± 1.3	2.9 ± 0.2^{bc}
	0.5	4.1 ± 0.1^{i}	6.80 ± 1.1	2.5 ± 0.1^{c}
	1.0	4.1 ± 0.2^{i}	8.80 ± 0.8	2.5 ± 0.1^{c}
	2.0	4.0 ± 0.1^{i}	7.80 ± 1.1	2.9 ± 0.2^{bc}
NAA	0.1	4.4 ± 0.1^{gh}	6.20 ± 0.8	2.0 ± 0.1^{e}
	0.5	4.4 ± 0.1^{gh}	7.50 ± 0.6	2.7 ± 0.4^{bc}
	1.0	4.2 ± 0.1^{hi}	8.90 ± 0.4	2.5 ± 0.2^{c}
	2.0	4.1 ± 0.1^{i}	7.20 ± 0.7	2.4 ± 0.1^{cd}
0.5 KIN + 0.25 IAA		6.4 ± 0.3^{d}	9.90 ± 0.5	2.0 ± 0.2^{de}
0.5 BAP + 0.25 IAA		7.8 ± 0.5^{abc}	7.50 ± 0.1	2.2 ± 0.2^{cd}
0.5 TDZ + 0.25 IAA		6.8 ± 0.3^{cd}	6.90 ± 0.2	2.4 ± 0.2^{cd}
0.5 GA_3 + 0.25 IAA		4.4 ± 0.3^{ghi}	3.90 ± 0.8	4.3 ± 0.4^{a}
0.5 KIN + 0.25 IBA		5.6 ± 0.3^{e}	9.20 ± 0.1	2.1 ± 0.2^{de}
0.5 BAP + 0.25 IBA		6.3 ± 0.3^{d}	6.00 ± 0.2	2.1 ± 0.2^{de}
0.5 TDZ + 0.25 IBA		7.2 ± 0.3^{c}	7.10 ± 0.3	2.5 ± 0.4^{cde}
0.5 GA_3 + 0.25 IBA		4.6 ± 0.2^{g}	3.10 ± 0.6	4.1 ± 0.5^{ab}

In terms of root organogenesis, auxin-containing treatments were found more productive resulting in higher numbers of root formation. Of the tested auxins, IAA was the most effective for root induction in this plant, which resulted in a mean of 8.1 to 9.0 roots per cultured explant with a 100% rooting frequency (Table 2.1; Figure 2.3A). IBA was also effective for adventitious root formation producing 6.2 to 7.4 roots per explant and with a high frequency of rooting response (ranging from 95.0 to 98.5%; Figure 2.3B). Similarly, NAA also induced high rooting at low concentration (0.1 and 0.5 mg/l); however, at high concentration callus and root hair formation were predominant (Figure 2.3C). It was also observed that the number of roots increased with increase in concentrations of auxins, except for NAA-containing medium in which the reverse was the case. In addition, other PGRs including KIN, TDZ and zeatin also produced considerable amount of roots; however, root formation decreased with increasing concentrations of these PGRs (Table 2.1; Figure 2.3D, E). Of all the treatments, media containing BAP or GA_3 alone were found inefficient for rooting, producing a single root per explant with a maximum rooting frequency of 62.5% and 56.3%, respectively. When BAP or GA_3 concentration was increased from 0.1 to 2.0 mg/l, the percentage of explants forming roots decreased (from 62.5% to 16.3% for BAP and from 56.3% to 12.5% for GA_3). Likewise, the mean number of roots also decreased with increasing concentrations of these PGRs (Table 2.1).

The effects of auxins (IAA or IBA) when combined with other PGRs were found to be promising for root formation. For example, while MS medium containing 0.5 mg/l GA_3 produced 1.4 roots, 0.5 mg/l GA_3 in combination with 0.25 mg/l IAA produced twice more roots (a mean of 2.8 roots) with a rooting frequency of 86.7%. Similarly, the number of roots also increased from 1.4 to 3.0 roots once 0.5 mg/l BAP was combined with 0.25 mg/l IAA or from 1.4 to 2.9 when combined with 0.25 mg/l IBA (Table 2.1). For the control group, in spite of having low shooting response, it was found highly effective for root formation, producing up to 5.0 roots per explant after four weeks of culture (Figure 2.2F). Nevertheless, the frequency of explant forming roots in control groups was lower than treatments having 0.25 mg/l IAA or IBA. Due to the effectiveness of all treatments including the control group as regards to root formation,

no additional rooting stage was needed, since almost all the regenerated shoots produced a sufficient number of roots for acclimatization on their respective treatments.

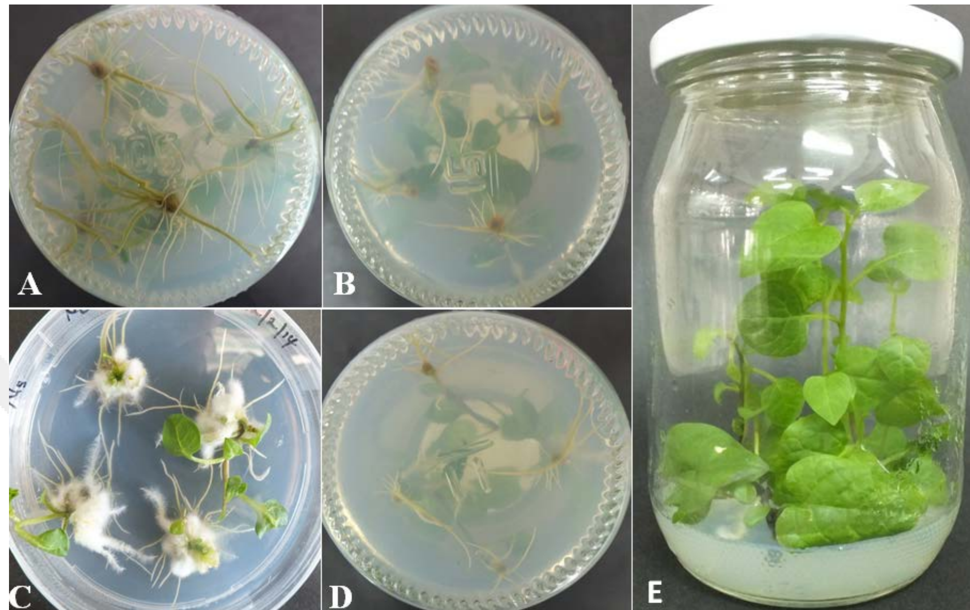

Figure 3.3. Adventitious root development from *P. peruviana* cultured on MS medium containing different concentrations of PGRs: multiple root production on MS medium supplemented with IAA (**A**); root formation on MS medium supplemented with IBA (**B**); multiple root production together with root hairs on MS medium supplemented with NAA (**C**); root development on MS medium supplemented with TDZ (**D**); and further shoot and root development (**E**).

For shoot size, there was no significant difference between the treatments except for those containing GA_3, which promoted higher internode elongation than others. The longest shoot length (4.6 cm) was obtained from MS medium containing 2.0 mg/l GA_3 while producing less number of leaves, which were also small in size (Table 2.2). This finding revealed that there was an inverse relationship between leaf number and shoot length of the plantlets when GA_3 was used (Figure 2.4A). The highest leaf number was obtained from BAP containing treatments followed by TDZ and KIN treatments. However, there was no significant difference observed in treatments containing auxins (IAA, IBA or NAA) or zeatin in terms of the mean leaf number (ranging from 4.0 to 5.5). In addition to number of leaves produced, KIN, TDZ and IAA treatments also produced leaves which were larger in size compared to other treatments. The largest leaf

area was obtained on MS medium containing 0.1 mg/l KIN, developing the leaf size up to 19.4 cm^2 within four weeks of culture (Figure 2.4B). The smallest leaf area was observed on MS medium containing GA_3 at all concentrations. For the other treatments, there was no considerable difference observed regarding leaf expansion.

Figure 3.4. Morphological differences: plantlets regenerated on MS medium containing GA_3 (**A**) or KIN (**B**).

An observation concerning the effects of the tested plant growth regulators on total biomass accumulation of *in vitro* regenerated goldenberry revealed that treatments containing BAP promoted high biomass accumulation in fresh samples followed by IAA, TDZ and KIN as compared to control groups (Figure 2.5). However, the total fresh weight of plantlets regenerated on media containing IBA, NAA or GA_3 was lower compared to control plants. Moreover, combinations of 0.5 mg/l KIN, BAP, TDZ or GA_3 with 0.25 mg/l IAA or IBA decreased the total fresh weight of this plant. Regarding total dry weight, except for treatments containing GA_3 which resulted in lower dry weight, there was no significant difference between the plantlets regenerated on medium supplemented with PGRs and control (Figure 2.5).

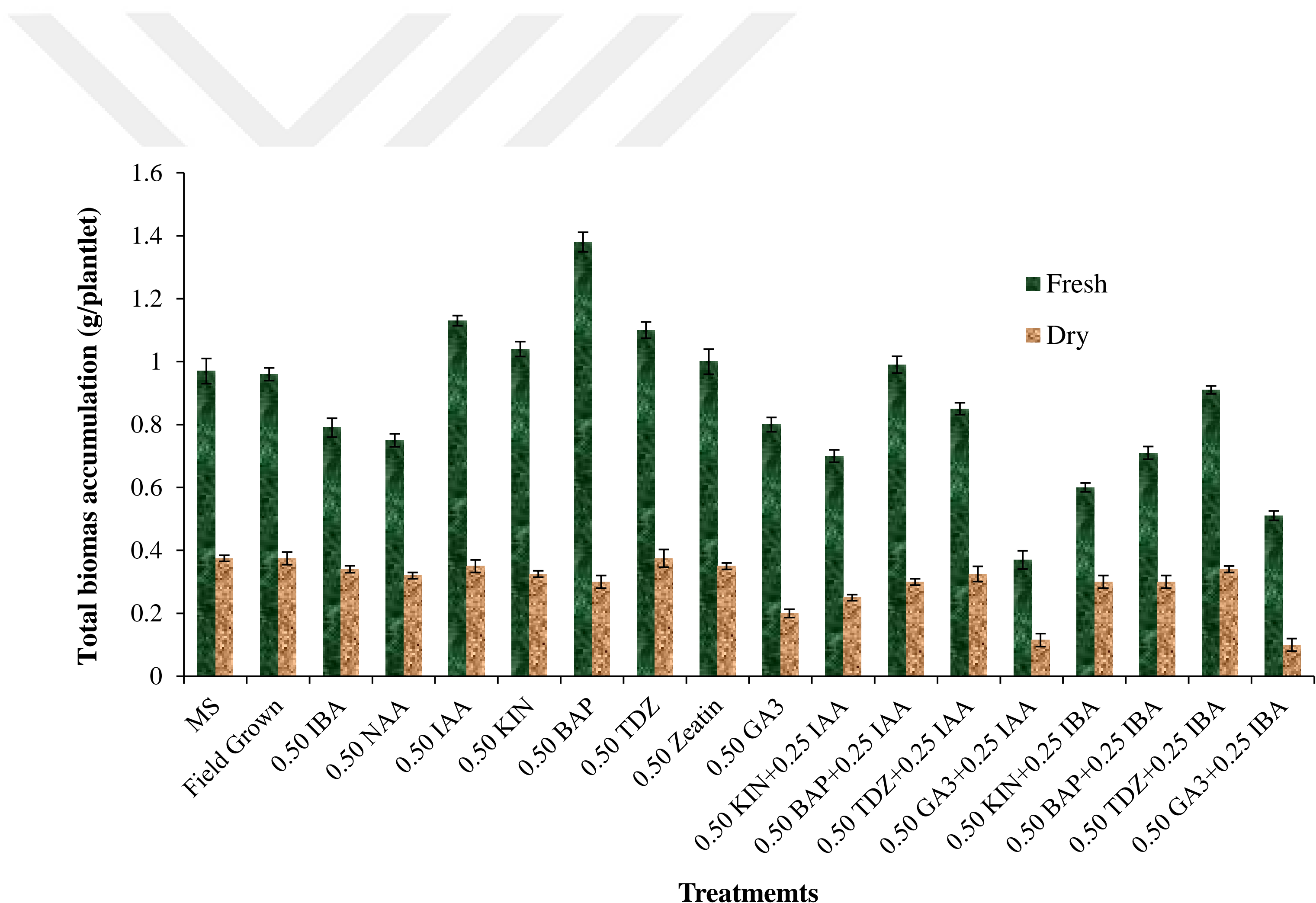

Figure 3.5. Effect of plant growth regulators on total biomass accumulation of *in vitro* regenerated plantlets collected after four weeks of culture.

For the achievement of callus induction and subsequent plant regeneration from the callus clumps, it appears that NAA and BAP combinations were more productive in comparison with other treatments (Table 2.3). The highest frequency of callus formation (100%) was achieved on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP (Figure 2.6A). However, no callus formation was noticed on media containing either NAA or 2,4-D alone, instead, necrosis occurred (Figure 2.6B). On the other hand, shoot regeneration was also achieved on the same medium without subculturing (Figure 2.6C, D). The highest mean number of shoots was also obtained on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP (Figure 2.6D). Regardless of NAA or 2,4-D concentration, an increase in BAP concentration decreased the frequency of callus induction as well as the number of shoots produced per callus clump (Table 2.3).

Table 2.3. Effects of NAA or 2,4-D alone or in combination with BAP on indirect organogenesis from leaf explant of *P. peruviana*. Mean numbers with the same letter within the same column are not significantly different at $P>0.05$.

Treatments (mg/l)		**Frequency of callus induction**	**Mean number of shoots per callus clump**
MS (control)	-	0.0	0.0 ± 0.0
NAA	**BAP**		
1.0	0.0	0.0	0.0 ± 0.0
2.0	0.0	0.0	0.0 ± 0.0
1.0	0.5	96.1	4.3 ± 0.3^{bc}
2.0	0.5	100.0	5.7 ± 0.5^{a}
1.0	1.0	95.3	4.1 ± 0.1^{c}
2.0	1.0	98.7	4.6 ± 0.4^{b}
2,4-D	**BAP**		
1.0	0.0	0.0	0.0 ± 0.0
2.0	0.0	0.0	0.0 ± 0.0
1.0	0.5	76.4	2.3 ± 0.0^{d}
2.0	0.5	87.0	2.1 ± 0.6^{d}
1.0	1.0	75.3	1.2 ± 0.4^{e}
2.0	1.0	79.0	1.5 ± 0.2^{e}

All the regenerants that performed normal growth and appearance were singled out and then successfully acclimatized under non-axenic conditions in a greenhouse for 4 weeks before transferring to the field. More than 90% of the plantlets survived after being transferred to the soil. Plantlets (~20 cm in height) were directly transferred to the field for fruit setting. Fruit formation having similar taste and appearance to the mother plants was achieved (Figure 2.7, 2.8).

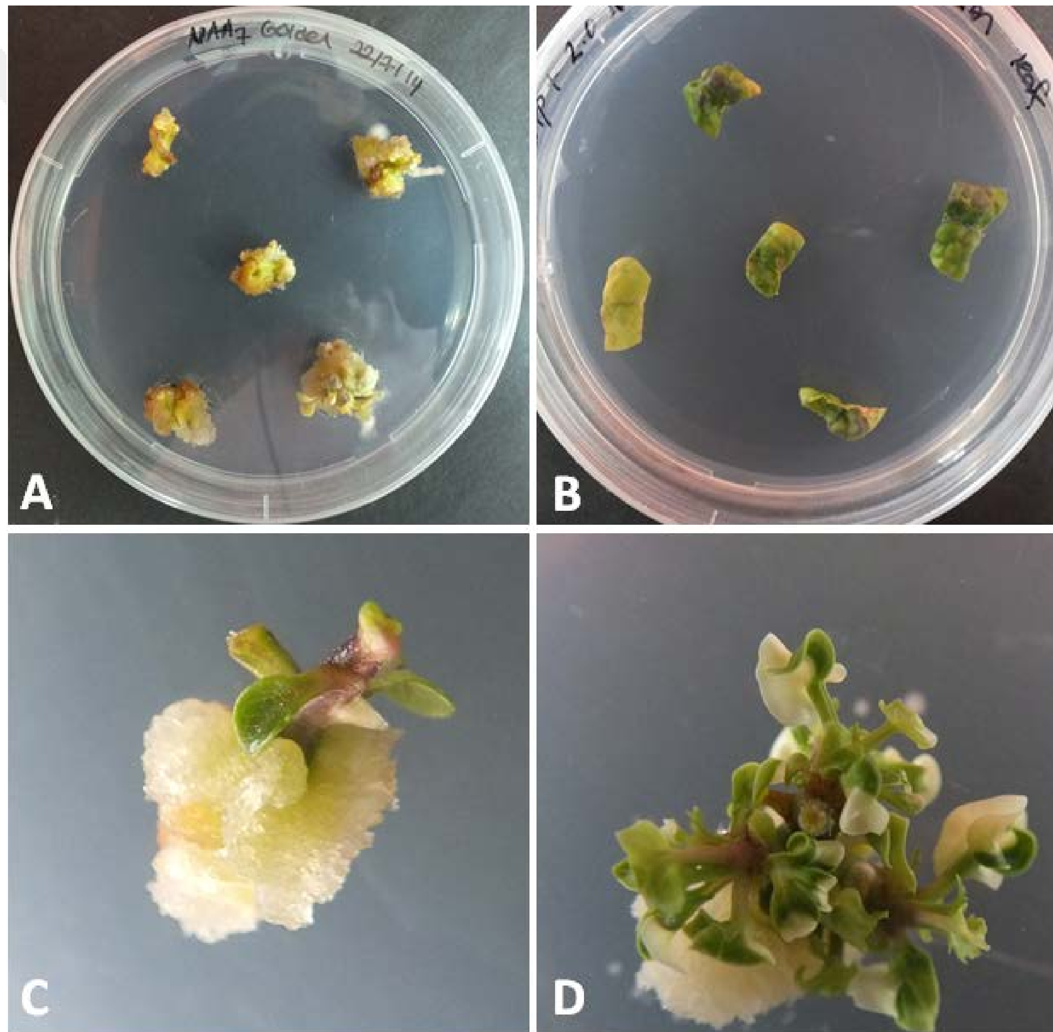

Figure 3.6. Callus induction and subsequent plantlet regeneration from leaf explants of *P. peruviana*: callus formation on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP after five weeks of culture (**A**); leaf explants cultured on MS with 2.0 mg/l NAA alone (**B**); single shoot formation on MS medium containing 2.0 mg/l 2,4-D and 0.5 mg/l BAP after 7 weeks (**C**); and multiple shoots production on MS medium containing 2.0 mg/l NAA and 0.5 mg/l BAP after 7 weeks (**D**).

Figure 3.7. Regenerated shoots cultured in glass jars in a growth chamber at 24±1 °C under 16/8 hours light/dark photoperiod provided by cool-white fluorescent light having an average of 2000 lux, and at 60% relative humidity.

Figure 3.8. Fruit production from *in vitro*-derived plants in the field.

3.4 Discussion

Plant tissue culture techniques offer an effective solution for true-to-type plant propagation compared to the time consuming and cumbersome conventional techniques such as grafting, air layering, stooling etc. Hence, regeneration capacity of *P. peruviana* under *in vitro* conditions was investigated ensuring the preservation of genetically identical plant materials, protection of explants against environmental factors including pests and diseases, etc. Although *Physalis peruviana* is an important medicinal plant species, there are no detailed regeneration studies involving different concentrations and combinations of plant growth regulators. Therefore, this study aimed to develop simple and efficient *in vitro* propagation system for high-frequency regeneration of goldenberry through adventitious organogenesis and thus examined the effects of several concentrations and combinations of plant growth regulators.

Even though earlier studies showed successful regeneration in different *Physalis* species from callus cultures including *P. minima* (Bapat and Rao, 1977; Sipahimalani et al., 1981; Sheeba et al., 2010) and *P. pubescens* (Rao et al., 2004), there are only few reports on *in vitro* regeneration of *P. peruviana*. Of those, Bapat and Schieder (1981) developed a protoplast culture in several *Physalis* species including *P. peruviana*, however, none of them resulted in shoot formation at all. Instead, protoplasts isolated from *P. peruviana* resulted in callus formation on MS medium supplemented with a combination of 1.0 mg/l 2,4-D and BAP. Similarly, Torres (1991) reported some difficulties in terms of organogenic response in tissue cultures studies, addressing the recalcitrant pattern of leaf, stem and root explants of this species. Recently, Rodrigues et al. (2013b) established a regeneration system using nodal explants on different concentrations of BA without testing synergistic effects of other PGRs. In general, shoot tips and nodal segments are known as the best explants for *in vitro* propagation of most plants species without any intervening callus formation (Otroshy et al., 2013). Thus, it is still a major challenge performing efficient regeneration protocol for this crop. In contrast to these shortcomings, this study was focused on rapid clonal regeneration system and development of very simple protocol regarding micropropagation of this plant.

In this finding, TDZ was found most effective PGR concerning direct shoot organogenesis from nodal explants of goldenberry, inducing more shoot formation when used at 0.50 mg/l in MS medium. The promoting effect of TDZ has been reported for many economically important plant species (Yucesan et al., 2007; Kumar and Reddy, 2012; Lata et al., 2013). The effectiveness of TDZ on shoot formation in several tissue culture studies might be attributed to its stability (not degradable) and high persistence in the culture medium, demonstrated by a carbon isotope study (Mok and Mok, 1985). Due to this high stability, Huetteman and Preece (1993) reported that low concentrations of TDZ have been found to be useful for micropropagation whereas higher concentrations induce callusing in woody plants. It was also proposed that TDZ increases the levels of nucleosides (Laloue and Pethe, 1982), or the accumulation and synthesis of purine cytokinins as well as promoting the conversion of adenine to adenosine (Capelle et al., 1983), which may be needed to trigger initiation of shoot meristems proliferation (Faisal et al., 2014). Based on previous reports on the effect of TDZ, it is noteworthy to say that the synergistic effect of TDZ and endogenous auxins might have also played a critical role in altering the auxin:cytokinin ratio within plant tissue and eventually enhancing axillary regeneration. Unlike natural cytokinins, TDZ has the potential to fulfill some actions of both auxins and cytokinins at different regenerative pathways (Guo et al., 2011). Therefore, it can be suggested that the use of TDZ alone was sufficient not only for axillary regeneration but also for root formation in goldenberry. This result is in consistence with the finding of Yucesan et al. (2007) that TDZ was more effective than BAP, KIN and zeatin for shoot regeneration in *Cichorium intybus* L. In spite of this high popularity of TDZ, its exact biological role has been still a mystery due to its metabolic roles including primary signaling event, storage, and passage of endogenous plant signals which may or may not be established as organogenesis (Guo et al., 2011).

The results of these experiments further showed that the rate of shoot or root proliferation was not only influenced by the types of plant growth regulators but also the concentrations. Therefore, using TDZ at moderate concentrations is more favorable for shoot organogenesis. Among cytokinins tested, BAP application also appeared to be effective for shoot proliferation even though resulted in weak rooting response. In accordance with this finding, Farhana et al. (2009) also observed that the presence of

BAP in a medium is important for shoot proliferation in *P. minima*. Hence, tissue culture protocol based on the use of BAP for *P. peruviana* regeneration is cost effective for shoot induction. In relation to cytokinin effect, it is worth noting that BAP was ineffective for root organogenesis. Otroshy et al. (2013) also reported a similar result concerning the negative effect of BAP on rhizogenesis.

On the other hand, when shoot growth is taken into account, GA_3 played a crucial role growing the shoots up to 4.6 cm, but less effective in terms of leaf number. Likewise, small leaflets were predominant pattern in the presence of GA_3 in the medium. This might be due to the negative influence of fast internodes elongation while retarding the leaf number and size (Bostrack and Struckmeyer, 1967). As for root formation, it is known that GA_3 in tissue culture mimics like an inhibitor during rhizogenesis (Sutter, 1996; Yıldırım and Turker, 2009), as seen in this present work producing single root per explant with a frequency of 56.3% explants forming roots. Due to these negative effects on leaf length, leaf number, root formation and biomass accumulation, all advantages of fast growth mediated by gibberellins were taken away in regeneration system of goldenberry.

Furthermore, all the auxins used produced less number of shoots, and also reduce the shooting response of cytokinins when used together. This inhibitory effect of auxins on multiple shoot regeneration has been previously demonstrated in many plants (Gulati and Jaiwal, 1992; Khalafalla and Hattori, 2000; Abdellatef and Khalafalla, 2007). Nevertheless, the rooting effect of all the PGRs used, IAA was found more effective. Similar results were also reported by many authors that presence of auxins (especially IAA) in a medium is crucial for root induction in *Physalis* species (Farhana et al., 2009; Sheeba et al., 2010). Auxin is usually considered necessary for the attainment of meristematic competence of the responsive cells. However, once this competence has been established, high auxin concentrations were often found to have an inhibitory effect on adventitious root development (Gurel and Wren, 1995). Therefore, using auxin in an optimum concentration is preferable in order to avoid its inhibitory effect and callus formation on cultured tissue. In contrast, PGR-free MS medium was also found effective regarding root formation.

In this experiment, all PGRs tested and their respective combinations clearly show that clonal propagation of *P. peruviana* is plausible via axillary regeneration using nodal explants. Unlike nodal segments, root or stem segments were not effective for *in vitro* regeneration of goldenberry (data not shown), as also mentioned by Torres (1991) due to the recalcitrancy of aforesaid tissues under *in vitro* conditions. Therefore, these findings show that variability of physiological responses including shoot and root formation were dependent on specific interactions of plant growth regulators and explant source (Rout et al., 2000; Gurel et al., 2011; Gurel and Gurel, 2014).

The highest percentage of callus induction took place in the treatments that include NAA and BAP. When either of the auxins (NAA or 2,4-D) was used alone no callus formation was noticed; hence, addition of BAP is required for improved response. However, 2,4-D and BAP combinations also induced callogenesis, the yields was not satisfactory, particularly at low concentrations of BAP. The results clearly indicated that appropriate amounts of auxin (NAA) and lower concentration of cytokinin (BAP) are crucial for callus induction in this plant. Ilahi et al. (2007) also reported the effect of NAA and BAP combination for embryonic callus formation in *Chrysanthemum*. More recently, Seyyedyousefi et al. (2013) described a similar result while working with *Alstroemeria*, indicating that 0.5 mg/l of BAP and 2.0 mg/l of NAA induced more callus compared to other treatments. From present results and literature review, it is evident that exogenous auxin is the most important growth regulator for the induction of callus in the majority of plants. Besides, as observed in the present study, many species require supplementary cytokinin along with auxin for optimum response and in some cases to prevent necrosis of callus. This requirement for exogenous cytokinin could be related to the maintenance of a proper balance between auxin and cytokinin, which act synergistically to regulate cell division (Johri and Mitra, 2001), a process essential for callus formation. This synergism also has a vital role in controlling the meristematic cells within the explants towards a particular development pathway. Therefore, callus induction is highly influenced not only by types of plant growth regulators but also concentrations.

The present study is the first report describing a simple and efficient *in vitro* regeneration protocol for *P. peruviana* using both direct and indirect organogenesis.

Therefore, this protocol is expected to contribute to the future efforts for large scale production of secondary metabolites from *in vitro* propagated materials as well as genetic manipulation studies in *P. peruviana*. Micropropagation of this plant can also provide a mass production of pesticide-, herbicide- and disease-free material on a commercial scale and unlimited plant materials can consistently be obtained throughout the whole year. Moreover, this protocol can provide plant material for future pharmacological, physiological and biochemical studies. Hence, plant tissue culture is a good alternative for commercial propagation of a large number of plant species, including many medicinal plants (Rout et al., 2000).

CHAPTER III

4 SYNTHETIC SEED PRODUCTION

4.1 Introduction

Although plants are propagated traditionally through seed, propagation using seed is usually season-dependent. Therefore, in order to provide adequate plant material at the industrial level, there is a need for an incessant supply of plant throughout the year, a good way of storage and better means of transporting healthy plant materials. To achieve these, considerable researches in the area of plant biotechnology has provided a greater opportunity for the improvement of many plant species including ornamental, medicinal and other economically important crops. In this perspective, encapsulation technology may be a useful alternative for conservation and germplasm exchange since this technology has opened new understandings in the field of agriculture and plant biotechnology.

This technology started from Haberlandt's early hypothesis in 1902, of artificial embryo cultivation to the concept proposed by Murashige (1977), artificial seeds have evolved from a futuristic idea into a real field of experimental research. The term "artificial seed", which was first suggested by Murashige, is now known by different names such as "manufactured seed", "synthetic seed" or "synseed". The original definition of an artificial seed, as proposed by Murashige (1978), was "an encapsulated single somatic embryo", that is a clonal product that could mimic real seed for transport, storage, sowing and would eventually grow either *in vitro* or *ex vitro* into an intact plantlet (conversion). Gray and Purohit (1991) also defined synthetic seed as "a somatic embryo that is engineered for the practical use in commercial plant production".

Thus, synthetic seed production was previously restricted to those plants in which somatic embryogenesis had been documented whereas many plant species remain recalcitrant to somatic embryogenesis (Verma et al., 2010). In recent years, the encapsulation of non-embryogenic vegetative propagules such as axillary buds, apical shoot buds, nodal segments, protocorm-like bodies, embryogenic or organogenic callus etc. has also been employed as a suitable alternative to somatic embryos, provided that it can imitate as seed during storage, sowing and possesses the ability to convert into an intact plant under *in vitro* or *ex vitro* conditions (Ara et al., 2000; Chand and Singh, 2004; Naik and Chand, 2006; Micheli et al., 2007; Rai and Jaiswal, 2008; Rai et al., 2008a,b, 2009; Sharma et al., 2009; West and Preece, 2009; Ahmad and Anis, 2010; Verma et al., 2010; Ozudogru et al., 2011). For instance, Verma et al. (2010) proposed synthetic seed by encapsulating shoot tips of *Solanum nigrum* L. as an alternative to somatic embryo.

Generally, *in vitro*-derived plant materials are used in most synthetic seed experiments for encapsulation, it is also possible to encapsulate plant materials excised directly from *ex vitro* cultivated plants. Pattnaik et al. (1995) successfully encapsulated the quiescent vegetative buds of *ex vitro*-grown mulberry tree. Recently, Banerjee et al. (2012) produced synthetic seed containing young vegetative micro-shoots together with a small basal rhizome segment excised from *in vivo*-derived rhizomes of *Curcuma amada*.

Over the past few years, encapsulation technology has attracted the interest of many researchers for germplasm delivery and for various analytical studies (Ara et al., 2000). This technology is highly promising for the conservation and management of seedless plant species, endangered plants, sterile unstable genotypes, elite genotypes, rare valuable hybrids, transgenic plants which seeds are either not viable or that require some fungal association for their germination as in the case of orchids and plant lines that are difficult to propagate using conventional methods (Singh et al., 2006; Rai et al., 2009). Saiprasad (2001) described the applications, prospects and limitations of synthetic seed technology while Rai et al. (2009) reported a brief overview of synthetic seed technology in several industrial fruit plants. However, there are some basic obstacles associated with this technology such as lack of a natural endosperm, the

absence of resting phase, inability to undergo dehydration and lack of protective coat which makes them inconvenient to handle and store (Redenbaugh et al., 1993). All these limit the utilization of somatic tissues as a source of synthetic seed production. Nonetheless, advancement in this technology has been extended by various research groups using several plant species including fruits, cereals, vegetables, orchids, medicinal plants, forest trees and other ornamentals plants to overcome these problems (Ipekci and Gozukirmizi, 2003; Utomo et al., 2008; Rai and Jaiswal, 2008; Rai et al., 2008a, b, 2009; Verma et al., 2010; Yucesan et al., 2014). Based on the literature available to date, synthetic seeds are classified into two categories; desiccated synthetic seeds and hydrated synthetic seeds.

Desiccated synthetic seeds are produced naked or polyethylene glycol encapsulated somatic embryo. The somatic embryos are first hardened to withstand desiccation before encapsulation, which induces quiescence in the embryos and provides more handling flexibility (Pond and Cameron, 2003). Thus, the ability of somatic embryos to withstand drying to low moisture content is an important factor for storage and also play a significant role in the developmental transition between maturation and conversion (Sharma et al., 2013). Such types of synthetic seeds can only be produced in plants whose somatic embryos are desiccation-tolerant. This desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the Petri dishes and leaving them overnight to dry (Ara et al., 2000). Sundararaj et al., (2010) reported that desiccation tolerance can also be induced by growth medium with high osmotic potential generated by either increased levels of permeating osmoticants (e.g., sucrose, mannitol) or non-permeating osmoticants (e.g., polyethylene glycol). While working with ginger Sundararaj et al. (2010) found that sucrose-dehydration was more effective than air-dehydration in terms of re-growth ability by providing required nutrients; moreover, rapid moisture loss during air dehydration resulted in poor conversion frequency.

On the other hand, hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogels. Redenbaugh et al. (1984) were the first to developed hydrogel encapsulation of individual somatic embryos of alfalfa (*Medicago sativa*). Since then it remains the most studied strategy for synthetic seed production (Ara et al.,

2000; Rai et al., 2009). A number of coating agents such as potassium alginate, agar, sodium pectate, sodium alginate, carrageenan, gelatin, sodium alginate with carboxymethyl cellulose, gelrite, tragacanth gum, guargum, etc. have been tested as hydrogels (Ara et al., 2000; Rai and Jaiswal, 2008). Among all, sodium alginate has been frequently selected because of its moderate viscosity, quick gelation, low cost and bio-compatibility (Saiprasad, 2001; Swamy et al., 2009). Sodium alginate and calcium salt are reported to be the best combination for encapsulation since these ions are non-damaging, have a low price, easy to use and result in a high embryo-to-plant conversion. The capsule gel potentially serves as a reservoir of nutrients that helps in the survival and speedy growth of the encapsulated plant tissues (Redenbaugh et al., 1987).

Production of synthetic seeds by encapsulating somatic embryos, shoot buds or any other meristematic tissue helps in minimizing the cost of micropropagated plantlets for commercialization and transportation. In addition, cryopreservation of encapsulated germplasm has now been used as a means of *ex vitro* conservation with the possibility of minimizing adverse effects of cryoprotectants and post-preservation damages (Sharma et al., 2013). Synthetic seeds technology can also provide seed throughout the year whereas most of the plants produce seeds only in certain months of the year (Rai et al., 2009). Through this technology, germplasm exchange between countries could be accelerated because of the aseptic condition of the plant material and easy to transport.

Therefore, this study aimed at the production of synthetic seeds using nodal segments excised from the stock plantlets as well as verifying their wellness after storing at +4 °C for different time periods (up to 70 days) in terms of germination, regrowth and development on different sowing media *in vitro*.

4.2 Materials and Methods

For the multiplication of stock plants, nodal segments (~5 mm) excised from four-weeks-old seedlings were cultured on MS medium containing 3% (w/v) sucrose. The medium was solidified with 0.8% (w/v) plant-agar and the pH was adjusted to 5.7 ± 0.02 prior to autoclaving at 121 ˚C for 15 min. Nodal segments aseptically excised from

four-weeks-old proliferated shoots were used as explants for the synthetic seed production.

To achieve synthetic seed production via encapsulation technique, 1.5% (w/v) sodium alginate (NaAlg) was used as a gelling agent whereas 1% (w/v) calcium chloride ($CaCl_2$) was used as the complexing agent. The gelling matrix of 1.5% NaAlg and 1% $CaCl_2$ was found the most suitable for the formation of firm, clear and isodiametric beads in our previous study, hence, these concentrations were used for encapsulation of nodal segments throughout the experiment. Sodium alginate was used in four different compositions as follows; NaAlg only as a control group, NaAlg together with MS medium with or without 3% (w/v) sucrose, and finally NaAlg matrix augmented with MS medium, 3% (w/v) sucrose and 0.50 mg/l abscisic acid (ABA). Both gelling and complexing agents were first autoclaved at 121 ˚C for 15 min before using.

Under aseptic condition, encapsulation in NaAlg beads was accomplished using a modified protocol of Winkelmann et al. (2004): the nodal segments (~3 mm) excised from stock plants were singly sucked up in 250 µL of either of the four different 1.5% (w/v) NaAlg composition using a micropipette (1000 µL) with a cut tip to provide a wider opening. Each droplet containing one randomly selected nodal explant was dropped into 250 ml of 1% (w/v) $CaCl_2$ solution in a 500 ml beaker. The solution mix and gel beads were stirred at 100 rpm for 25 min in an orbital shaker at room temperature in order to induce further polymerization. After hardening of the gel beads, they were washed with sterile distilled water 4-5 times in order to remove $CaCl_2$. After which, they were collected on a metal sieve, and then transferred to sterile filter paper in Petri plates for 5 min under the laminar airflow hood to eliminate the excess water. Later, the alginate beads were transferred into sterile 50 ml falcon tubes and kept at low temperature (+4 °C) for seven different time periods (0, 7, 14, 21, 28, 35 or 70 days).

To investigate the viability of encapsulated explants and the effect of different sowing media, after each storage period encapsulated nodal segments were cultured either in a Petri plate containing full-strength PGR-free MS basal medium or in glass jars containing sterile soil moistened with liquid MS medium or with sterile distilled water for conversion into plantlets. Encapsulated nodal explants sowed on sterile soil moistened with liquid MS medium were sprayed with liquid MS while those sowed on

sterile soil moistened with sterile water were sprayed with sterile water at four days intervals.

After four weeks, plantlets with well-developed shoot and root system obtained from encapsulated nodal segment were removed from the sowing medium, washed gently under running tap water and transferred to plastic pots filled with sterile commercial compost containing a mixture of soil, manure, moss and sand at a ratio of 1:2:2:1. The potted plantlets were cover up with a transparent plastic cover in order to maintain high humidity under greenhouse condition and irrigated at two days interval. The covers were removed after 10 days so as to acclimatize the plants to the field conditions. After four weeks, the acclimatized plants were finally transferred to the field under full sunlight.

All *in vitro* cultures were either grown in (90 × 15 mm) disposable Petri plates or 300 ml transparent glass jars and kept at 23±1 °C under 16/8 hours light/dark photoperiod provided by cool-white fluorescent light with an irradiance of 2000 lux and at 60% relative humidity. All materials and media used throughout the experiment were first autoclaved before using, and the culture was done in an aseptic condition under laminar flow cabinet. All tissue culture media and PGRs used were purchased from Duchefa Biochemie (Netherlands).

Data concerning percentage of regrowth and mean shoot length were recorded after two weeks of culture on sowing medium for each treatment. For the observation of regrowth patterns, 25 synthetic seeds were used for each treatment and each experiment was repeated thrice. The frequency of conversion was estimated as the percentage of encapsulated nodal segments developed into whole plantlets out of a total number of encapsulated nodal segments inoculated in a particular medium. Mean shoot length was recorded by measuring the distance (cm) between shoot-base and shoot-tip of 25 randomly selected plantlets for each treatment. The survival rate was also determined by calculating the number of plantlets survived after acclimatization out of a total number of plantlets transfer to the field. The data were analyzed statistically using ANOVA and the significant differences between means were determined using Duncan's multiple range test at $P < 0.05$ level.

4.3 Results

Different chemical compositions of sodium alginate matrix were tested for the effective conservation of viable synthetic seeds. For the conversion of encapsulated nodal segments into plantlets, the encapsulated nodal segments were sown in either i) PGR-free MS medium or ii) soil moistened with liquid MS or iii) soil moistened with sterile water. Of the three different sowing media tested, the best response for shoot development with the highest frequency (100%) was found when the seeds were sown on PGR-free MS medium; irrespective of the gel bead chemical compositions (Table 3.1; Figure 3.1). In spite of the low conversion frequency, plantlet regeneration was also achieved from synthetic seeds incubated on soil after short-term storage (Figure 3.2). However, for the synthetic seeds which were sown on soil moistened with liquid MS after storing at +4 °C for more than 28 days, or 14 days for those cultured on soil moistened with sterile water, neither shoot nor root development was observed (Table 3.1). On the other hand, conversion of synthetic seeds into plantlets was observed even from those stored up to 70 days at +4 °C when cultured on MS medium. However, the rate of conversion was inversely related to the storage period; as the time of storage increased, the rate of shoot development decreased.

Based on the results obtained from the effect of different sowing media, the analysis of different gel matrix compositions was done base on synseeds incubated on MS sowing medium. When considering chemical compositions of sodium alginate matrix, the highest percentage of shooting response of encapsulated nodal explants was observed from sodium alginate matrix containing sucrose and MS basal medium with a frequency of 60% regrowth after storing for 70 days (Table 3.1). In this gel bead composition, all synthetic seeds performed an efficient regrowth (100%) until 28th day of storage, after which the percentage reduced to 60% with longer storage. The difference was only observed on shoot length, it was noticed that long storage of the seeds affect the shoot size (Table 3.2). For instance, when nodal explants encapsulated in NaAlg + MS + sucrose were taken into account, the shoot growth from the synthetic seeds reduced after storing for 70 days at +4 °C (from 1.1 to 0.4 cm). Hence, there is also an inverse relationship between the shoot length and the storage duration.

Sodium alginate matrix alone was also found effective for regrowth until 28th day of storage, but regrowth of this sample sharply decreased at longer storage. Gel beads containing abscisic acid (0.50 mg/l) had 73% germination rate at 28th day, however, this value gradually decreased to 26.7% at 70th day of storage (Table 3.1; Figure 3.3). Therefore, in this study, the presence of sucrose was found effective for prolonged storage as can be seen in all the data presented. For example; in samples of day 70, the frequency was 60% in sucrose containing treatment while it was 40% in the absence of sucrose. In terms of shoot elongation, there was no significant difference among different compositions of gel matrix tested; the difference depends only on duration of storage (Table 3.2). Irrespective of storage time and gel matrix composition, all plantlets obtained from synthetic seeds exhibited new growth and normal morphological characteristics were hardened and successfully established to the field with a survival frequency of 90-100% under *ex vitro* conditions.

Table 4.1. The effect of different compositions of gelling matrix and sowing media on percentage of synthetic seed germination after storing for different time periods (0, 7, 14, 21, 28, 35 and 70 days) at +4 °C. The data was collected after two weeks of incubation on sowing medium.

Sowing medium	Chemical composition of gel matrix	Percentage (%) of shoot formation						
		Day 0*	Day 7	Day 14	Day 21	Day 28	Day 35	Day 70
MS medium	NaAlg	100.0	100.0	80.00	80.00	67.70	20.00	13.30
	NaAlg + MS	100.0	100.0	100.0	100.0	100.0	73.30	40.00
	NaAlg + MS + Sucrose	100.0	100.0	100.0	100.0	100.0	80.00	60.00
	NaAlg + MS + Sucrose + ABA	100.0	100.0	73.30	73.30	73.30	33.30	26.70
Soil moistened with liquid MS	NaAlg	30.00	28.00	20.00	11.00	5.000	00.00	00.00
	NaAlg + MS	35.00	31.00	30.00	15.00	6.000	00.00	00.00
	NaAlg + MS + Sucrose	50.00	44.00	41.00	20.00	7.000	00.00	00.00
	NaAlg + MS + Sucrose + ABA	33.00	30.00	30.00	10.00	4.000	00.00	00.00
Soil moistened with sterile water	NaAlg	20.00	20.00	10.00	00.00	00.00	00.00	00.00
	NaAlg + MS	30.00	21.00	11.00	00.00	00.00	00.00	00.00
	NaAlg + MS + Sucrose	34.00	30.00	12.00	00.00	00.00	00.00	00.00
	NaAlg + MS + Sucrose + ABA	15.00	13.00	10.00	00.00	00.00	00.00	00.00

* Day 0 samples were immediately placed on sowing medium.

Table 4.2. The effect of different compositions of gelling matrix and sowing media on shoots growth of *P. peruviana* derived from synthetic seeds after storing for different time periods (0, 7, 14, 21, 28, 35 and 70 days) at +4 °C. The data was collected after two weeks of incubation on sowing medium.

Sowing medium	Chemical composition of gel matrix	Mean number of shoot length (cm)						
		Day 0*	Day 7	Day 14	Day 21	Day 28	Day 35	Day 70
MS medium	NaAlg	1.0 ± 0.3^{ab}	1.0 ± 0.3^{ab}	0.8 ± 0.1^{ab}	0.7 ± 0.0^{c}	0.6 ± 0.1^{b}	0.5 ± 0.0^{ab}	0.3 ± 0.0^{b}
	NaAlg + MS	1.0 ± 0.4^{ab}	1.0 ± 0.3^{ab}	0.9 ± 0.2^{ab}	0.9 ± 0.3^{abc}	0.8 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.1^{a}
	NaAlg + MS + Sucrose	1.1 ± 0.4^{ab}	1.1 ± 0.3^{a}	1.0 ± 0.3^{a}	1.0 ± 0.0^{a}	0.8 ± 0.0^{a}	0.6 ± 0.1^{a}	0.4 ± 0.1^{ab}
	NaAlg + MS + Sucrose + ABA	1.0 ± 0.3^{ab}	1.0 ± 0.3^{ab}	0.7 ± 0.1^{b}	0.7 ± 0.1^{c}	0.4 ± 0.1^{c}	0.4 ± 0.0^{c}	0.3 ± 0.0^{b}
Soil moistened with liquid MS	NaAlg	0.9 ± 0.1^{ab}	0.9 ± 0.2^{ab}	0.9 ± 0.1^{ab}	0.8 ± 0.1^{bc}	0.5 ± 0.0^{bc}	0.0 ± 0.0	0.0 ± 0.0
	NaAlg + MS	1.0 ± 0.1^{a}	1.0 ± 0.2^{ab}	0.9 ± 0.2^{ab}	0.9 ± 0.0^{b}	0.6 ± 0.0^{b}	0.0 ± 0.0	0.0 ± 0.0
	NaAlg + MS + Sucrose	1.1 ± 0.3^{ab}	1.0 ± 0.4^{ab}	1.0 ± 0.1^{a}	0.9 ± 0.3^{abc}	0.6 ± 0.1^{b}	0.0 ± 0.0	0.0 ± 0.0
	NaAlg + MS + Sucrose + ABA	0.8 ± 0.1^{b}	0.8 ± 0.1^{b}	0.7 ± 0.1^{b}	0.8 ± 0.1^{bc}	0.4 ± 0.1^{c}	0.0 ± 0.0	0.0 ± 0.0
Soil moistened with water	NaAlg	0.8 ± 0.2^{b}	0.7 ± 0.2^{b}	0.6 ± 0.2^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	NaAlg + MS	0.9 ± 0.1^{ab}	0.9 ± 0.1^{ab}	0.8 ± 0.3^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	NaAlg + MS + Sucrose	0.9 ± 0.1^{ab}	0.9 ± 0.4^{ab}	0.8 ± 0.3^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	NaAlg + MS + Sucrose + ABA	0.8 ± 0.1^{b}	0.8 ± 0.2^{b}	0.7 ± 0.2^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Mean numbers with the same letter within the same column are not significantly different at P>0.05.
[*] Day 0 samples were immediately placed on sowing medium.

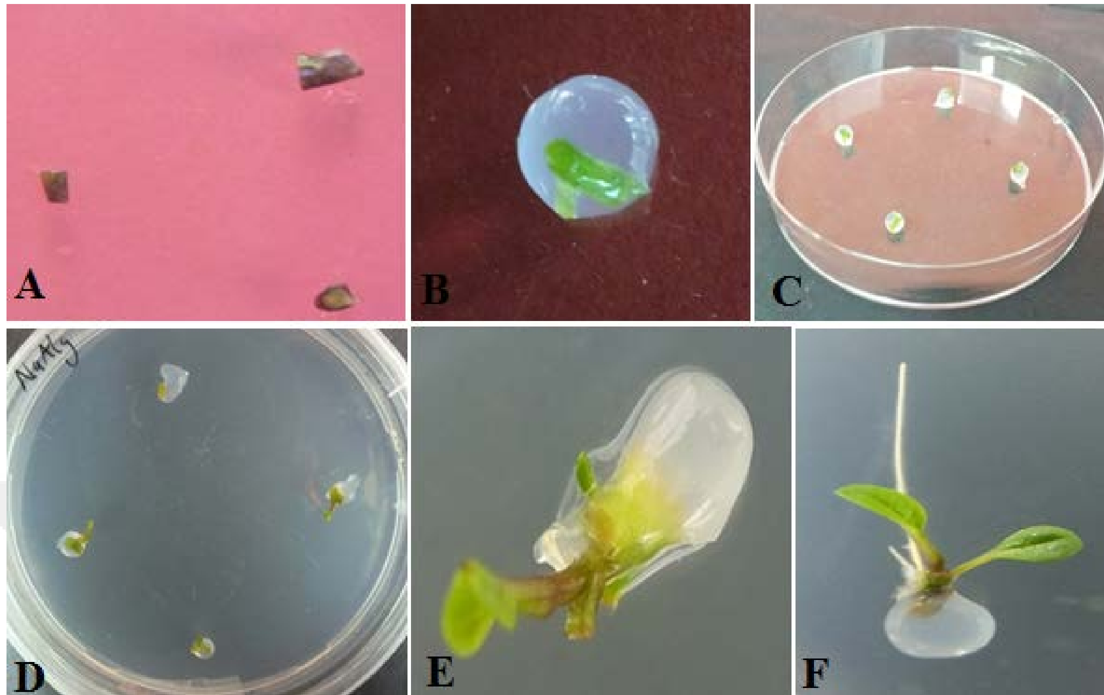

Figure 4.1. Synthetic seeds of *P. peruviana* and their subsequent conversion into plantlets on MS medium: nodal segments excised from *in vitro* stock plants (**A**); isodiametric gel bead of NaAlg consisting one nodal explant (**B**); synthetic seeds incubated on MS medium at day 1 (**C**); micro-shoot development from nodal explants encapsulated with NaAlg + MS + sucrose germinated after one week of incubation on MS sowing medium (**D**); and (**E**); regrowth of synthetic seeds with well-established shoot and root system after two weeks of incubation (**F**).

Figure 4.2. Synthetic seeds of *P. peruviana* and their subsequent conversion into plantlets on soil: synthetic seeds sown on soil at day 1 (**A**); micro-shoot induction after 3 days (**B**); and well-developed shoot derived from synthetic seed after two weeks of culture on soil (**C**).

Figure 4.3. Effect of ABA as growth retardant (see black arrows) in contrast to a well regrown synthetic seed encapsulated with NaAlg + MS + sucrose.

4.4 Discussion

Synthetic seed production using nodal segments was established via encapsulation in different NaAlg compositions. In the present study, nodal segments obtained from *in vitro* regenerated shoots were used for the synthetic seeds production because somatic embryogenesis has not yet achieved in *P. peruviana*. Nodal segments of this species contain meristematic tissues with high capacity of cell division, which make it better alternative than the other non-embryogenic vegetative propagules.

Although direct planting of synthetic seeds in the soil was not as successful as those cultured on MS medium, it was the first attempt to introduce synthetic seeds directly into the soil for conversion into plantlets. This gives a new insight that sowing synthetic seeds directly into the soil is possible with further advancement of the protocol. The synseeds planted on soil showed that conversion into plantlets is possible after short-term storage, but completely lost their viability after long storage. However, synseeds incubated on MS medium showed a high rate of plantlet regeneration even after long-term storage. This effect of MS medium on plantlets formation was also reported previously by Rai et al. (2008b), suggesting that full-strength MS medium was the best medium for shoot development from encapsulated tissues in comparison to half- and quarter-strength medium. This might be due to sufficient nutrients available in the culture media, which promote cell division and induced bud proliferation. Nevertheless, direct sowing of synseeds into the soil or other substrates may help to avoid the acclimatization procedure required for tissue culture-raised plantlets (Sharma et al., 2013). Mandal et al. (2000) also suggested that the successful conversion of synseeds into plantlets on a simple planting substrate such as sand, soil, soilrite or vermicompost is necessary for their use in commercial-scale propagation.

The analysis of data showed that long preservation of encapsulated nodal segments at +4 °C caused a significant reduction in plantlet recovery. A similar result was also reported for the encapsulated shoot tips of guava after storing for 30 days at +4 °C, which showed about four times decrease in retrieval rate as compared to control (Rai et al., 2008b). The decline in plant recovery from stored encapsulated vegetative propagules may be due to oxygen deficiency in the calcium alginate bead (Redenbaugh

et al., 1991). Likewise, the conversion frequency of encapsulated nodal segments of *Punica granatum* L. also decreased after storing at +4 °C (Naik and Chand, 2006).

In addition, it was clearly shown that lack of nutrients and presence of ABA as a growth retardant can be ascribed as a limiting factor for the regrowth pattern of the synthetic seeds after storage at +4 °C. Similarly, in guava, 1.0 mg/l ABA was reported to show a slow-growth pattern in encapsulated somatic embryos (Rai et al., 2008a). However, in contrast to our findings, Wang et al. (2004) reported that B5 medium containing 0.1 mg/l ABA extended the storage time up to 90 days without loss of viability in grapes. The use of ABA as a growth retardant might contribute a new understanding for the optimization of storage conditions in certain plant species having highly active meristematic tissues for germination within the hollow gel beads after being capsulated.

Although there was no significant difference in terms of shoot length between MS with or without sucrose in NaAlg matrix, the highest frequency of synthetic seed regrowth was observed in the presence of sucrose. This finding was also consistent with the promising effect of sucrose on artificial endosperm formation in such a way that sucrose and the meristematic tissue mimicked zygotic embryos during storage (Germena et al., 2011; Sharma et al., 2013). Beside these, there are several reports on synthetic seed production of fruit plants based on *in vitro* conservation and storage at +4 °C, pomegranate (Naik and Chand, 2006), mulberry (Kavyashree et al., 2006), raspberry (Piccioni and Standardi, 1995) and black nightshade (Verma et al., 2010).

It is also important to mention that combination of several parameters such as plant genotype, explant type, concentration of mineral contents and/or sucrose in medium, storage conditions and growth regulators are crucial for testing viability as well as the conservation of synthetic seeds (Benelli et al., 2013; Sharma et al., 2013; Berjak et al., 2014). Preservation of plant materials at low temperature for a long time may result in losing their genetic integrity, and in some cases, malformed shoot tips were observed. To circumvent these drawbacks, NaAlg encapsulation containing MS medium with or without sucrose is feasible to obtain high potential regrowth after storage at +4 °C. Moreover, for encapsulation, the use of *in vitro* raised plantlets as an explant source

rather than field grown plants possibly minimize the recalcitrance under aseptic condition (Rai et al., 2009).

The present protocol highlights the development of high-frequency shoot recovery in *P. peruviana* from encapsulated nodal segments after 70 days of storage. Therefore, based on the results presented, it can be understood that synthetic seed production through encapsulation technique will be of promising effect on germplasm conservation and in providing plant material throughout year-round. In addition, the introduction of synthetic seeds directly into the soil as described here will surely be of great importance for near future agricultural practices with some improvement or modification of the protocol. Synthetic seeds are also expected to offer an appropriate recipient system for alien gene transfer in micro-projectile based gene delivery system. The present protocol also explores the possibility of preserving the genetic stability of the selections and promotes true to type genotype for the exchange of germplasm between laboratories. Hopefully, this work will contribute new understandings in synthetic seed technology of Solanaceous and other plant species.

CHAPTER IV

5 ANTIOXIDANT ACTIVITY

5.1 Introduction

Reactive oxygen species (ROS) are continuously produced by our body's normal use of oxygen such as respiration and some cell-mediated immune functions. ROS include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH•), and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2$) (Gülçin, 2006a). When these free radicals (ROS) attack biological molecules, they usually cause cell or tissue injury, since they induce some oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates. And damaging these biomolecules can cause ageing, cancer, and many other diseases (Aruoma, 1994).

All aerobic organisms have antioxidant defenses, including antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxide) and antioxidant food constituents to remove or repair the molecules damaged by free radicals. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for the deterioration of food and pharmaceutical products during processing and storage (Halliwell, 1997). Antioxidants can protect human body from free radicals and ROS effects. Many small molecules are also involved in ROS detoxification, such as glutathione (GSH), α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Sies, 1997). They retard the progress of many chronic diseases as well as lipid peroxidation. Hence, a need has appeared to identify alternative natural and safe sources of food antioxidants, especially of plant origin, which has notably increased in recent years. Antioxidants are often added to the foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and

propagation steps, leading to the termination of the reaction and delaying the oxidation process (Shahidi et al., 1992; Gülçin, 2002a).

When the mechanism of antioxidant protection becomes unbalanced by exogenous factors such as smoking, ionizing radiation, certain pollutants, organic solvents, pesticides and endogenous factors such as normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, these may result in some diseases such as malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and cancer with accelerating aging (Hertog et al., 1993; Tanizawa et al., 1992; Duh, 1998; Yıldırım et al., 2001; Büyükokuroğlu et al., 2001). However, antioxidant supplements or food with high antioxidant capacity may be used to help the body in reducing oxidative damage by free radicals and active oxygen (Halliwell and Gutteridge, 1984; Ho et al., 1994, Mau et al., 2001; Gülçin, 2002b). From the past few decades, various phytochemicals and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas, spices and herbs have also been intensively studied (Ho et al., 1994).

Many antioxidant compounds, naturally occurring in plant have been identified as free radical or active oxygen scavengers (Yen and Duh, 1994; Duh, 1998). Interest has considerably increased in finding naturally occurring antioxidant in foods or medicinal materials to replace synthetic antioxidants, which protect the human body from free radicals and delay the process of many chronic diseases such as carcinogenicity (Kinsella, 1993; Pryor, 1991; Zheng and Wang, 2001; Lai et al., 2001). Natural antioxidants are also known for their wide range of biological activities, including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic, and vasodilatic activity (Liyana-pathirana and Shahidi, 2006). Most of the plant tissues synthesize antioxidant compounds in form of phenols or polyphenols such as flavonoids, carotenoids, tocopherol derivatives, lignin, and related isoprenoids, which have been isolated from different kinds of plants, including oilseeds, cereal crops, vegetables, herbs, and seaweeds (Loliger, 1991; Wettasinghe and Shahidi, 1999; Gülçin, 2006b).

Flavonoids belong to a large group of plant-derived low-molecular weight polyphenolic compounds biosynthesized from both shikimic acid and acetic acid pathways (Bruneton, 1999). These compounds deserve attention because of their important biological properties, such as vascular protective and venous tonic, anti-inflammatory, diuretic, antispasmodic, hepatoprotective, and hypocholesterolemic (Bruneton, 1999; Cazarolli et al., 2008). There has been a lot of interest in the antioxidant activity of flavonoids and other plant phenolic compounds due to their potential in health promotion and disease prevention (Dillard and German, 2000; Su et al., 2007; Pereira et al., 2009; Parr and Bolwell, 2000). Antioxidants are able to scavenge free radicals by various mechanisms (Parr and Bolwell, 2000; Pereira et al., 2009), and thus contribute to the protection of biologically important cellular components, such as DNA, proteins, and membrane lipids from free radical attacks, which may lead to cell damage, and this may cause ageing, inflammation, atherosclerosis, ischemic injury, and cancer (Bruneton, 1999; Su et al., 2007; Stanojevic et al., 2009; Siatka and Kasparova, 2010).

Diets rich foods containing antioxidant compounds, such as fruits and vegetables, could help prevent pathologies caused by oxidative stress (Lampe, 1999). Therefore, it is important to evaluate the antioxidant potential of fruits and vegetables in order to be certain of their quality and to develop methods for their optimal conservation. Several methods such as ORAC (oxygen radical absorbing capacity), TRAP (total radical-tapping antioxidant parameter) and TEAC (trolox equivalent antioxidant capacity) are used for the evaluation of the antioxidant activities of fruit and vegetable extracts (Moreno-Sanchez, 2002; Pellegrini et al., 2003; Proteggente et al., 2002; Wu et al., 2006). *In vitro* tests such as the ORAC assay do not, however, take into account the physiological conditions of the cell, the bioavailability of the antioxidant molecule as well as general cellular metabolism (Liu and Finley, 2005). Moreover, antioxidant molecules present in fruits and vegetables are heterogeneous and multifunctional (Frankel and Meyer, 2000). Many factors can affect their antioxidant potential, such as the affinity of the molecules for the aqueous or lipid phase, the oxidation conditions in the cell as well as the nature of the oxidizable substrate used in the assay. Therefore, one-dimensional assay protocol is not enough to test all relevant parameters (Frankel

and Meyer, 2000). A complementary method used to evaluate the antioxidant activities of plant extracts could be more useful. Furthermore, it has been generally known that bioactive substances are synthesized or accumulate in different types of secretory structures including the dark glands, translucent glands, and secretory canals located in different organs of the plants (leaf, stem, flower etc.). Hence, it is important to determine phytochemical compositions from different parts of the plants.

In the present study, antioxidant activities were determined from regenerants (*in vitro* derived plantlets) using leaf, stem, fruit, calyx, and callus extracts; and from seed derived seedlings grown under *ex vitro* conditions using leaf, stem, fruit and calyx, testing three different antioxidant methods as follow: free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteau) and total flavonoid content test (aluminium chloride colorimetric).

5.2 Materials and Methods

5.2.1 Plant Material and Extraction

Analysis of antioxidant activity of *P. peruviana* was conducted using fruit, leaf, stem, calyx and callus samples obtained from two different plant sources; 1) regenerants produced from nodal explants under *in vitro* conditions, and 2) seed-derived seedlings grown under *ex vitro* conditions. The *in vitro*-derived plantlets were regenerated according to previously described protocol (Chapter 2), by culturing nodal explants (~3 mm) on MS medium containing 0.5 mg/l TDZ, which were maintained for 4 weeks before transferring to the soil in a greenhouse for acclimatization. However, the seedlings were obtained from the seeds germinated in soil under greenhouse conditions. The plantlets from both sources were transferred to the field progressively. The leaf and stem samples of both plant sources were collected from 3-months-old plantlets while fruit and calyx samples were collected at the end of fruit maturity. The callus sample was obtained from leaf explants cultured on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP for 6 weeks.

In order to determine the best conditions for the extraction of antioxidants in this plant, the following parameters were evaluated: type of solvent and proportion, extraction temperature, maceration period and numbers of extraction stages. Following protocol validation, the best protocol suitable for the samples extraction was applied for the analysis of antioxidant potential from as described below:

Collected samples were shade dried at room temperature to avoid sunlight effect and then grounded into fine powder with mortar and pestle. The powder of each sample (5 g) was added to 50 ml methanol and kept in dark at room temperature for 24 hours. After extraction at 35 °C in an ultrasonic bath for 30 minutes, the samples were centrifuged at room temperature for 10 minutes at 8,000 rpm. The supernatant was collected and transferred into new Falcon tube then re-centrifuged again. Afterwards, the supernatant was collected and filtered with 45 µm disposable syringe filter (Chromafil® Xtra PVDF). The pellet was re-extracted once more and then centrifuged as described above until filtration step. The first and second extracts were combined and evaporated the methanol at 40 °C using rotary evaporator (IKA® RV 8). The methanolic extracts were kept in laboratory refrigerator at +4 °C until used.

5.2.2 Free Radical Scavenging Activity (DPPH Method)

The free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteau) and total flavonoid content (aluminium chloride colorimetric) were used in this assessment. DPPH, as stable free radical with a characteristic absorbance at 517 nm, was used as a free radical in this experiment. This wavelength was chosen after conducting absorbance scan, which showed that 517 nm is suitable for measuring DPPH absorbance without any intervention of plant pigments (Figure 4.1). Ascorbic acid (vitamin C), which is a well-known antioxidant in phenolic structure and widely used in DPPH experiments was chosen as a positive control in this study, because of its very high antioxidant capacity and it's abundant in *P. peruviana*.

Antioxidant activities of methanolic extracts (leaf, stem, fruit, calyx or callus) of *P. peruviana* were assessed against 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-

Aldrich Chemie, Steinheim, Germany) with ascorbic acid (vitamin C) as a positive control, using modified protocol described by Brand-Williams et al. (1995). Briefly, ascorbic acid and each methanolic extract of both regenerants and seedlings were diluted into five different concentrations (final concentrations; 10, 20, 40, 80 and 160 µg/ml) with methanol, each containing a final volume of 2 ml. The methanolic DPPH solution (1.5×10^{-4} M) was prepared in a daily basis before starting the experiment. To determine the antioxidant capacity of the aforesaid extracts, 1.5 ml of each diluted concentration (sample or ascorbic acid) was mixed with 0.5 ml methanolic DPPH solution in 5 mL glass test tubes. The solutions were mixed vigorously, and incubated at room temperature for 30 minutes in a dark chamber. Afterwards, the change in colorations (from deep-violet to light-yellow) was measured at 517 nm wavelength using a spectrophotometer (Hitachi U-1900, UV-VIS Spectrophotometer 200V, Tokyo, Japan) against the blank samples (all reagents without tested sample).

Free radical scavenging activities of the samples and ascorbic acid were calculated according to their ability to reduce the initial absorbance of DPPH solution. The results were given in terms of percentage of inhibition along with IC_{50} values, which represent the amount of antioxidants needed to reduce 50% of original DPPH reagent. The IC_{50} was determined by plotting a graph with the sample concentration on the abscissa and free radical inhibition capacity as the ordinate. DPPH scavenging capacity of the extracts was calculated using the following equation:

$$\text{DPPH scavenging effect (\% inhibition)} = \left(A\Delta - \frac{A1}{A\Delta}\right) \text{x } 100$$

Where A_{Δ} is the absorbance of the control solution; and

A_1 is the absorbance of tested samples.

5.2.3 Total Phenolic Assay (Folin-Ciocalteau Method)

Total phenolic contents of methanolic extracts from *in vitro*- and of *ex vitro* derived *P. peruviana* were determined using Folin-Ciocalteu reagent according to modified protocol of Slinkard and Singleton (1977) using gallic acid as a standard phenolic compound. For the preparation of stock solution, 3 mg methanolic extract from each sample was added into 3 mL methanol (1 mg/mL) in 15 ml falcon tubes and use as a stock solution throughout the experiment. To obtain the standard calibration curve, gallic acid was diluted to have final concentrations as 0, 12.5, 25, 50, 100, 150, 200 or 250 mg/l.

For appropriate determination of phenolic concentrations, 20 µl from each calibration solution (gallic acid) or sample was added to 5 ml glass test tube containing 1.58 ml distilled water, then 100 µl of Folin-Ciocalteu reagent was added to the solution mixture, and carefully mixed. After waiting for 2 minutes at room temperature in dark chamber, 300 µl of 20% (w/v) Na_2CO_3 solution was added to the tube and then mixed. The solutions were incubated at room temperature for 2 hours in a dark chamber. Finally, the absorbance of each solution was measured at 765 nm wavelength against the blank samples (all reagents without tested sample) using the spectrophotometer. After plotting the calibration curve with gallic acid standard, the total phenolic content was determined, and the results were presented as milligram (mg) gallic acid equivalent (GEA) per gram dry weight (DW).

5.2.4 Total Flavonoid Assay (Aluminum Chloride Colorimetric Method)

The total flavonoid content of methanolic extracts was also determined using aluminium chloride ($AlCl_3$) colorimetric assay, with catechol as a reference flavonoid. The catechol solution was prepared in eight different concentrations to have final concentrations as 0, 5, 10, 20, 40, 60, 80 or 100 mg/l in order to obtain standard calibration curve.

The total flavonoid content was determined according to protocol described by Karakas and Turker (2013). In brief, 250 µl from each stock solution (1 mg/ml) or standard solution of catechol was added to 5 mL glass test tube containing 1 mL distilled water and added 75 µl of 5% (w/v) sodium nitrite ($NaNO_2$). After waiting for 5 minutes at room temperature, 75 µl of 10% (w/v) AlCl3 was added to the solution. At the sixth minute, 500 µl of 1M NaOH was added to the solution mixture, after which the reaction tubes were diluted to a final volume of 2.5 ml with the addition of 600 µl distilled water and mixed thoroughly. After waiting for 30 minutes at room temperature in a dark chamber, the absorbance of the solution mixture was determined using spectrophotometer at 510 nm wavelength against the blank sample. Disposable plastic cuvettes (12.5 × 12.5 × 45 mm) purchased from Interlab (Istanbul, Turkey) were used for the absorbance measurement. The total flavonoid content of all samples was calculated as milligram of catechol equivalents (CE) using an equation obtained from catechol calibration curve and the results were given in terms of milligram catechol equivalent per gram dry weight.

All experiments (including antioxidant activity, total phenolic content and total flavonoid content) were repeated three times. The calibration curves (gallic acid and catechol) were constructed from the mean of three independent repeats. The data were expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) was used to determine statistical significance, and significant differences between the means ± SD values were calculated using Duncan’s multiple range tests at $P < 0.05$ level.

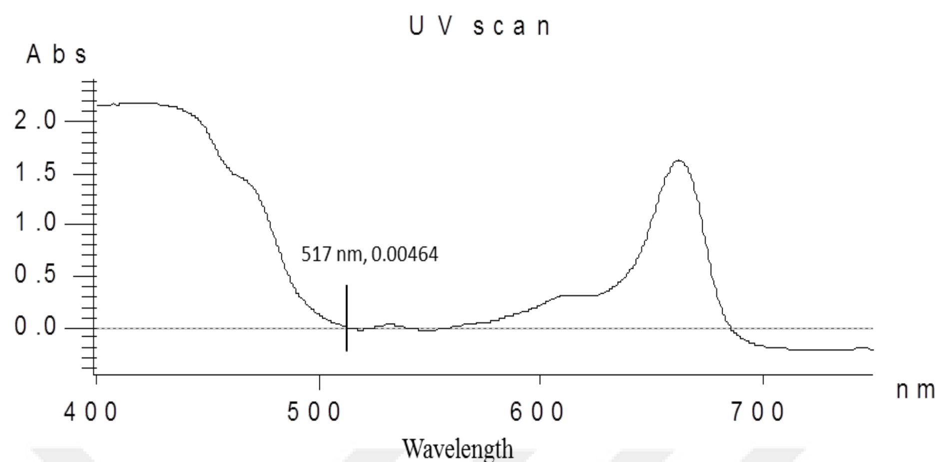

Figure 5.1. Absorbance scan of methanolic extract at 160μg/ml.

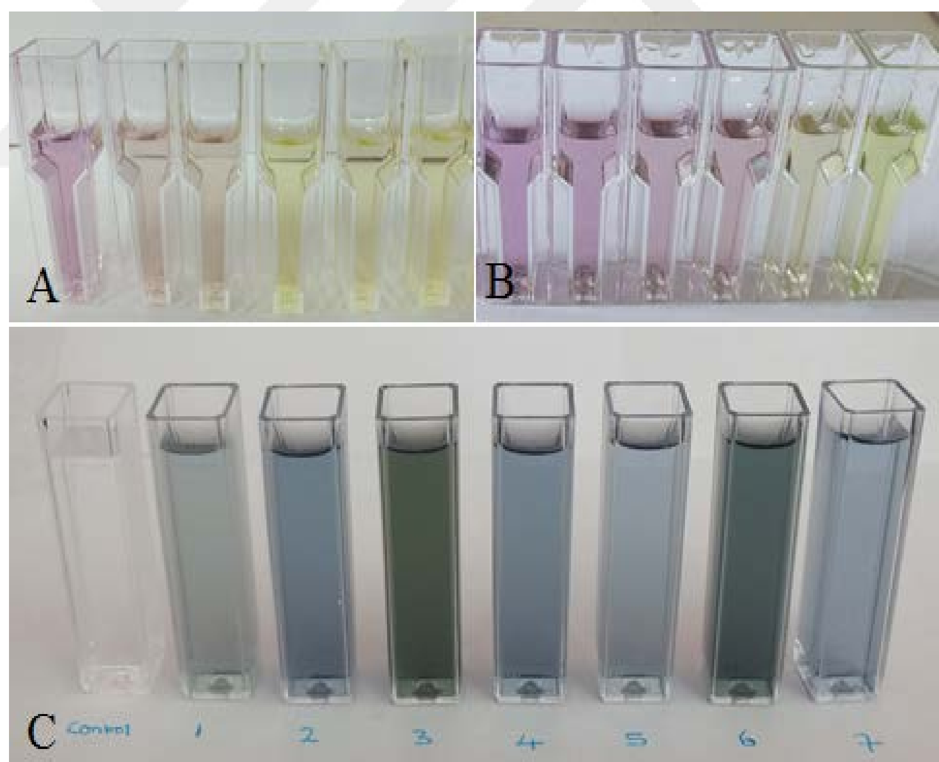

Figure 5.2. Change in coloration during DPPH reaction from purple to yellow: high antioxidant activity from positive control (ascorbic acid) changing the colour from purple to yellow (**A**); tested sample (**B**); and change from green to violet for phenolic compounds (**C**).

5.3 Results

In this study, regardless of plant source (*in vitro*-derived or *ex vitro*-derived), the lowest IC_{50} values of 35.60 and 36.30 µg/ml, which means the highest radical scavenging activity 95.3 and 94.2%, respectively, were obtained from the fruit extracts with the maximum scavenging activity at 160 µg/ml concentration (Table 4.1; Figure 4.3). On the other hand, the highest IC_{50} value 166.7 µg/ml indicating the lowest radical scavenging activity was obtained from callus extract at all tested concentrations. Although, at low concentrations (10 or 20 µg/ml) extracts of calyces collected from both regenerants and seedlings exhibited higher scavenging activity than other extracts, starting from 40 µg/ml fruit extracts showed more antioxidant activity compared to other extracts. Furthermore, regarding fruit and calyx extracts there was no significant difference observed between those collected from regenerants and those of seed-derived seedlings in terms of their anti-radical efficiencies (Table 4.1; 4.2).

In addition, following fruit extracts, high antioxidant activity was also noted from stems extracts. However, when leaf and stem extracts were taken into account, the extracts of the samples collected from regenerants show higher radical scavenging activity compared to those collected from seedlings. For example, the stem extracts derived from regenerants have IC_{50} value of 44.80 µg/ml and 90.1% radical scavenging capacity at 160 µg/ml whereas at the same concentration the stem extracts obtained from seedlings have IC_{50} value of 48.2 µg/ml and 86.4% radical scavenging capacity, respectively. Similarly, the leaf extracts of regenerants have higher free radical scavenging potential compared to those derived from seedlings (compare 77.0% for leaf extract of regenerants with 59.4% for that of seedlings). These results indicate that leaves and stems obtained from regenerants possessed more antioxidant compounds than those collected from seedlings (Table 4.1; Figure 4.3). In this contribution, all methanolic extracts showed a concentration dependent free radical scavenging activity. The free radical scavenging activity of these extracts elevate when concentration increase from 10-160 µg/ml (Figure 4.4).

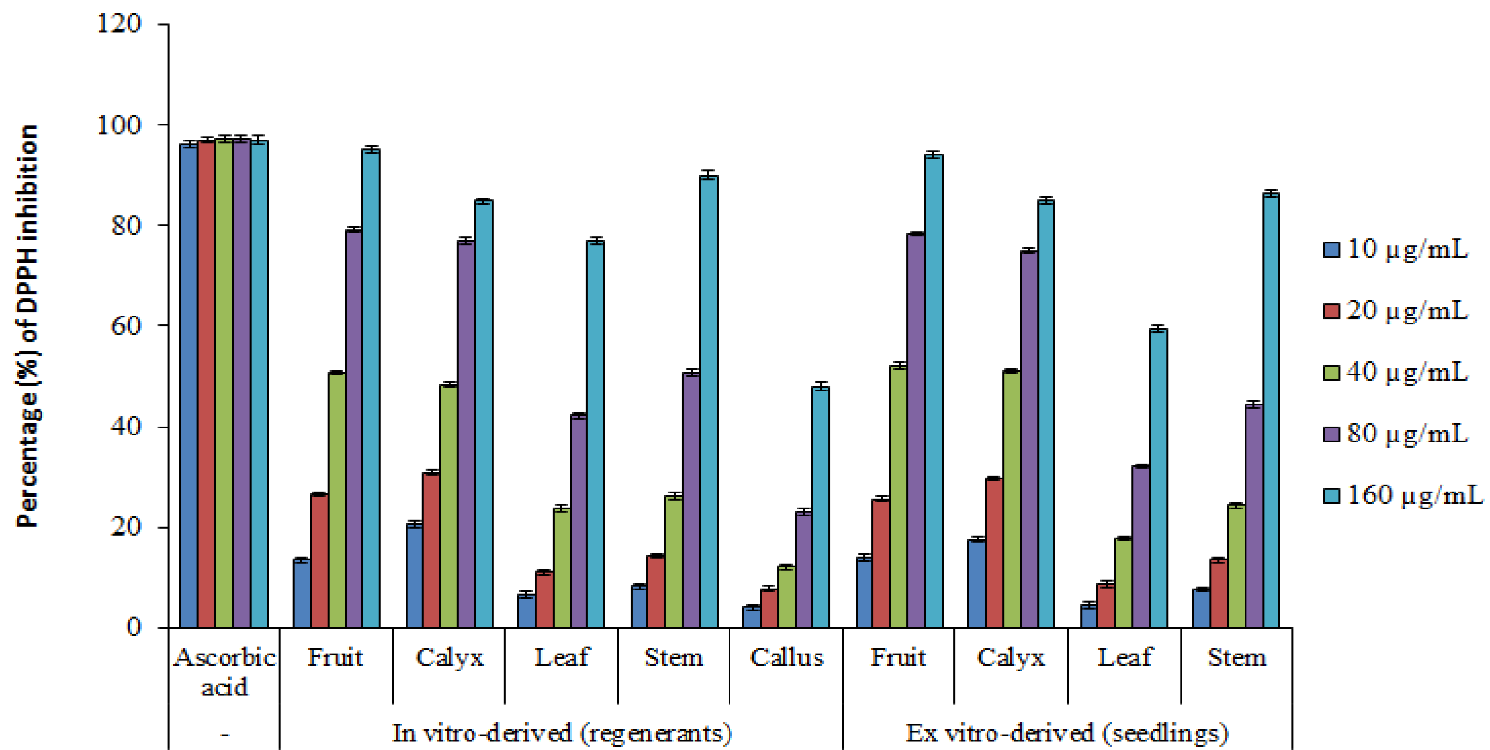

Figure 5.3. Free-radical scavenging activity (%) of methanolic extracts of leaf, stem, fruit, calyx and callus derived from regenerants; and leaf, stem, fruit and calyx derived from seedlings of *P. peruviana* at different concentrations and ascorbic acid (positive control).

Table 5.1. DPPH radical scavenging activity (%) of different extracts from *in vitro*-derived regenerants and *ex vitro*-derived seedlings of *P. peruviana* at different concentrations and ascorbic acid (positive control).

Plant materials	DPPH % inhibition					
	Extract	**10 µg/ml**	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**	**160 µg/ml**
	Ascorbic acid	96.4 ± 0.1	97.1 ± 0.1	97.3 ± 0.2	97.4 ± 0.2	97.1 ± 0.1
***In vitro*-derived (regenerants)**	Fruit	13.4 ± 0.2	26.5 ± 0.4	50.6 ± 0.3	79.2 ± 0.5	95.3 ± 0.7
	Calyx	20.6 ± 0.2	30.8 ± 0.2	48.4 ± 0.2	77.0 ± 0.6	84.9 ± 0.4
	Leaf	6.60 ± 0.1	11.0 ± 0.1	23.7 ± 0.1	42.2 ± 0.1	77.0 ± 0.1
	Stem	8.20 ± 0.1	14.3 ± 0.1	26.2 ± 0.1	50.7 ± 0.3	90.1 ± 0.3
	Callus	4.00 ± 0.0	7.70 ± 0.2	12.1 ± 0.0	23.0 ± 0.1	48.0 ± 0.2
***Ex vitro*-derived (seedlings)**	Fruit	14.0 ± 0.2	25.6 ± 0.3	52.1 ± 0.4	78.4 ± 0.2	94.2 ± 0.4
	Calyx	17.6 ± 0.3	29.6 ± 0.5	51.0 ± 0.3	75.0 ± 0.4	85.0 ± 0.5
	Leaf	4.40 ± 0.2	8.70 ± 0.2	17.8 ± 0.1	32.1 ± 0.1	59.4 ± 0.1
	Stem	7.50 ± 0.1	13.4 ± 0.1	24.3 ± 0.1	44.3 ± 0.1	86.4 ± 0.2

Table 5.2. 50% inhibition concentration (IC_{50}) of different extracts of *P. peruviana* and ascorbic acid (positive control).

Samples	Extracts	IC_{50} (µg/ml)
	Ascorbic acid	6.50 ± 0.6^{h}
In vitro-derived (regenerants)	Fruit	35.60 ± 0.4^{g}
	Calyx	50.60 ± 0.6^{d}
	Leaf	72.90 ± 0.3^{c}
	Stem	44.80 ± 0.7^{f}
	Callus	166.7 ± 0.5^{a}
Ex vitro-derived (seedlings)	Fruit	36.30 ± 0.3^{g}
	Calyx	49.00 ± 0.5^{de}
	Leaf	88.70 ± 0.8^{b}
	Stem	48.20 ± 0.4^{e}

*Mean numbers with the same letter within the same column are not significantly different at P>0.05.

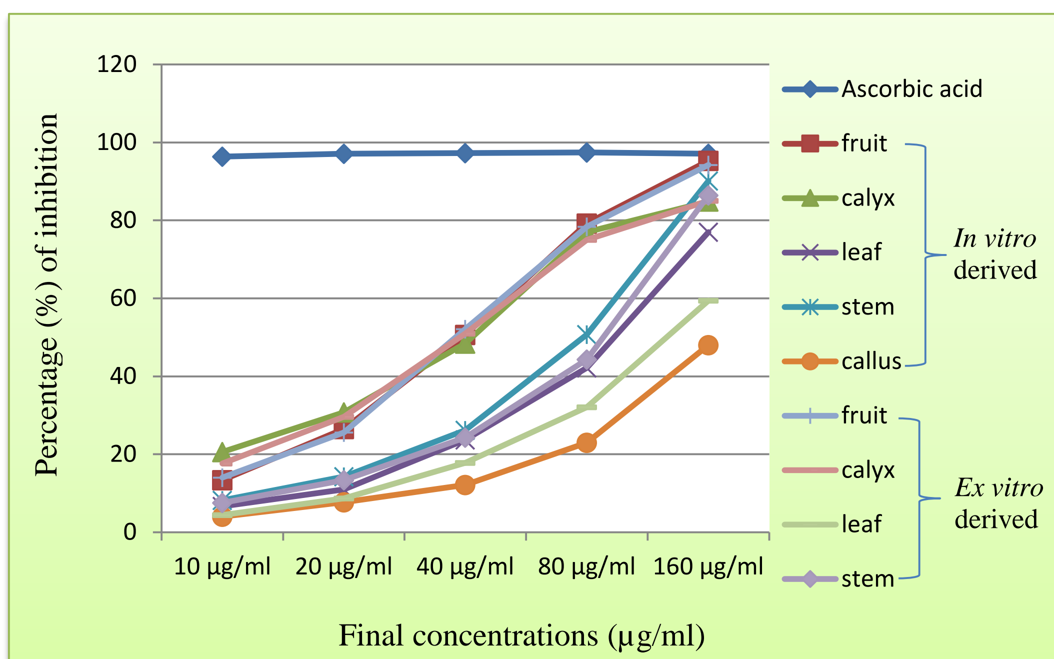

Figure 5.4. Dose-response curves of *P. peruviana* extracts on their antioxidant activity.

The total phenolic contents of methanolic extracts of fruit, calyx, leaf, stem or callus tissue produced from regenerants and of *ex vitro*-grown seedlings of *P. peruviana* were assessed using Folin-Ciocalteu reagent. The results were expressed as milligram of gallic acid equivalents (GAE) per gram dried weight (DW). The data presented (Table 4.3; Figure 4.5, 4.6), indicated that fruit extracts of all plant sources (*in vitro*-derived and *ex vitro*-derived) contained high concentration of phenolic compounds, which are 31.38 and 30.90 mg GAE/g DW, respectively. Similarly, following fruit extracts, calyx extracts also contained high concentration of phenolic compounds, resulted in 26.46 and 25.69 mg GAE/g DW respectively. However, the lowest concentration of phenolic compounds was observed from callus extracts (4.03 mg GAE/g DW). Based on these findings, there was no considerable difference ascertained between fruit extracts and calyx extracts of both regenerants and seedlings in terms of their total phenolic contents. On the other hand, leaf and stem extracts of regenerants contained more phenolic compounds compared to those of *ex vitro* seedling materials. Leaf extracts of regenerants contained 20.01 mg GAE/g DW while that of seedlings contained 12.14 mg GAE/g DW. Likewise, stem extract of regenerants contained 13.56 mg GAE/g DW whereas seedlings had 8.65 mg GAE/g DW. Therefore, based on these results, it was observed that (except for stem extracts) there was a significant correlation in between free radical scavenging activity and the total phenolics in all samples.

Furthermore, the total flavonoid contents of the aforesaid methanolic extracts were also determined spectrophotometrically by aluminium chloride method. The content of flavonoids was expressed as milligram catechol equivalents (CE) per gram dried weight. The results of total flavonoid contents show a similar pattern with those found in total phenolic assay. The results (Table 4.3; Figure 4.5, 4.7) indicated that, irrespective of the plant source, the methanolic extracts of fruits contained three to four-fold higher concentration of flavonoid compounds, 16.96 mg CE/g DW (fruit extract obtained from regenerants) and 16.51 mg CE/g DW (fruit extract obtained from seed-derived plant) than other methanolic extracts tested. Nevertheless, very low concentration of flavonoids was identified from methanolic extract of callus (1.87 mg CE/g DW). No significant difference was determined between extract of fruit and calyx obtained from regenerants and seedlings for their total flavonoid contents. However, the

significant difference was obtained in leaf and stem extracts, which showed higher flavonoid content in samples obtained from regenerants than those obtained from seedlings (compare 4.38 with 3.00 mg CE/g DW for leaf extracts, and 2.98 with 2.47 mg CE/g DW for stem extracts). In general, the total flavonoid contents of all extracts tested were found very low as compared to total phenolic contents. Except for fruit extracts which contained twice more phenolics than flavonoids, other extracts contained four to five-fold more phenolics than flavonoid compounds.

Table 5.3. Total phenolics content (TPC) and total flavonoids content (TFC) of different methanol extracts of *P. peruviana*. Gallic acid equivalent was used as mg GAE/g dry weight of plant material for phenolic compounds while catechol equivalent was used as mg CE/g dry weight of plant material for flavonoid compounds. The results were expressed as means ± SD of three independent experiments.

Plant materials	Extracts	TPC (mg/g DW)	TFC (mg/g DW)
In vitro-derived	Fruit	31.38 ± 1.2[a]	16.96 ± 0.22[a]
	Calyx	26.46 ± 1.1[b]	4.99 ± 0.24[b]
	Leaf	20.01 ± 1.3[c]	4.38 ± 0.23[bc]
	Stem	13.56 ± 1.7[d]	2.98 ± 0.27[c]
	Callus	4.03 ± 2.0[e]	1.87 ± 0.11[g]
Ex vitro-derived	Fruit	30.90 ± 1.0[a]	16.51 ± 0.22[a]
	Calyx	25.69 ± 1.6[bc]	5.01 ± 0.25[b]
	Leaf	12.14 ± 3.0[de]	3.00 ± 0.25[c]
	Stem	8.65 ± 2.1[f]	2.47 ± 0.20[d]

*Mean numbers with the same letter within the same column are not significantly different at P>0.05.

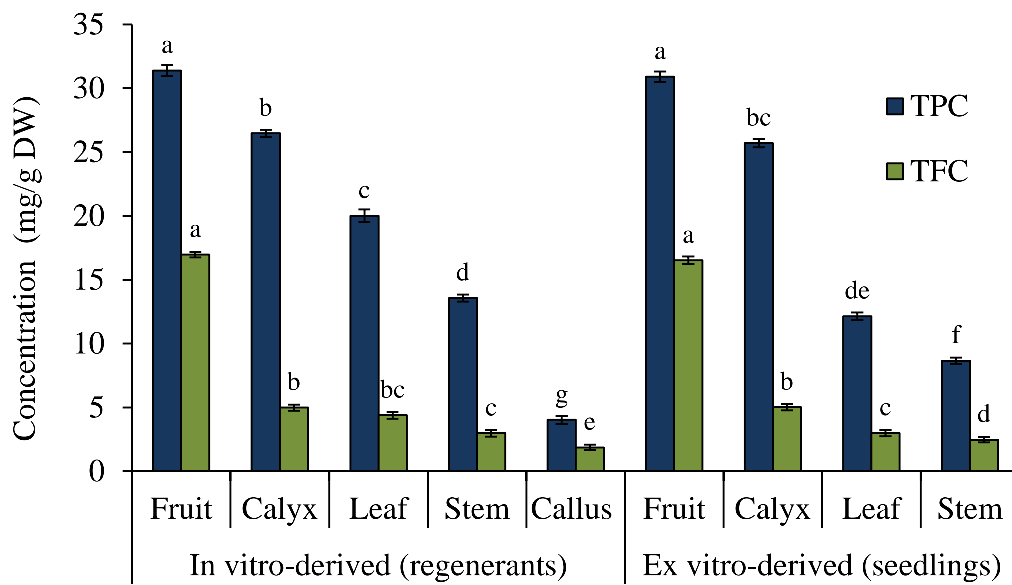

Figure 5.5. Total phenolic (TPC) and flavonoid (TFC) contents of different extracts of *in vitro*-derived and *ex vitro*-derived *P. peruviana*.

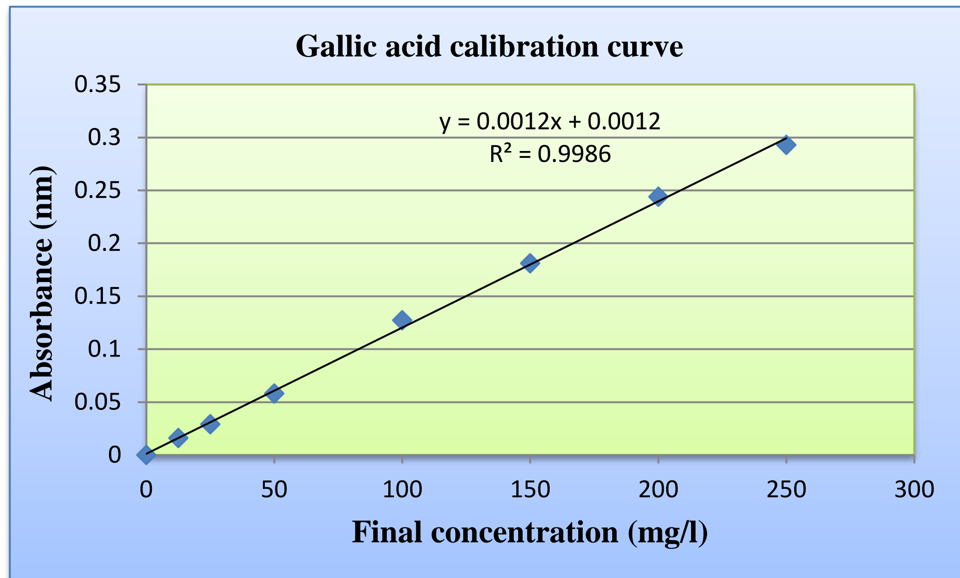

Figure 5.6. Gallic acid calibration curve.

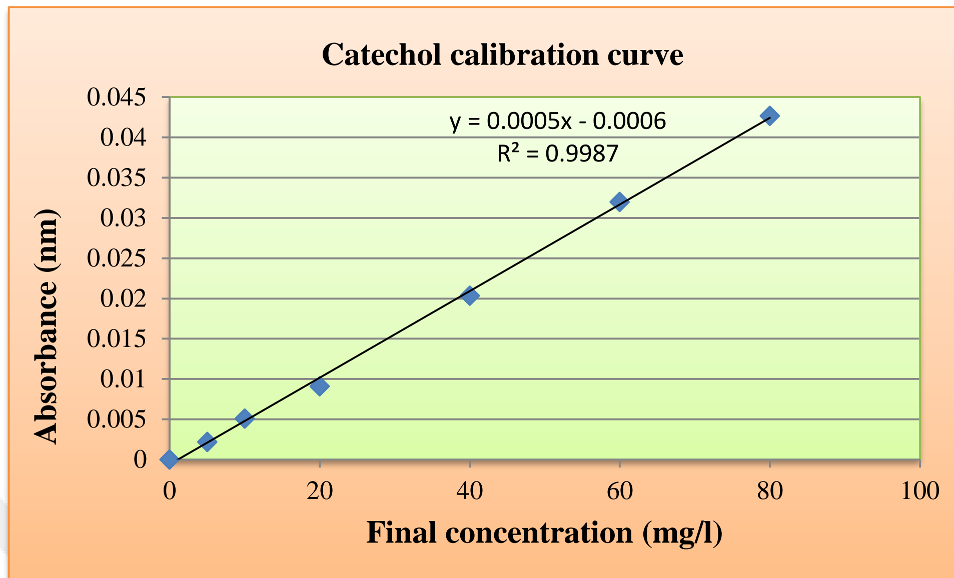

Figure 5.7. Catechol calibration curve.

5.4 Discussion

Antioxidant capacity of *P. peruviana* was reported in previous studies (Wu et al., 2006; Ramadan and Moersel, 2007; Vasco et al., 2008; Puente et al., 2011). It was demonstrated that *P. peruviana* leaves had potent antioxidant activity (Wu et al., 2006). Ramadan and Moersel (2007) investigated the antioxidant activity in *Physalis* juice with respect to reaction time with DPPH. They noted that the antioxidant capacity was related to its fat-soluble bioactive contents such as tocopherols, sterols and carotenoids. Vasco et al. (2008) analyzed antioxidant capacities of major fruits in Ecuador and determined that *P. peruviana* has the highest antioxidant activity at low concentration compared to other fruits. However, antioxidant activities of different *P. peruviana* extracts as mentioned here from different plant sources along with their phenolic and flavonoid composition have not been studied so far. Therefore, in this study antioxidant capacities of different extracts from different plant source were explored by using DPPH radical scavenging and in terms of their total amount of phenolics and flavonoids, which are well-known compounds with strong antioxidant properties. DPPH radical scavenging activity assay was chosen since it is one of the most favorable methods for determination

of antioxidant capacity of plant samples due to its simplicity and reproducibility (Demir et al., 2014).

The result of this work clearly indicates the highest antioxidant activity in fruit extracts, underline with high total phenolic and flavonoid contents. This high anti-radical effect of fruit extracts might be due to high concentrations of phenolics and flavonoids found in these extracts. It is known from previous studies that phenols and flavonoids are actively involved in plant cell antioxidant defense mechanism (Narváez-Cuenca et al., 2014). Furthermore, Benavente-García et al. (2000) reported that free radical scavenging activity of plant extracts may also depend upon the amount of polyphenols present in the extracts. The antioxidant activity of *P. peruviana* plant extracts is also attributed to its bioactive compounds, mostly phenolics (Casanova et al., 2008; Saito et al., 2008; Narváez-Cuenca et al., 2014). Demir et al. (2014) also reported high antioxidant capacity of *P. peruviana* fruit extract, presented the IC_{50} value of 0.430 mg/ml, which is nearly consistent with our results. When addressing the antioxidant activity of *P. peruviana* fruits, Licodiedoff et al. (2013) suggested that antiradical is influenced not only by phenolics but also flavonols rutin and myricetin. In addition, it was previously reported that the antioxidant mechanism of flavonoids may also come from the interaction between transition-metal ions and flavonoids to produce complexes that keep the metal ions from participating in the free-radical generation (Zayova et al., 2013). These results characterize the fruit of *P. peruviana* L. as a good source of natural phenolic compounds.

Physalis peruviana calyx extracts were also found to be good antioxidant with high concentration of total phenolic compounds. Although different kinds of research have been conducted on calyx extract of some solanaceous and many other plant species (Hirunpanich et al., 2006; Yin and Chao, 2008; Ge et al., 2009), up to now there is no study on evaluation of antioxidant activity of *P. peruviana* calyx. This is the first report concerning antiradical capacity of *P. peruviana* calyx extract as well as its phenolic and flavonoid compositions. The results of this investigation have demonstrated that screening of antioxidant activity from extracts of different isolated plant part rather than

whole plant give new understanding to which plant part is more preferable for a particular medicinal purpose as well as their potential used in pharmaceutical industries.

Even though stem extract of this plant contain low concentrations of phenols and flavonoids, it shows high free radical scavenging activity even more than leaf and calyx extracts. This protective effect is probably due to the presence of other several components that have high antioxidant action, rather than phenols and flavonoids; these may include vitamins (vitamin A), sterols and carotenoids. Therefore, the antioxidant activity of plant extract is not always attributed to their total phenolics or flavonoids, but total bioactive compounds present (Casanova et al., 2008; Saito et al., 2008). In contrast to this finding, Çakir et al. (2014) reported higher antioxidant capacity in leaf than shoot extracts. However, it important to note that there are many different antioxidants present in plants and it is very difficult to measure each antioxidant component separately. Therefore, some other antioxidants (rather than phenols and flavonoids) may be involved in inhibiting and quenching free radicals to terminate the radical chain reaction, acting as reducing agents.

On the other hand, callus extract was found ineffective for radical scavenging and also contained low concentrations of phenolics and flavonoids. This is probably because of the fact that some metabolites are not produced in dedifferentiated cells due to loss of production capacity. Moreover, some natural products are only being produced by whole plants, but not by isolated parts, due to the uncoupling of the enzymatic machinery (George, 2008). Another evident proving this, was observed in *Saccharum officinalis*, in which production of antioxidant diterpenes was only detected in shoots extracts not in callus and suspension cultures (Grzegorczyk et al., 2007). Similar results were also observed in callus and cell suspension cultures of *Crataegus monogyna*, where low rates of phenolic antioxidants were detected (Rakotoarison et al., 1997). Kim et al. (2011) as well observed lower phenolic content in callus tissue compared to leaves in *Stevia rebaudiana*. Nonetheless, these low antioxidants production levels can be overcome using genetic engineering approaches, through modulation of synthesis pathways by gene silencing, knockout, duplication etc. In addition, tissue culture methods can also be used through manipulation of culture media, including the addition

of growth regulators and precursors, manipulation of the environmental factors, elicitation, and selection of high production lines (Bernabe-Antonio et al., 2010; Hussein et al., 2010).

Another important aspect observed in this work was the higher antioxidant potential, phenolics and flavonoids from leaf and stem extracts of *in vitro*-derived plants as compared to those of *ex vitro*-derived plants. This difference may be associated directly or indirectly to the culture conditions and use of exogenous plant growth regulators which are also known to affect metabolite content (Coste et al., 2011). Hence, this finding indicates how plant tissue culture systems are influential not only on plant growth but also on metabolite production. However, regarding fruit and calyx extracts there was no difference observed between those collected from *in vitro*-derived plants and *ex vitro*-derived plants in terms of their antioxidant activity, total phenolic and flavonoid contents. These similarities in bioactive constituents are probably due to their exposure to field condition for a long time while waiting for fruit setting.

The quantitative estimation of the phytochemical constituents of different *P. peruviana* extracts shows that this plant is rich in phenols and flavonoids. It is also known that these compounds are highly effective not only as free radical scavengers but also in prevention and cure of some diseases related to free radicals. The positive influence of certain flavonoids and phenols or their potential anti-radical activities, particularly when they are available in large quantities in the edible parts, resulted in supplementation of natural antioxidants through an effective balanced diet, which is of great importance to consumers. Therefore, it can be concluded that fruits, stems, calyces and leaves of *P. peruviana* are good source of natural antioxidants.

CHAPTER V

6 CONCLUSIONS

The overall results of our study regarding medicinal plant *P. peruviana* show that *in vitro* regeneration of this plant via nodal segment is possible using plant growth regulator as growth promoters. Plant growth regulators have a decisive role in the regulation of morphological characteristic in this plant. The results from tissue culture experiment have proven that nodal explants cultured on media containing different concentration and types of cytokinin or auxin- or their combinations possess the potential to undergo adventitious regeneration. Regeneration occurred via meristemoids, demonstrating that the nodal explants cultured on a medium supplemented with TDZ were the most favorable for multiple shoot regeneration from a single nodal explant. In addition, medium containing different BAP concentrations also show multiple shoot induction, which can also be used as an efficient alternative for shoot regeneration. On the other hand, for root formation, media containing IAA were more favorable when compared to other plant growth regulators tested.

Moreover, callus culture was also observed to be possible in *P. peruviana*, and this finding will play an important role in gene transformation studies and production of many bioactive metabolites having medicinal properties by using bioreactors, elicitors, PGRs etc. The result obtained from callus culture may also give insight for further research on somatic embryo production in this plant which has not yet documented.

Furthermore, a simple and effective protocol for synthetic seed production in *P. peruviana* via encapsulation of nodal segment and subsequent conversion into plantlets was achieved in this study. Thus, it is plausible to use encapsulation technology for preservation of vegetative propagules of plants having economic importance. Encapsulation of the plant tissue in sodium alginate matrix is a useful technique for long-term storage and facilitates germplasm exchange between laboratories. This

protocol could not only be used for large-scale production and conservation of *P. peruviana*, but also for various plant species. Besides, direct planting of the synthetic seeds into the soil conform practical application of synthetic seed as an alternative to real seeds. This will probably give new awareness for the production of artificial seeds in the future.

Commercial production of bioactive secondary metabolites by traditional agriculture is an inefficient process. Strategies, based on *in vitro* culture methods, have been extensively studied to improve the production of specific plant-derived chemicals. In this report, the antioxidant activities of different methanolic extracts of *P. peruviana* were investigated. The results revealed that the fruit extracts have high antioxidant activity than other extracts, which make it a good source of natural antioxidant. Besides having high antioxidant capacity, the fruit extracts also contain high concentrations of both phenolic and flavonoids compounds, which are the main antioxidant compounds. The protocol described here can have an important contribution to the future efforts for a large scale production of natural compounds in this crop plant. In addition, the use of plant materials obtained from *in vitro*-derived regenerants was evident to be preferable compared to those obtained from seedling grown under *ex vitro* condition. Hence, plant tissue culture techniques provide plant material with increased accumulation of bioactive compounds. Moreover, the growing demand in today's marketplace for natural, safe and renewable products from the plant has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products. Therefore, *in vitro* techniques have been used as an alternative for the production of secondary metabolites including phenolics and flavonoids.

Hopefully, these findings would contribute to the future studies concerning this plant and other Solanaceous species in general. We expect that further analysis of this plant will give more attention to isolation of individual compounds and determination of their roles as antioxidant. We also expect that more effort will be given to the introduction of encapsulated propagules directly to the soil in further research.

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EDUCATION

1992-1995 **Nursery School** (3 years) – Sir Kashim Ibrahim College of Education Nursery and Primary School, Maiduguri, Barno State, **Nigeria**.

1995-2001 **Primary School** (6 years) – Ramat Primary School, Gashua, Bade Local Government, Yobe State, **Nigeria**.

2001-2007 **Secondary School** (6 years) – Government Secondary School Goneri, Gujba Local Government, Yobe state, **Nigeria**.

2008-2009 **University Foundation Program** (1 year) – Nigerian-Turkish International College, Abuja, **Nigeria**.

2009-2010 **English proficiency** (1 year) – Abant Izzet Baysal University, School of Foreign Languages, Bolu, **Turkey**.

2010-2013 Undergraduate Degree (3 years) – Abant Izzet Baysal University, Faculty of Art and Science, Department of Biology, Bolu, **Turkey**.

2013-2016 Master's Degree (2 years) Abant Izzet Baysal University, Faculty of Art and Science, Department of Biology, Bolu, **Turkey**.

RESEARCH PROJECTS PARTICIPATED:

1. **Stevia'nın (*Stevia rebaudiana* Bertoni) *in vitro* koşullarda üretimi, tatlandırıcı bileşiklerinin karşılaştırmalı analizi, izolasyonu, saflaştırılması ve maliyet unsurlarının hesaplanması (Eng: *In vitro* propagation of honey leaf (*Stevia rebaudiana* Bertoni), comparative analysis of sweeter compounds, isolation, purification and estimation of cost analysis).** A scientific project supported by TÜBİTAK 3001-programme, Project no: 114-O-006, Date: 2014-2016, Position: *Researcher*
2. ***Digitalis ferruginea* L. Türünün Kardenolit Sentez Metabolizmasının Moleküler Yöntemlerle Arastırılması ve Kardenolit içeriğinin Karsılastırmalı Analizi (Eng: Investigation of Cardenolide Synthase Metabolism via Molecular Techniques and Comparative Analysis of Cardenolide Contents in *Digitalis ferruginea* L. Species):** A scientific Project supported by TÜBITAK (1001-programme, Project no: 112-O 134), Date: 2012-2015, Position: *Researcher*
3. **The genus Digitalis: Molecular taxonomy, preservation, active constituents and therapeutic applications. A European Union Project funded under the international research staff exchange scheme (IRSES) from Marie Curie Foundation** (Grant Agreement Number: PIRSES-GA-2011-295251). Project Members (Countries): Germany, Portugal, Turkey, Brazil. Date: 2012-2016, Position: *Researcher*

WORKING EXPERIENCE

2012-2016 Worked in Plant Biotechnology laboratory, Department of Biology, Abant Izzet Baysal University, Bolu, **Turkey**.

Training/Skills

Plant Tissue Culture and Biotechnology: Micropropagation, artificial seed production, callus cultures, somatic embryogenesis, organogenesis, secondary metabolite production.

Chromatographical techniques: Thin layer chromatography (TLC), High performance liquid chromatography (HPLC).

Molecular genetic techniques: DNA/RNA extraction, Polymerase chain reaction (PCR)

Statistics: SPSS and Excell Data analyzer.

ACHIEVEMENTS

2009-2013 **Scholarship Award** for undergraduate studies provided by Yobe State Government, **Nigeria.**

2012/2013 **Best Student Award,** Department of Biology**,** Faculty of Art and Science, Abant Izzet Baysal University, Bolu, **Turkey**.

2013-2015 (20 months) research fellowship awarded by **Tübitak.**

2015-2016 (9 months) research fellowship to Brazil awarded by the **European Union.**

SCIENTIFIC ACTIVITY

Poster Presentations

1. **Mohammed A**, Arslan M, Gürel E, Yücesan B (2013) Altın Çilek (*Physalis peruviana* L.) Bitkisinin Doku Kültürü Yöntemleriyle Klonal Çoğaltımı. National **Congress** on garden and ornamental plants, Yalova, **Turkey**.

Oral Presentations

2. Yücesan B, **Mohammed A**, Ilal T, Gürel E. (2014) An Efficient *in vitro* regeneration system and synthetic seed production in Stevia" 6th World Convention on Stevia. Stevia Tasteful copyright, pp. 12, June 19-20 2014, Berlin, **Germany**.
3. Yücesan B, **Mohammed A**, Kılıçsaymaz B, Arslan M, Cihangir C**,** Ilal T, Muhammad S, Büyükgöçmen R, Gürel E (2014) Synthetic seed production of economically

important plants. Turkey 5th seed congress with international participation and sectorial business forum, Diyarbakır, **Turkey**.

4. Kılıçsaymaz B, Yücesan B, **Mohammed A**, Gürel E (2014) Mini tuber production from purple potato (*Solanum tuberosum* L. subsp. andigenum). Turkey 5th seed congress with international participation and sectorial business forum, Diyarbakır, **Turkey**.
5. Yücesan B, Eker I, Sameeullah M, **Mohammed A**, Cihangir C, Demir-Ordu Ö, Gürel E (2015) Türkiye'de doğal yetişen kardiyotonik glikozitlerce zengin yüksükotlarının (Digitalis L. spp.) in vitro tekniklerle üretimi. 11. Tarla Bitkileri Kongresi, 7-10 Eylül 2015 Çanakkale, **Turkey.**

PUBLICATIONS:

Book Chapter

1. Yücesan B, **Mohammed A**, Büyükgöçmen R, Kavas Ö, Kılıçsaymaz B, Arslan M, Cihangir C, İlal AT, Mohammed S, Sağırlı A, Gürel S, Eker İ, Altuğ C, Gürel E (2015) Comparision of conventional and Biotechnological approaches for *Stevia rebaudiana* production with elavated Rebaudioside A content. Proceedings of the 8th EUSTAS Stevia symposium, JMC Geuns and S Ceunen (Eds), Eu print, Heverlee, 2015, pp. 183-196.

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1. Yücesan BB, **Mohammed A**, Arslan M, Gürel E (2015) Clonal propagation and synthetic seed production from nodal segments of Cape gooseberry (*Physalis peruviana* L.), a tropical fruit plant. Turk J Agric For, 39: 797–806.
2. **Mohammed A**, Yücesan B, Demir-Ordu Ö, Cihangir C, Eker I, Kreis W, Gürel E (2015) In vitro regeneration and cardenolide determination of an endemic foxglove, *Digitalis cariensis* (Aegean Foxglove). In vitro Cell Dev Biol–Plant, 51: 438–444.
3. Yücesan B, **Mohammed A**, Sameeullah M, Eker I, Demir-Ordu Ö, Cihangir C, Şahbaz N, Kaya Ö, Müller-Uri F, Kreis W, Gürel E (2016) In vitro propagation and cardenolide profiling of *Digitalis ferruginea* subsp. *schischkinii*, a medicinally important foxglove species with limited distribution in Turkey. In vitro Cell Dev Biol–Plant, DOI: 10.1007/s11627-016-9759-4.

4. Yücesan B, **Mohammed A**, Büyükgöçmen R, Altuğ C, Kavas Ö, Gürel S, Gürel E (2016) *In vitro* and *ex vitro* propagation of *Stevia rebaudiana* Bertoni with high Rebaudioside-A content – A commercial scale application. Sci Hort 203: 20–28.
5. Moreira CM, De Andrade HB • Bertolucci SKV, Lameira OA, **Mohammed A**, Pinto JEBP (2016) Plantlets regeneration from young leaf segments of *Ananas erectifolius* - an Amazon species. Turk J Biol (in press).
6. Yücesan B, Büyükgöçmen R, Mohammed A, Sameeullah M, Altuğ C, Gürel S, Gürel E (2016) An efficient regeneration system and steviol glycoside analysis of *Stevia rebaudiana* Bertoni, a source of natural high intensive sweetener. In vitro Cell Dev Biol–Plant (accepted)
7. **Mohammed A,** Karakas F.P, Yücesan B, Sameeullah M, Gürel E (2016) Determination of antioxidant activities of *in vitro*-derived regenerants and field-grown seedlings of goldenberry (*Physalis peruviana* L.). Food Chemistry (accepted).