DEVELOPMENT OF IN VITRO MICROPROPAGATION TECHNIQUES FOR SAFFRON (CROCUS SATIVUS L.)

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

EVRİM YILDIRIM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

AUGUST 2007

Approval of the Thesis:

DEVELOPMENT OF IN VITRO MICROPROPAGATION TECHNIQUES FOR SAFFRON (CROCUS SATIVUS L.)

submitted by **EVRİM YILDIRIM** in partial fulfillment of the requirements for the degree of **Master of Science in Department of Biology, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Zeki Kaya _____
Head of the Department, **Biology Department, METU**

Prof. Dr. Zeki Kaya _____
Supervisor, **Biology Department, METU**

Examining Committee Members:

Assoc. Prof. Dr. Sertaç Önde (*) _____
Biology Department, METU

Prof. Dr. Zeki Kaya (**) _____
Biology Department, METU

Assoc. Prof. Dr. Murat Ekici _____
Biology Department, Gazi University

Assist. Prof. Dr. Ayşe Gül Gözen _____
Biology Department, METU

Assist. Prof. Dr. Fatih Temel _____
Faculty of Forestry, Artvin Çoruh University

Date: 27.08.2007

(*) Head of committee
(**) Supervisor

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.

Name, Last name : Evrim Yıldırım

Signature :

ABSTRACT

DEVELOPMENT OF IN VITRO MICROPROPAGATION TECHNIQUES FOR SAFFRON (CROCUS SATIVUS L.)

Yıldırım, Evrim

M. S. Department of Biology
Supervisor: Prof. Dr. Zeki Kaya
August, 2007, 76 pages

In vitro micropropagation of saffron (*Crocus sativus* L.) by using direct and indirect organogenesis was the aim of this study. Also, the effect of plant growth regulators on growth parameters, such as corm production, sprouting time and germination ratio were investigated in *ex vitro* conditions.

For *in vitro* regeneration of saffron, the effects of 2,4-D (2,4-dichlorophenoxyacetic acid) and BAP (6-benzylaminopurine) were tested initially. It was observed that 0,25 mg/L 2,4-D and 1 mg/L BAP combination was superior for indirect organogenesis while 1 mg/L 2,4-D and 1 mg/L BAP combination was favorable for direct organogenesis. During the improvement of direct organogenesis experiments, BAP (1 mg/L) without 2,4-D stimulated further shoot development.

For adventitious corm and root induction, NAA (naphthaleneacetic acid) and BAP combinations were tested. Although a few corm formations were achieved, root development was not observed with these treatments. Further experiments with the culture medium supplemented with 1 mg/L IBA (indole-3-butyric acid)

and 5% sucrose was effective on obtaining contractile root formation and increasing corm number. As a result, the overall efficiency was calculated as 59.26% for contractile root formation, 35.19% for corm formation and 100% for shoot development.

In *ex vitro* studies, 50 mg/L IAA (indole-3-acetic acid) , 50 mg/L kinetin and 200 mg/L GA_3 (gibberellic acid) were used. These applications were not as efficient as expected on assessed growth parameters.

Keywords: *Crocus sativus* L.; saffron; corm; *in vitro* micropropagation; direct organogenesis; indirect organogenesis; 2,4-D; BAP; NAA; IBA.

ÖZ

SAFRAN BİTKİSİNDE (CROCUS SATIVUS L.) IN VITRO KOŞULLARDA MİKROÜRETİM TEKNİKLERİNİN GELİŞTİRİLMESİ

Yıldırım, Evrim

Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi: Prof. Dr. Zeki Kaya
Ağustos, 2007, 76 sayfa

Bu çalışmada, doğrudan ve dolaylı organ gelişimi metodları kullanılarak safran bitkisinin *in vitro* koşullarda üretimi hedeflenmiştir. Ayrıca bitki büyüme düzenleyicilerinin; korm üretimi, çimlenme zamanı ve çimlenme oranı gibi gelişim parametreleri üzerine etkileri *ex vitro* koşullarda araştırılmıştır.

Safranın *in vitro* koşullarda üretimi için ilk olarak 2,4-D (2,4-Diklorofenoksiasetik asit) ve BAP (benzilaminopürin) büyüme düzenleyicilerinin etkileri test edilmiştir. 0,25 mg/L 2,4-D ve 1 mg/L BAP kombinasyonunun dolaylı organ gelişimi için, 1 mg/L 2,4-D ve 1 mg/L BAP uygulamasının ise doğrudan organ gelişimi için uygun olduğu anlaşılmıştır. Doğrudan organ gelişiminin iyileştirilmesi çalışmalarında, 2,4-D bileşeninin besiyerlerinden çıkarılması ve sadece BAP (1 mg/L) içermesi sürgün gelişimini arttırmıştır.

Korm ve kök oluşumu üzerinde, NAA (naftelen asetik asit) ve BAP büyüme düzenleyicilerinin değişik kombinasyonlarının etkileri araştırılmıştır. Bu uygulamalarla az sayıda korm oluşumu gerçekleşirken, köklenme meydana gelmemiştir. Sonraki deneylerde kullanılan 1 mg/L IBA (indol-3-bütirik asit) ve

%5 şeker içeren büyüme ortamı kontraktil kök oluşumunda ve korm sayısının arttırılmasında oldukça etkili olmuştur. Sonuç olarak, toplam başarı kontraktil kök oluşumu için %59.26, korm oluşumu için %35.19 ve sürgün oluşumu için %100 şeklinde hesaplanmıştır.

Safranın *ex vitro* çalışmalarında, 50 mg/L IAA (indol-3-asetik asit), 50 mg/L kinetin ve 200 mg/L GA_3 (giberellik asit) kullanılmıştır. Bu uygulamalar gelişim parametreleri üzerinde beklenen düzeyde etkili olmamıştır.

Keywords: *Crocus sativus* L.; safran; korm; *in vitro* mikropropagasyon; doğrudan organ gelişimi; dolaylı organ gelişimi; 2,4-D; BAP; NAA; IBA.

To My Family and My Love,

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Zeki Kaya for his guidance, encouragements and advice throughout the research. I would also like to express my regards to Assoc. Prof. Dr. Sertaç Önde for his great support and guidance all through my thesis. I would like to thank to Neslihan Parlakgöz and Taner Parlakgöz from Biopatent Company and İrfan Kandemir for providing the saffron corms.

I express my sincere appreciation to all members of thesis examining committee for their comments and criticism.

I would like to thank Assist. Prof. Dr. Tolga Yıldırım, who helped me with his broad knowledge about the topic. Assist. Prof. Dr. Fatih Temel is also gratefully acknowledged for his great help and patience through statistical analysis.

I am thankful to all my lab partners Ayten Dizkırıcı, Cansu Özge Tozkar, Hatice Elif Özcan, Zeynep Özkurt, Kubilay Yıldırım, Aslı Özdilek, Melis Or Akman, Yasemin Parastouk and Aysun Demet Güvendiren for their friendship and help.

Finally, I would like to express my deepest thanks to my family, especially my mom, for their endless love, patience, insight, support and encouragements all through my life. I would also like to express my special thanks to my sweetheart, Serdar, for his true love, support and patience.

This study was supported by the State Planning Organization (DPT) Grant No: BAP-08-11-DPT-2002K120/510.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
2,4-D	2,4 Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
GLM	General linear model
HCL	Hydrogen chloride
$HgCL_2$	Mercuric chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
MS	Murashige and Skoog basal medium
NAA	Naphthaleneacetic acid
NaOH	Sodium hydroxide
PGR	Plant growh regulator

CHAPTER I

INTRODUCTION

1.1 Saffron (*Crocus sativus* L.)

Saffron, *Crocus sativus* L., is an important crop cultivated as the source of its spice for at least 3,500 years. Dried stigmas of saffron flowers compose the most expensive spice which has been valuable since ancient times for its odoriferous, coloring, and medicinal properties (Plessner *et al*., 1989). The name saffron is commonly used to refer both to the spice and the plant itself.

Some archaeological and historical studies indicate that domestication of saffron dates back to 2,000-1,500 years BC (Grilli Caiola, 2004).

The origin of saffron is obscure, but the plant is believed to have originated in the eastern Mediterranean, probably in Asia Minor and Persia. The name 'saffron' is derived from Arabic *zá-faran* which means 'be yellow' (Winterhalter and Straubinger, 2000).

1.1.1 Saffron Taxonomy

Saffron is classified into Magnoliophyta division, class Liliopsida and order Asparagales. It is a member of the Iridaceae family and the *Crocus* L. genus (Table 1.1).

Iridaceae family includes about 60 genera and 1,500 species. The plants in this family are herbs with rhizomes, corms or bulbs. The *Crocus* genus includes approximately 80 species worlwide. There are about 32 species of *Crocus* genus in Turkey (Vurdu and Güney, 2004). Among these, the cultivated species saffron is well known with its high economic value (Fernández, 2004).

Table 1.1 Scientific classification of *Crocus sativus* L.
(Judd *et al*., 2002; Davis, 1984)

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Subclass	Monocots
Order	Asparagales
Family	Iridaceae
Genus	*Crocus*
Species	*Crocus sativus* L.

Most of the *Crocus* species grow naturally in fields between shrubs and grasses or in light woodlands. The species in the genus *Crocus* have underground fleshy corms and basal, grass-like, dark green leaves with whitish median stripe. Their leaves appear with or after flowers. A plant may have one or several flowers. The fruit is a capsule and they have numerous seeds with brownish or reddish color.

There are several features which help to identify *Crocus* species from each other. The lobe numbers of styles, the character of corm tunics, appearance of foliage before or after flowering, number of leaves, the flower color, and the anther color

are some of these features. The distinctive characteristics of saffron are given in the following sections.

1.1.2 Saffron Morphology and Biology

Saffron is a sterile geophyte with underground fleshy corms. These corms are three to five cm in diameter and covered by tunics. Corms consist of nodes and are internally made up of starch-containing parenchyma cells. A corm survives for only one season, reproducing via division into "cormlets" that eventually give rise to new plants.

Each corm produces six to nine leaves. The photosynthetic activity of the leaves during the early winter and the early spring months contributes to the formation of the replacement corms at the base of the shoots. Leaves are grass-like and dark green with whitish median stripe. The leaves wither at the onset of the dry season. The plant shows no aboveground organs or roots during late spring and most of summer.

Flowering spans from late autumn until December according to climatic conditions. Each corm yields one to three purple flowers that have three violet sepals and three petals similar together; i.e., tepal. The flowers of *Crocus* are bisexual. Pistil is central with a tubular ovary that a thin style comes out of it. Style is long and pale yellow that is branched to an orange red three-branch stigma which is 20 to 35mm long. Dried stigmas are the source of the commercial spice saffron. Androecium consists of three distinct stamens. Anthers are on top of the filaments. Saffron flowers are sterile; i.e., the plant is not able to set viable seed. Therefore, corms are indispensable for the propagation of saffron (Warburg, 1957).

Saffron corms produce both fibrous roots and contractile roots. The fibrous roots emerge from a single ring at the base of the corms. These roots are straight and thin (about 1mm in diameter). The contractile root has the appearance of a tuber

organ, very large and whitish. This type of roots enable corms to dig into the ground, so corms rest in optimum depth and position in the soil (Chio-Sang, 1996). A general structure of saffron and organs are shown in Figure 1.1.

Figure 1.1 Saffron morphology.

a. General appearance of saffron; b. Purple flowers have three violet sepals and three petals similar together; i.e., tepal; c. Three distinct yellow stamens; d. Orange red three-branch stigma; e. Grass-like and dark green leaves with whitish median stripe; f.Saffron corms with contractile roots (Index of Zukan web page-available at http://aquiya.skr.jp/zukan/Crocus_sativus.jpg. and Webshots Home&Garden web page-available at www.webshots.com/explains/home-and-garden/saffron.html, Last access date: August 16, 2007)

Saffron has a life span of about 220 days. Rainfall in the autumn, warm summers and mild winters are the favorable climatic conditions for high yields of saffron. Water requirement of saffron is low. Garden soil (clay sand) is suitable for optimum growth of saffron. A relatively low water use, growth and development during fall and winter and a very low harvest index are some of remarkable characteristics of saffron

1.1.3 Reproductive Biology of Saffron

The saffron is a sterile geophyte that produces annual renewal corms and is propagated only by them (Warburg, 1957; Mathew, 1982).

Cytological studies indicate that saffron is a sterile triploid (2n=3x=24) plant (Brighton, 1977; Mathew, 1982). Although it has been considered to be an autotriploid, there may still be some uncertainty whether it is an alloploid or an autoploid. Many authors have hypothesized the origin of saffron by autotriploid (Brighton, 1977; Mathew, 1977; Ghaffari, 1986). The probable origin is a cross between diploids derived from the fertilization of an unreduced egg cell by haploid sperm or a haploid egg by two haploid sperms. On the basis of overall length and centromeric position, the somatic chromosomes could be assembled in seven triplets, one pair and one single chromosome. The karyotype consists of a series of chromosome triplets with the exception of one group of three chromosomes of which one is different from the others. Because of this unique chromosome in saffron genotype, selfing can be excluded (Chichiricco, 1984). Since *Crocus sativus* is sterile, the presence of this chromosome may be explained by the existence of chromosomal polymorphism at diploid level in the progenitors, most probably *C. thomasii* Ten or *C. cartwrightianus* Herb. (Brighton, 1977; Mathew, 1999, Grilli Caiola, 2004).

Triploid nature of the species allows for vegetative multiplication, but not for regular sexual reproduction. This is because in triploids meiosis and gamete development are irregular resulting into many anomalies in sporogenesis and gametophyte development (Chichiricco, 1984). Saffron infertility is mainly related to the male gametophyte (Chichiricco, 1989). Saffron does not produce viable seed; therefore corms are indispensable for its propagation.

1.1.4 Saffron Uses

The use of saffron goes back to the ancient times. It is most commonly used in medicine, as well as dye and spice in food industry (Basker and Negbi, 1983). Saffron has been also used as a drug to treat various human health conditions such as coughs, stomach disorders, colic, insomnia, chronic uterine haemorrhage, femine disorder, scarlet fever, smallpox, colds, asthma and cardiovascular disorders (Basker and Negbi, 1983; Giacco, 1990; Winterhalter and Straubinger, 2000; Abdullaev, 2003).

It has been shown that saffron is a protective agent against chromosomal damage (Premkumar *et al*., 2003 and 2004), a modulator of lipid peroxidation, and an antioxidant and detoxifying spice (Premkumar *et al*., 2003). Antinociceptive and anti-inflammatory (Hosseinzadeh and Younesi, 2002), antiseizure (Hosseinzadeh and Khosravan, 2002) and blood-pressure reducing (Fatehi *et al*., 2003) effects in animals were also reported. Saffron extract or its active constituents, crocetin and crocin, could be useful as a treatment for neurodegenerative disorders accompanying memory impairment (Zhang *et al*., 1994). Several other studies have reported that these main ingredients improved memory and learning skills in mice and rats (Sugiura *et al*., 1995; Abe and Saito, 2000). Picrocrocin has a sedative effect on spasms (Giaccio, 1990). Crocin extracts have been used for the treatment of nervous, cardiovascular and respiratory systems (Abe and Saito, 2000). Crocin is also unique antioxidant that struggle with oxidative stress in neurons (Ochiai *et al*., 2004). An antidepressant effect of saffron petal and stigma in mice was showed (Karimi *et al*., 2001). The positive effect of saffron

water extracts has also been described in patients suffering from allergic asthma (Haggag *et al*., 2003). Hartwell (1982) reported that saffron extracts were used against different kinds of tumors and cancers in ancient times. Therefore, liver, spleen, kidney, stomach and uterus tumors have been treated with pharmaceutical preparations of saffron. A number of studies in animal model systems have showed an antitumor effect of saffron on different malignant cells. Some researchers also indicated that saffron might be a potential anticancer agent (Nair *et al*., 1991; Escribano *et al*., 1996; Premkumar *et al*., 2001; Abdullaev, 2002; Abdullaev and Espinosa-Aquirre, 2004).

Despite wide use of this unique spice, it is highly priced due to high demand and low supply. Saffron prices at wholesale and retail rates range from US$1100–US$11,000 per kilogram. In Western countries, the average retail price is US$2200 per kilogram (Hill, 2004). It is considered to be the highest priced spice in the world (Winterhalter and Straubinger, 2000). The main reason for its great cost is that saffron is still propagated by human help (Figure 1.2). The cultivation processes, such as planting, flower harvesting and separation of the stigmas are carried out manually. Each saffron flower has three stigmata and one stigma weights about 2mg. It takes 150,000-200,000 flowers and over 400 h of hand labor to produce 1kg saffron stigma (Plessner *et al*., 1989). That is why there are some differences in quality between saffron of different origins and the subsequent fluctuation of prices. Its high value makes saffron the object of adulteration. The addition of artificial colorants is one of the most common means of deception. Such a practice is expected to improve the appearance of the dried stigmas or even to give rise to the coloring strength of the aqueous extract. Other methods include mixing in extraneous substances like beet, pomegranate fibers, red-dyed silk fibers, or the saffron's tasteless and odorless yellow stamens, and submerging saffron fibers with viscid substances like honey or vegetable oil. Saffron consumption is rising but production is not enough to meet this demand. In order to ensure the future of saffron crop, it is necessary to improve cultivation techniques. The production should be increased due to rising in utilization of saffron as natural product through worldwide.

Figure 1.2 The cultivation processes of saffron

a. Planting; b. and c. Flower harvesting; d. Separation of the stigmas (Saffron Spain web page-available at www.saffron-spain.com/ingles/azafran.html, European Saffron webpage-available at www.europeansaffron.eu/ftp/raccolta.jpg and www.europeansaffron.eu/ftp/donne_al_lavoro.jpg, last access date: August 16, 2007)

1.2 Saffron Production in the World and Turkey

Saffron is currently being cultivated more or less intensely in Iran, India, Greece, Morocco, Spain, Italy, Turkey, France, Switzerland, Israel, Pakistan, Azerbaijan, China, Egypt, United Arab Emirates, Japan and recently in Australia (Tasmania) (Figure 1.3). The world's total annual saffron production is estimated as 205 tons per year. Iran is said to produce 80 percent of this total; i.e., 160 tons, and Khorasan province alone 137 tons of the totals. The Kashmir region in India

produces between 8 to 10 tons mostly dedicated to India's self-consumption. Greek production is 4 to 6 tons per year. Morocco produces between 0.8 and 1 ton. Saffron production has decreased rapidly in many traditionally producing countries, and is abandoned in others such as England and Germany. Spain was used to be the traditional world leader and most reputed saffron producer for centuries in areas of La Mancha and Teruel. Nowadays, the production is only about 0.3-0.5 tons. Productions of Italy (Sardinia, Aquila, Cascia) 100kg; Turkey (Safranbolu) 10kg; France (Gâtinais, Quercy) 4-5kg; and Switzerland (Mund) 1kg are nearly insignificant. All saffron producers in the European Union, as well as in Turkey, suffer from increasing labour costs (Fernández, 2004).

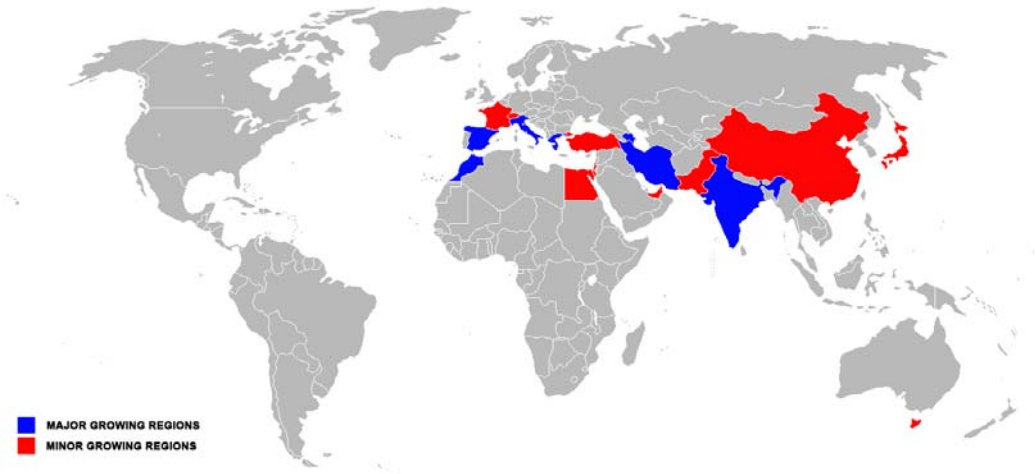

Figure 1.3 Distribution and major producers of saffron
(Adopted from Saffron web page-available at
http://en.wikipedia.org/wiki/Saffron, Last access date: August 16, 2007)

1.3 Tissue Culture Studies of Monocots

As mentioned in the previous sections, saffron is a monocotyledon member of the large family Iridaceae. Comparatively, bulbous and cormous monocotyledons are regarded as difficult *in vitro* material. Contamination is a serious problem during micropropagation of monocots especially if below ground organs, such as corms, bulbs, rhizomes and tubers, are used as an explant source. The size of geophyte, physical damage and dormancy are the other problems which make tissue culture studies difficult.

Schenk and Hildebrandt (1972) reported the importance of medium composition and techniques for induction and growth of monocotyledonous and dicotyledonous plants in cell culture. They found that a high level of auxin-type growth regulating substances generally favored cell cultures of monocotyledonous plants, while low levels of cytokinin were essential for most dicotyledonous cell cultures.

Within the last few decades, an increasing number of bulbous and cormous monocotyledons have been successfully cultured. Tissue culture technology was greatly influenced by the demand of rapid multiplication and clonal propagation of slow-growing monocots. Several economically important monocot species constituting nutritional, medicinal or ornamental groups of plants were used for *in vitro* clonal propagation (Sutter, 1986) and production of secondary metabolites (Aslanyants *et al*., 1988). Organogenesis and somatic embryogenesis from differentiated tissues of bulbous and cormous monocots, such as *Crocus sativus* L., are also reported in the literature as detailed in the following sections.

1.3.1 Organogenesis of Saffron

Organogenesis means the development of adventitious organs or primordia from an explant source. Direct and indirect are the two types of this method. In direct organogenesis, a cell or a group of cells differentiate to form organs. Indirect

organogenesis is the development of adventitious organs originating from an intervening callus phase. Callus is an unorganized or undifferentiated mass of proliferative cells produced in culture and also in nature. It is made up of a mass of loosely arranged thin walled parenchyma cells arising from the proliferating cells of parent tissue.

Many plant regeneration studies concerning saffron via direct and indirect organogenesis have been reported as explained in the following sections.

1.3.1.1 Organogenesis Studies for *in vitro* Propagation Purposes

Plessner *et al*. (1990) reported *in vitro* corm production in saffron. Corms smaller than 1cm in diameter and isolated apical buds from larger corms were used as explants. The nutrient medium consisted of MS (Murashige and Skoog, 1962) minerals, supplemented with sucrose (3%), nicotinic acid (5mg/L), pyrodoxine-HCL (1mg/L), thiamine-HCL (0.5mg/L), myo inositol (100mg/L), adenine sulphate (160mg/L) and casein hydrolysate (500mg/L). The medium was solidified with 0.9% agar. 1 mg/L 2,4-D (Dichlorophenoxyacetic acid), 3-12 mg/L kinetin and 3 mg/L zeatin were used as growth regulators. They reported that cytokinins, particularly zeatin, and the auxin 2,4-D were essential for regular bud development *in vitro*. They also examined the effects of ethylene and ethapon on organogenesis. Ethylene and ethaphon pretreatments inhibited leaf development, but on the other hand, induced corm production as well as dormancy. However, rooting could not be achieved.

Direct adventitious shoot regeneration from ovary explants of *Crocus sativus* L. was revealed by Bhagyalakshmi (1999). Media components, incubation conditions, and age of the explants were the factors influencing the regeneration. Full strength MS medium supplemented with NAA (naphthelene acetic acid) and BA (benzyladenine) produced the best response towards caulogenesis (28%) with highest shoot numbers per ovary. On the whole, the best response toward shoot growth, both in terms of leaf length and number, was on the medium with

0.54μM NAA and 2.22μM BA. Ovaries of different growth stages having stigmas of pale yellow, pale orange and bright orange regenerated a maximum mean number of shoots per ovary. Further development of ovary-derived shoots was influenced by the composition of basal salts in the culture medium where full strength MS salts gave the best response of those tested. Regenerated shoots produced normal photosynthetic leaves and corms.

Plant regeneration studies of saffron via indirect organogenesis from callus cultures have also been reported. Ilahi *et al.* (1986) described the morphogenesis in saffron tissue culture. Corms of saffron were cultured on half strength MS medium supplemented with different combinations of growth regulators; i.e., auxin and cytokinins, and coconut milk. Callus was induced in a medium containing 0.5 mg/L each of 2,4-D and BAP, and 2% coconut milk. The same culture was used for differentiation of callus into buds. They reported that an increase in 2,4-D also enhanced callus formation but suppressed shoot-bud formation. These shoots were induced to root when inoculated on a medium containing 2 mg/L NAA for 24h. However, further growth of these roots was slow when reinoculated on half strength MS containing 0.1 mg/L each of 2,4-D and BAP. In another set of experiments when a piece of callus, growing in similar conditions, was transferred to MS medium containing 0.5 mg/L NAA, 0.1 of either BAP or kinetin and 2% coconut milk, the nodules gave rise to roots after 4 weeks of culture with subsequent suppression of the shoot development.

1.3.1.2 Organogenesis Studies for Secondary Metabolite Production Purposes

In vitro production of stigma-like structures of *Crocus sativus* for the purpose of crocin, picrocrocin and safranal induction was also reported. Stigma-like structures were reported to be induced from almost every part of floral organs, including half ovaries (Himeno and Sano, 1987; Sano and Himeno, 1987; Loskutov *et al.*, 1999), stigmas (Koyama *et al.,* 1988; Sarma *et al*., 1990), petals

(Lu *et al*., 1992; Jia *et al*., 1996), anthers (Fakhrai and Evans, 1990) and stamens (Zhao *et al*., 2001).

Himeno and Sano (1987) described the synthesis of crocin, picrocrocin and safranal by saffron stigma-like structures proliferated *in vitro*. One young half-ovary (0.3 mg, fresh weight) was placed on each medium and incubated at 20 ºC, in the dark. Some of the stigma-like structures were formed on Linsmaier-Skoog medium (LS) supplemented with NAA (10ppm) and kinetin (1ppm), and the others were formed on Nitsch medium supplemented with NAA (1ppm) and BA (1ppm). The stigma-like structures were formed directly on the explants after 10 weeks. They found that the average of concentration of crocin and picrocrocin in the stigma-like structures grown on Nitsch medium were about 3-fold higher than those grown on LS medium, while the total contents in each structure were about the same.

Loskutov *et al*. (1999) studied the optimization of *in vitro* conditions for stigma-like structure production from half-ovary explants of *Crocus sativus*. The optimum proliferation of stigma-like structure was observed on B5 basal medium (Gamborg B5 medium) containing NAA (5.4 μM), BA (44.4 μM), MS organics, casein hydrolysate (0.05%) and L-alanine (11.2 μM). They reported that the amounts of crocin, crocetin, picrocrocin and safranal in stigma-like structure, as determined by high performance liquid chromatography analysis, were similar to those found in natural saffron.

Sarma *et al*. (1990) also reported *in vitro* production of stigma-like structures from stigma explants of *Crocus sativus* L. MS medium supplemented with NAA (10mg dm^{-3}) and BA (1mg dm^{-3}) induced the optimum response. NAA was found to be an important additive to achieve a good response.

Zeng *et al.* (2003) recorded the increased crocin production and induction frequency of stigma-like structures from floral organs of *Crocus sativus* by precursor feeding. MS medium supplemented with 5 mg/L kinetin and 4 mg/L

NAA was used as the basal medium. Almost all of the stigma-like structures formed directly from explants, instead of from callus.

Induction of crocin, crocetin, picrocrocin and safranal synthesis in callus cultures of saffron was reported by Visvanath *et al*. (1990). Callus cultures were obtained from floral buds on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin. The cultures could be induced to produce red globular callus and red filamentous structures which produced crocin, crocetin, picrocrocin and safranal.

Crocin production using *Crocus sativus* callus by two stage culture system was reported by Chen *et al*. (2003). Saffron callus was grown in a two-stage culture on B5 medium supplemented with casein hydrolysate (300mg/L) at 22 ºC in dark with 2 mg/L NAA and 1mg/L BA to give maximum biomass (16g dry wt/L), and with 2 mg/L IAA (indole-3-acetic acid) and 0.5 mg/L BA for crocin formation. The maximum crocin production (0.43 g/L) was achieved by this two-stage culture method.

Chen *et al*. (2004) also examined the promotion of growth of *Crocus sativus* cells and the production of crocin by rare earth elements. They reported that La^{3+} and Ce^{3+}, either individually or as a mixture, promoted crocin production of *Crocus sativus* callus but Nd^{3+} (40μM) had little effect and all metal ions were toxic above 100μM. La^{3+} (60μM) promoted growth of callus significantly but increased crocin only slightly. Ce^{3+} (40μM) significantly promoted crocin production but had little effect on cell growth. They showed that La^{3+} (60μM) and Ce^{3+} (20μM) together gave the highest dry weight biomass (20.4g/L), crocin content (4.4mg/g) and crocin production (90mg/L).

Although most of the studies about *in vitro* production of saffron secondary metabolites via direct and indirect organogenesis were reported in the literature, there were very few studies related to the achievement of a whole plant. All these studies make clear that there were no problems with the callus and shoot

formation of saffron. However, corm and especially root formation were very rare events in organogenesis of saffron.

1.3.2 Somatic Embryogenesis of Saffron

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathways that lead to reproductive regeneration of non-zygotic embryos capable of germinating to form complete plants.

There are few studies in the literature about the regeneration of saffron via somatic embryogenesis. Ahuja *et al*. (1994) indicated the somatic embryogenesis and regeneration of plantlets in saffron. Somatic embryogenesis was initiated in *Crocus sativus* from shoot meristems on LS medium containing $2x10^{-5}$M BA and $2x10^{-5}$M NAA. They observed the various stages of somatic embryogenesis in the same medium and the development was asynchronous. Maturated embryos could be germinated on half strength MS medium containing 20 mg/L gibberellic acid (GA_3). Complete plantlets with well developed root system and corm formation were obtained on transferring germinated embryos to half strength MS supplemented with $5x10^{-6}$M BA, $5x10^{-6}$M NAA and 2% activated charcoal.

Somatic embryogenesis in saffron was described by also Blázquez *et al*. (2004). They used MS culture medium supplemented with 0.5 mg/L BAP and 0.1 mg/L 2,4-D for induction of somatic embryogenesis. Embryogenic calli were subcultured in MS medium containing 1 mg/L BAP and 0.05 mg/L NAA for multiplication in solid medium. Temporary immersion systems (TIS) were used for this purpose. A four-fold increase in the production of embryogenic calli (fresh weight increase) was observed in TIS culture when compared to solid medium. They obtained the best result when 1 mg/L of paclobutrazol was added. They also improved the development of somatic embryos on solid medium supplemented with 0.5 mg/L jasmonic acid (JA) and obtained plant regeneration via somatic embryogenesis after eight weeks of treatment of JA in combination with sucrose.

Karamian (2004) indicated the plantlet regeneration via somatic embryogenesis in four species of *Crocus*. Shoot meristem culture on LS medium containing 4 mg/L NAA and 4 mg/L BA or 1 mg/L 2,4-D and 4 mg/L kinetin was used for somatic embryogenesis in *C. sativus, C. cancellatus, C. michelsonii* and *C. caspius.* Asynchronous somatic embryogenesis in all of the four species was investigated and various stages of somatic embryo development were observed when embryogenic calli with globular somatic embryos were transferred into half strength MS medium containing 1 mg/L abscisic acid. According to Karamian (2004), maturated embryos could be germinated on half strength MS medium supplemented with 25 mg/L GA_3. Finally, complete plantlets were obtained by transferring germinated embryos into half strength MS medium supplemented with 1 mg/L NAA and 1 mg/L BA at 20ºC under 16/8 h (light/dark) cycle.

1.4 *Ex vitro* Studies of Saffron

The effect of gibberellin on functional activity of dormant saffron corms were reported by Azizbekova *et al.* (1982). At the time of transplantation, saffron corms were immersed in a solution of gibberellin (100 mg/L) for 4h. Corms immersed in water for 4h served as the control. After these treatments, corms were planted in soil. They demonstrated that treating saffron corms with gibberellin leads to subsequent acceleration of growth and development processes, increase of leaf and root length, and increase in the number of flowers.

Unfortunately, there were not many *ex vitro* studies about saffron in the literature. However, *ex vitro* studies of *Gladiolus* were reported more than saffron as explained in the following section. This species is also monocot with corms and is very similar to saffron. Therefore, it was thought that these studies could also be applicable for saffron.

The effectiveness of chemicals such as gibberellins (Arora *et al.*, 1992), BA (Goo *et al.*, 1998), ethephon (Suh, 1989) and methyl disulfide (Hosoki and

Kubara, 1989) in breaking the dormancy of *Gladiolus* corms and cormels has been studied extensively. Ram *et al*. (2002) indicated that plant growth regulators affect the development of both corms and cormels in gladiolus. Three plant growth regulators, BA (25, 50 and 100 mg/L), ethephon (100, 200 and 400 mg/L), and GA_3 (25, 50 and 100 mg/L) were tested on fresh cormels. The cormels were soaked in the solutions for 24h and they planted in the field. In conclusion, ethephon at 400 mg/L was most effective treatment for many of the growth parameters including days to sprouting, percentage sprouting, corm diameter and cormel production and weight.

Halevy (1986) also reported the induction of contractile roots in *Gladiolus grandiflorus.* Various growth substances applied to the leaves or corms did not induce contractile roots in dark-grown plants but roots were induced by IBA in both small and large corms grown at constant temperatures and light.

1.5 Justification of the Study

Saffron (*Crocus sativus* L.) has been cultivated for its red stigmas since ancient times. The saffron plant is a sterile geophyte and propagates by vegetative reproduction through the formation of daughter corms from the mother corm. Although corms are indispensable for its propagation, there are some risks. The saffron is known to be infested by pathogenic fungi and viruses which are carried over by the corms and may affect the crop under certain conditions (Plessner *et al*., 1990).

Saffron producers suffer from increasing labor costs. In the countries, where saffron is grown, cultivation is still performed by traditional methods. Corm planting, flower harvesting, stigma separation and corm lifting are carried out manually. Saffron also grows slowly as being a geophyte. All these factors contribute to the high price of saffron.

One of the possible means of reducing the production costs would be mechanized harvesting of saffron flowers. However, this method may cause the deleterious results by damaging the foliage and therefore drastically reducing the production of replacement corms. Also, it may reduce the quality of saffron by damaging the stigma (Plessner *et al*., 1989).

In order to ensure the future of saffron crop, it is necessary to improve cultivation techniques, plant material and quality evaluation methods. The worldwide increase in utilization of saffron as a natural product requires new biological and economical developments. To stabilize the production of saffron, new methods and techniques must to be developed urgently.

Compared to the traditional crop or chemical synthesis methods, plant tissue culture might offer a great potential for saffron production. Tissue culture has been employed for the propagation of rare and difficult species. For modernizing the cultivation of the saffron, efficient mass production of pathogen-free corms through tissue culture will become a prerequisite in the future.

Due to its expensive nature, majority of the *in vitro* studies are related to the production of secondary metabolites through direct and indirect organogenesis. Very rare studies progress to whole plant formation, complete with shoots, corms and roots. Another reason for the need of development efficient *in vitro* regeneration systems is that the saffron species is threatened of being extinct (Vurdu, 1993). Therefore, the attention should be given to conserve this species through *in vitro* systems. For the development of efficient *in vitro* regeneration systems, various parameters like plant growth regulators and media compositions must be investigated. Also *ex vitro* studies generally support *in vitro* studies. By this study, various gaps in tissue culture of saffron and in *ex vitro* studies are aimed to be filled.

1.6 Objectives of the Study

In vitro micropropagation of saffron (*Crocus sativus* L.) by using direct and indirect organogenesis was the main goal of this study. The effect of plant growth regulators on *ex vitro* growth parameters, such as corm production, sprouting time and germination ratio were also investigated. The specific objectives were:

- To induce adventitious shoot formation by using apical and lateral buds of saffron with direct organogenesis methods,

- To induce callus formation by using corm parts other than buds with indirect organogenesis methods,

- To achieve whole corms with roots by culturing adventitious shoots originated from direct and indirect organogenesis experiments,

- To acclimate regenerated (developed) plantlets for releasing to the field, and

- Through *ex vitro* studies, to increase the corm production, sprouting time and germination ratio.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

The corms of saffron plants (*Crocus sativus* L.) were obtained from Turkey (Kastamonu) and Iran. The only corms coming from Kastamonu were used in *in vitro* studies, because the Iranian source-corms were infested with microorganism and wounded that made them difficult to be used as an explant source. However, corms obtained from Kastamonu and Iran were used together for *ex vitro* studies.

Corms were stored in shallow trays at 20-25 ºC in the dark until use. During this period, corms grow continuously and produced apical and lateral buds which were later used as explant sources for *in vitro* studies.

2.1.2 Chemicals

All chemicals were plant tissue culture tested and purchased from Duchefa Biochemie (Haarlem, The Netherlands) and Sigma-Aldrich Chemical Company (St. Louis, USA).

Murahige and Skoog (MS) medium (Murahige and Skoog, 1962) including all vitamins (Duchefa Prod. No: M0222) was used in this study. The composition of this medium is given in Appendix A.1.

6-benzylaminopurine (BAP) (Duchefa Prod. No:B-0904), 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma Cat. No:D-4517), naphthaleneacetic acid (NAA) (Sigma Cat. No:N-0640), indole-3-butyric acid (IBA) (Sigma Cat. No:I-5386), kinetin (Sigma Cat. No: K-0753) , indole-3-acetic acid (IAA) (Sigma Cat. No:I-2886) and gibberellic acid (GA_3) (Duchefa Prod. No: G-0907) were used as plant growth regulators.

L-Ascorbic acid (Sigma Cat. No: A-7506), sucrose (Duchefa Prod. No: S-0809), and plant agar (Duchefa Prod. No: P1001) were used as media supplements.

Mercuric chloride ($HgCl_2$) (Sigma Cat. No: M-1136) and %97 ethanol (Botafarma Labaratuarı, Ankara, Turkey) were used for surface sterilization purposes.

2.1.3 Glassware

Glassware was purchased from Isolab-Interlab (Ankara, Turkey) and Sigma-Aldrich Chemical Company (St. Louis, USA). Baby jars (Sigma Cat. No:V-8630) with autoclavable caps (Sigma Cat. No:B-8648), test tubes (Sigma Cat. No: C-5916), standard 10 cm glass petri dishes (Isolab-Interlab) and polystyrene petri dishes (Isolab-Interlab) were used during the experiments.

2.1.4 Instrumentations

There were two laminar flow-hoods for all aseptic procedures, Holten Laminar TL2472 (Alerot, Denmark) supported with UV sterilization lamp (Philips TUV 30W G30T8 UV Longlife, Holland) and Bassaire Laminar 04HB (Southampton, UK).

Autoclave was used for sterilization of nutrient media, distilled water, baby jars and test tubes whereas dry oven was used for petri dishes sterilization.

Growth room was illuminated by cool white fluorescent lamps 58W/33 x 2/shelf and 36W/33 x 2/shelf (Philips TLD, Poland) as 16h light/8h dark cycle. Temperature of the growth room was adjusted at 24±1ºC.

2.1.5 Photography

HP Photosmart 735 Digital Camera with 3.2 mega pixel sensor resolution, Sony Cyber-shot DSC-T3/S Digital Camera with 5.1 mega pixel sensor resolution and Leica DFC 320 Digital Camera System were used for photography.

2.2 Methods

2.2.1 *Ex vitro* Propagation of Saffron

The purpose of this experiment was to obtain increased numbers of corms as a consequence of cytokinin, auxin and GA_3 treatments in *ex vitro* conditions. Corms coming from Kastamonu and Iran were used for these *ex vitro* experiments. After the saffron corms were soaked in solutions containing different concentrations of IAA, kinetin and GA_3, corm production, sprouting time and germination ratio traits were followed and data were collected.

IAA and kinetin efficiency were investigated in the first year experiment. Corms were soaked in 50 mg/L IAA and then 50 mg/L kinetin for 3 h each solution. Finally, corms were taken out and planted into pots containing light sandy-loam soil (Fleurelle®, M.S.S. Tic. Ltd. Şti., Türkiye). A control group was designed including corms that were not treated with any plant growth regulator. Plants were grown in a greenhouse under inductive conditions of light and fluctuating temperatures during 5 months. Only the numbers of corms; i.e., corm production data were recorded at the end of the experiment.

In the second year, corms were first treated with 200 mg/L GA_3 solutions for 6 hours in order to break up the dormancy. They were left to dry for overnight. Next day, just like the first year experiment, corms were first treated with 50 mg/L IAA solution for 3 hours, and then soaked in 50 mg/L kinetin solution for 3 hours. Corms were taken out and planted into pots containing a mixture of light sandy-loam soil (Güliş, Tarım Ürünleri İmalat Paz. İç ve Dış Tic.Ltd.Şti, Türkiye), garden soil and perlite. Some aphids were observed in first year experiment probably due to the soil type. Therefore, different trade mark soil with a mixture was used in second year. There were also control groups in this experiment. Plants were grown in a greenhouse under inductive conditions of light and fluctuating temperatures during 9 months. After first sprouting, numbers of leaves were recorded each week periodically. The numbers of corms, sprouting time and germination ratio data were collected. Experimental set up is displayed in Figure 2.1.

Figure 2.1 *Ex vitro* procedures of saffron corms

a. Corms were put into the pots including mixture of light sandy-loam soil, garden soil and perlite; b. Leaf numbers were recorded on each week periodically after the first sprouting; c. and d. New corms were obtained at the end of the cultivation time.

2.2.2 *In vitro* Propagation of Saffron

2.2.2.1 Surface Sterilization of Saffron Corms

The only corms coming from Kastamonu were used and thoroughly washed with running tap water for 30 minutes. The protective layer; i.e., tunics were removed gently from the corms and then they were surface sterilized with 70% (v/v) ethanol for 30 seconds in a laminar flow-hood. The surface sterilization of saffron corms was completed by dipping in 0.15% (w/v) $HgCl_2$ solution for 20

minutes. Finally, samples were rinsed with sterile distilled water for three times to remove residual $HgCl_2$. Each rinse was performed for 2 minutes.

2.2.2.2 Sterilization of Culture Media, Glassware and Laminar Flow-hood

All tissue culture media were sterilized by autoclaving at 121 ºC under the pressure of 1.1 kg/cm^2 for 20 minutes. Test tubes, baby jars with autoclavable caps and distilled water were sterilized by autoclaving for 25 minutes. Petri dishes, forceps and scalpels were heat sterilized at 180 ºC for two hours in a dry oven. Forceps and scalpels were flame-sterilized prior to use. According to explant type, the nutrient media were aseptically dispensed into sterile baby jars, test tubes or petri dishes.

Manipulations of the explants and transfer procedures were all performed in a sterile environment provided by a laminar flow-hood. Working surface area of the flow-hood were disinfected with UV light for 15 minutes and then sterilized with 70% ethanol. Laminar flow-hood was not used prior to 15-20 min after startup.

2.2.2.3 Explant Source

Corms of 2-3 cm in diameter were used as explant sources. All parts shown in Figure 2.2 were utilized for the propagation of saffron; i.e., the apical and the lateral buds were used for direct organogenesis, and the remaining parts of the corms which were sliced into small sections (5-6mm) were used for indirect organogenesis. All procedures were performed under aseptic conditions.

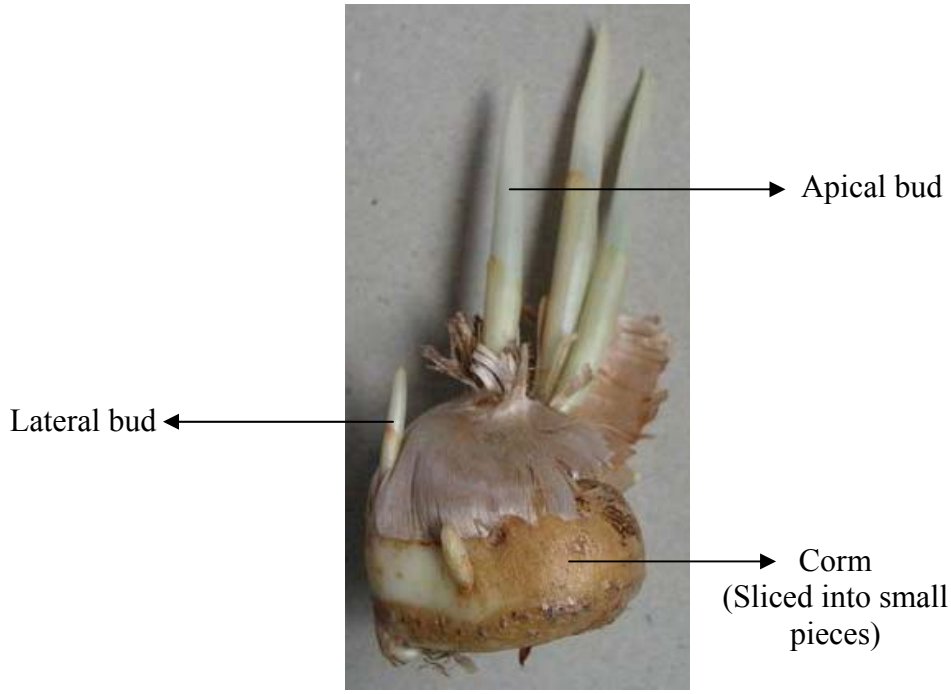

Figure 2.2 Explant source

2.2.2.4 Preparation of Nutrient Media and Culture Conditions

In vitro micropropagation of *Crocus sativus* L. was investigated through three groups of experiments in this study. These were indirect organogenesis, direct organogenesis and adventitious corm and root induction experiments. Various media compositions and culture conditions were designed for each set. Unless otherwise stated, the following media composition was used throughout all the three experimental sets. MS media including vitamins was supplemented with 3% sucrose, 100 mg/L ascorbic acid and various plant growth regulators (concentrations and types are given under each group of experiment). The pH of the media was adjusted to 5.8±0.1 by the addition of NaOH or HCI in a drop-wise manner. Finally, the nutrient media were solidified with 0.8% plant agar and then sterilized by autoclaving.

Explants were subcultured every three weeks and were incubated in growth room at 24±1 ºC. While the explants for direct organogenesis (shoot induction) were kept under 16h light/8h dark regime, callus induction explants (in indirect organogenesis experiments) were hold continuously under dark conditions. The first datum was recorded after an 8-week incubation period, and data collection was regularly repeated every two months.

2.2.2.5 Indirect Organogenesis Experiments

The effects of plant growth regulators on callus induction (indirect organogenesis) were studied.

Since the importance of 2,4-D and BAP combination on indirect organogenesis of saffron was reported in previous studies as explained in section 1.3.1.1 and 1.3.1.2, we also tested 2,4-D and BAP. Twenty five different treatments with three replicates were applied for callus induction experiments as shown in Table 2.1.

For explant source, corm parts other than apical and lateral buds were sliced into small sections and five were transferred into a petri dish. Five pieces were enough for one petri dish. They were incubated under dark conditions for about six months. Callus initiation, callus growth, meristemoid, shoot and root initiation data were recorded to evaluate indirect organogenesis (Table 2.2).

Table 2.1 Growth regulator combinations tested for indirect organogenesis

Treatments	2,4-D (auxin) (mg/L)	BAP (cytokinin) (mg/L)
1 (Control)	0.00	0.00
2	0.25	0.00
3	0.50	0.00
4	1.00	0.00
5	2.00	0.00
6	0.00	0.25
7	0.25	0.25
8	0.50	0.25
9	1.00	0.25
10	2.00	0.25
11	0.00	0.50
12	0.25	0.50
13	0.50	0.50
14	1.00	0.50
15	2.00	0.50
16	0.00	1.00
17	0.25	1.00
18	0.50	1.00
19	1.00	1.00
20	2.00	1.00
21	0.00	2.00
22	0.25	2.00
23	0.50	2.00
24	1.00	2.00
25	2.00	2.00

Table 2.2 Recorded traits and their units in indirect organogenesis experiments

Traits	**Units**
Callus Initiation	Count 0 = no callus formation 1 = callus formation
Callus Growth	Class 1 = 1-2cm in diameter 2 = 3-4cm in diameter
Meristemoid Initiation	Count 0 = no meristemoid formation 1 = meristemoid formation
Shoot Initiation	Count 0 = no shoot formation 1 = shoot formation
Root Initiation	Count 0 = no root formation 1 = root formation

2.2.2.6 Direct Organogenesis Experiments

Similar to the indirect organogenesis experiments, the effects of 2,4-D and BAP on direct organogenesis were tested based on previous studies explained in section 1.3.1.1 and 1.3.1.2. While three different levels of 2,4-D were used as the auxin type of growth regulator, five different levels of BAP were used as the cytokinin type, which add up to fifteen treatments with five replicates as shown in Table 2.3.

Table 2.3 Growth regulator combinations tested for direct organogenesis

Treatments	2,4-D (auxin) (mg/L)	BAP (cytokinin) (mg/L)
1 (Control)	0.0	0.0
2	0.0	0.5
3	0.0	1.0
4	0.0	3.0
5	0.0	5.0
6	0.1	0.0
7	0.1	0.5
8	0.1	1.0
9	0.1	3.0
10	0.1	5.0
11	1.0	0.0
12	1.0	0.5
13	1.0	1.0
14	1.0	3.0
15	1.0	5.0

Under sterile conditions, apical and lateral buds were carefully excised and were transferred into the test tubes containing the fifteen different treatments (shoot induction media). All cultured explants in these treatments were incubated in growth room for about six months. Shoot initiation, shoot growth, callus, meristemoid and root initiation data were recorded to evaluate direct organogenesis (Table 2.4). Shoot growth was visually scored on a scale from 1 (shortest) to 10 (longest).

Table 2.4 Recorded traits and their units in direct organogenesis experiments

Traits	**Units**
Shoot Initiation	Count 0 = no shoot formation 1 = shoot formation
Shoot Growth	Class 1 = barely open white shoots 2 = a small number of white shoots less than 2cm 3 = a few white shoots less than 4cm 4 = white shoots less than 6cm 5 = several green shoots with 6-10cm 6 = green shoots with 10-12cm 7 = a lot of green shoots with 12-14cm 8 = green shoots with 14-16cm 9 = many green shoots with 16-18cm 10 = longer green shoots with corms
Callus Initiation	Count 0 = no callus formation 1 = callus formation
Meristemoid Initiation	Count 0 = no meristemoid formation 1 = meristemoid formation
Root Initiation	Count 0 = no root formation 1 = root formation

To improve the adventitious shoot induction (direct organogenesis) further, new growth regulator combinations were generated based on the results obtained from the fifteen treatments applied in the previous experiment.

Eight treatments (two different levels of 2,4-D and four different levels of BAP) with two replicates were prepared as shown in Table 2.5. Shoots obtained so far from all previous experiments (indirect and direct organogenesis experiments) were used as explant sources. The excised shoots were transferred to baby jars randomly and they were incubated in the growth room about two months.

Table 2.5 Growth regulator compositions tested for improvement of direct organogenesis experiments

Treatments	2,4-D (auxin) (mg/L)	BAP (cytokinin) (mg/L)
1	0.0	0.5
2	0.0	1.0
3	0.0	1.5
4	0.0	2.0
5	0.1	0.5
6	0.1	1.0
7	0.1	1.5
8	0.1	2.0

Shoot initiation, shoot growth, callus, meristemoid and root initiation data were also recorded to evaluate improvement of direct organogenesis as previously shown in Table 2.4.

2.2.2.7 Adventitious Corm and Root Induction Experiments

Corm and/or root formation is a prerequisite for achieving success in indirect or direct organogenesis studies as explained in section 1.3.1.1. Therefore, such experiments were designed in this section.

2.2.2.7.1 Auxin Pulse Experiment for Corm and Root Induction

MS medium including all vitamins supplemented with two concentrations of NAA (0.1 or 0.5 mg/L) were prepared and adventitious shoots obtained from previous experiments (direct and indirect organogenesis) were transferred to these media randomly. Explants were cultured for a week in the growth room.

After this one week of auxin "pulse" experiment, explants were transferred to a new medium containing 1 mg/L BAP with two replicates and were grown approximately for six months. Due to lack of corm and root formation, only traits described in Table 2.4 were recorded in auxin "pulse" experiment as well.

2.2.2.7.2 Continuous NAA Experiment for Corm and Root Induction

"Continuous" auxin treatments were also prepared by keeping NAA constantly in the medium in combination with BAP. Two NAA levels (0.1 or 0.5 mg/L) and 1 mg/L BAP were used in these experiments and explants were incubated in growth room for four months as two replicates. Although subsequent shoot development and a few corm formations were achieved, root initiation was not observed. Traits described in Table 2.6 were recorded during the "continuous" auxin experiments.

Table 2.6 Recorded traits and their units in continuous NAA efficiency on corm and root induction

Traits	**Units**
Corm Initiation	Count 0= no corm formation 1= corm formation
Root Initiation	Count 0= no root formation 1= root formation
Shoot Growth	Class 1 to 10 (as described in Table 2.4.)

2.2.2.7.3 IBA and Increased Sucrose Experiment for Corm and Root Induction

Finally the effects of IBA and increased sucrose level were investigated to increase corm number and to induce root formation. MS medium including all vitamins was supplemented with 5% sucrose and three levels of IBA as shown in Table 2.7.

Table 2.7 Levels of IBA used for corm and root induction experiments

Treatments	IBA (auxin) (mg/L)
1	1
2	2
3	0 (No IBA)

Shoots obtained from previous corm and root induction experiments which produced limited corm initiation were subcultured in these three different treatments with three replicates. They were grown for two months and traits described in Table 2.8 were recorded. These explants could also be selected according to their genotypes in this experiment. Throughout the study, explants from thirty different genotypes were used to determine the effect of genotypes on *in vitro* regeneration capacity of saffron. Each corm was considered a separate genotype. Unfortunately, a large number of explants were lost due to contamination at previous experiments and there were not enough replicates for comparing the genotype effects. However, explants were available with respect to genotypes with adequate repetition in adventitious corm and root induction experiment. Twelve different genotypes could be used for this experiment. The effects of genotypes on traits described in Table 2.8 were also observed during this experiment.

Table 2.8 Recorded traits and their units in IBA and increased sucrose experiment for corm and root induction

Traits	**Units**
Shoot Growth	Class 1 to 10 (as described in Table 2.4)
Number of Shoots	Number 1 to 20
Corm Initiation	Count 0= no corm formation 1= corm formation
Number of Corms	Number 1 to 7
Root Initiation	Count 0= no root formation 1= root formation
Number of Roots	Number 1 to 22

2.2.3 Statistical Analyses

All of the statistical analyses were carried out using the SAS statistical package (SAS Inst. 9.1.3, 2002-2003). Analysis of Variances (ANOVA) with General Linear Model (GLM) was used to examine the effects of multiple factors (treatments, auxins, cytokinins and genotypes) on response variables (shoot, callus, root and corm formation).

In order to evaluate the effects of the treatment, Model 1 was applied.

$$Y_{ijk}=\mu+r_i+t_j+e_{ijk} \qquad \text{(Model 1)}$$

where; Y_{ijk} is the k^{th} individual in the j^{th} treatment and in the i^{th} replication, μ is the overall mean, r_i is the i^{th} replication, t_j is the j^{th} treatment and e_{ijk} is the experimental error.

To determine the effects of auxin, cytokinin and auxin-cytokinin interaction, Model 2 was used.

$$Y_{ijkl}=\mu+r_i+c_j+a_k+c*a_{(jk)}+e_{ijkl} \qquad \text{(Model 2)}$$

where; Y_{ijkl} is the l^{th} individual which was treated with j^{th} cytokinin level and k^{th} auxin level in the i^{th} replication, μ is the overall mean, r_i is the i^{th} replication, c_j is the j^{th} cytokinin level, a_k is the k^{th} auxin level, $c*a_{(jk)}$ is the interaction between the j^{th} cytokinin and the k^{th} auxin levels, and e_{ijkl} is the experimental error.

Finally to determine the genotype effects, Model 3 was employed.

$$Y_{ijkl}=\mu+r_i+g_j+t_k+g*t_{(jk)}+e_{ijkl} \qquad \text{(Model 3)}$$

where; Y_{ijkl} is the l^{th} individual in the k^{th} treatment and in the i^{th} replication, μ is the overall mean, r_i is the i^{th} replication, g_j is the j^{th} genotypes, t_k is the k^{th}

treatment, $g*t_{(jk)}$ is the interaction between the j^{th} genotypes and the k^{th} treatment, and e_{ijkl} is the experimental error.

The mean and standard error (SE) values were calculated for all independent variables and they were given in graphical forms by using Microsoft Office Excel 2003 to reveal their effects on recorded traits.

CHAPTER III

RESULTS AND DISCUSSION

In this study, the main objective was the *in vitro* regeneration of *Crocus sativus* via direct and indirect organogenesis. Either through callus initiation or directly through adventitious shoot formation, corm development with contractile roots were tried to be obtained. *Ex vitro* studies were also performed in greenhouse by investigating the effects of plant growth regulators on growth parameters, such as corm production, corm weight and sprouting time.

3.1 *Ex vitro* Propagation of Saffron

IAA (50 mg/L) and kinetin (50 mg/L) efficiency on corm production was detected in first year experiment and results are shown in Table 3.1.

Table 3.1 The effects of plant growth regulators on number of corms in the first year

Treatments	**Kastamonu Corms**		**Iran Corms**	
	Before Planting	End of Planting	Before Planting	End of Planting
PGRs	24	64	19	24
Control	24	55	10	13

As evident from the Table 3.1, treating corms with plant growth regulators for corm production were not as effective as expected. When the corms were treated with PGRs, the rate of increase was 167% in Kastamonu corms and 26% in Iran corms. The number of corms in control groups also increased as 129% in Kastamonu corms and 30% in Iran corms.

In the second year, the effects of GA_3 (200 mg/L), IAA (50 mg/L) and kinetin (50 mg/L) on corm production, sprouting time and germination ratio parameters were also recorded. Unfortunately, these treatments caused negative effects on corm production as shown in Table 3.2.

Table 3.2 The effects of plant growth regulators on number of corms in the second year

Treatments	**Kastamonu Corms**		**Iran Corms**	
	Before Planting	End of Planting	Before Planting	End of Planting
PGRs	63	34	23	18
Control	62	52	13	12

The rate of decrease was 46% in Kastamonu corms and 22% in Iran corms when PGRs were applied. The number of corms in control groups also decreased as 16% in Kastamonu corms and 8% in Iran corms.

Treating with GA_3 may be one of the reasons for the decrease. In some kind of plants, such as barley as a typical monocot, the dormancy of seeds and buds can be broken by GA and germination is promoted (Seeds and Seed Germination web page, 2007). The natural gibberellin composition of saffron corms may be overturned by GA application *ex vitro*. The other reason might be a mixture of

soil and perlite. The soil with different trademark from the first year was used. Perlite provides aeration and optimum moisture retention for plant growth. Water requirement of saffron is low. If the decreased numbers of control group were to be considered, the second reason was more probable.

Days to sprouting of saffron corms coming from Kastamonu and Iran are shown in Figure 3.1.

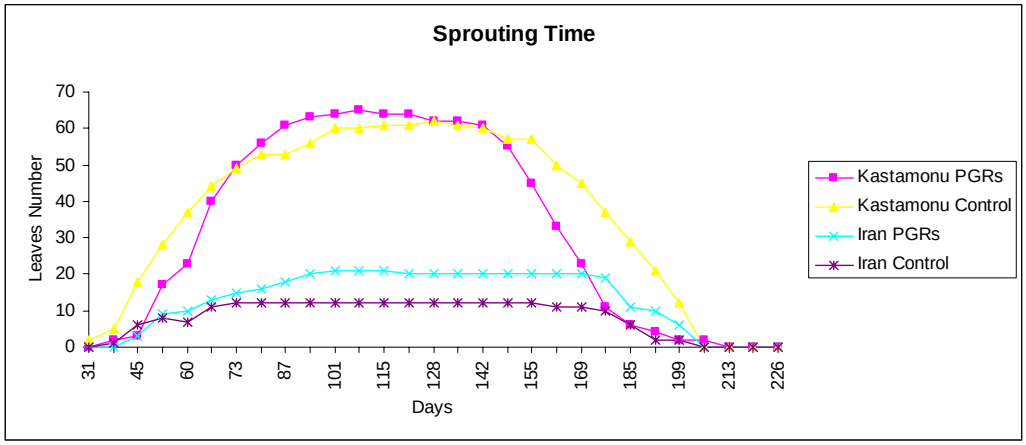

Figure 3.1 Sprouting times of saffron corms obtaining from Kastamonu and Iran

As shown in the figure, control groups generally germinated earlier than the treated ones with PGRs. The sprouting time was about 180 days. However, PGR applications were not effective on sprouting time.

Germination ratio of Kastamonu and Iran corms are shown in Figure 3.2. Towards germination rate, control groups of Kastamonu corms yielded more leaves. However, PGRs applications were favorable on Iran corms. Iran sources may still retain dormancy while Kastamonu sources may have lost this property.

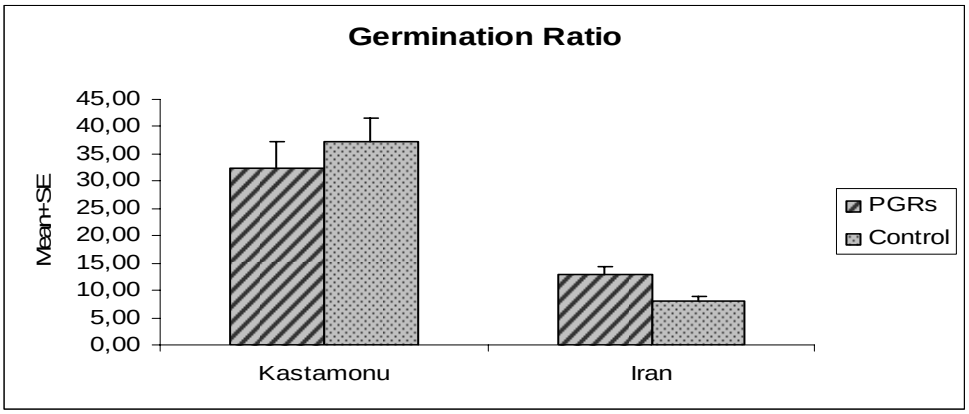

Figure 3.2 Treatment effects on germination ratio of saffron corms from Kastamonu and Iran

3.2 *In vitro* Propagation of Saffron

3.2.1 Indirect Organogenesis Experiments

Callus initiation, callus growth, meristemoid, shoot and root initiation data were recorded for indirect organogenesis experiments. Because data on meristemoid, shoot and root initiation were statistically insignificant, the indirect organogenesis capacity of saffron is based on callus initiation and callus growth data.

Twenty five different treatments including five levels of both auxin and cytokinin were applied in indirect organogenesis experiments as explained in the previous sections (Table 3.3). In order to find out the efficient combination of auxin and cytokinin level, Analysis of Variance (ANOVA) was performed with respect to treatments applied (Table 3.4).

Table 3.3 Treatments applied in indirect organogenesis (Numbers are given to treatments for easing the evaluation of ANOVA)

Treatments	Auxin 1 (0 mg/L 2,4-D)	Auxin 2 (0.25mg/L 2,4D)	Auxin 3 (0.5 mg/L 2,4D)	Auxin 4 (1.0 mg/L 2,4D)	Auxin 5 (2.0 mg/L 2,4D)
Cytokinin 1 (0 mg/L BAP)	1*	2	3	4*	5
Cytokinin 2 (0.25 mg/L BAP)	6**	7**	8*	9**	10
Cytokinin 3 (0.5 mg/L BAP)	11*	12	13	14	15
Cytokinin 4 (1.0 mg/L BAP)	16*	17	18	19	20
Cytokinin 5 (2.0 mg/L BAP)	21	22	23	24	25

* These treatments were not represented with bars in Figures 3.3 and 3.4 due to lack of any response.

** All explants in these treatments were lost due to contamination; therefore, they were not analyzed and were not shown in Figures 3.3 and 3.4.

Table 3.4 ANOVA for the traits studied in indirect organogenesis with respect to treatment levels (DF: degrees of freedom)

Traits assessed for Indirect Organogenesis		Replication	Treatment	Error
		(DF : 2)	(DF : 21)	(DF : 256)
Callus Initiation	Mean Square	0.0685	1.7413**	0.4705
Callus Growth		0.2981	1.5398**	0.4334

** Significant at $p < 0.01$

It was observed that treatments had significant effects (at $p < 0.01$) on callus initiation and callus growth. To understand which treatments were more effective than the others, the mean values for each treatment were given in graphical forms (Figures 3.3 and 3.4).

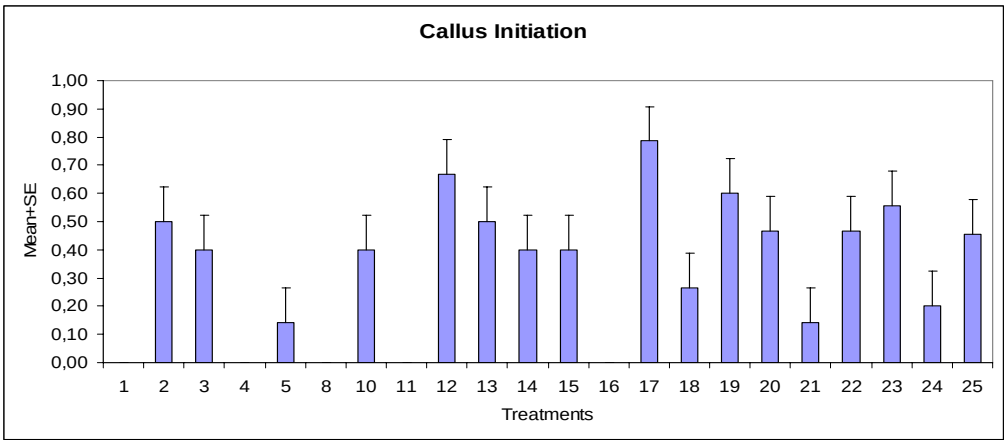

Figure 3.3 Treatment effects on callus initiation (vertical lines on the bar graphs indicate the standard errors)

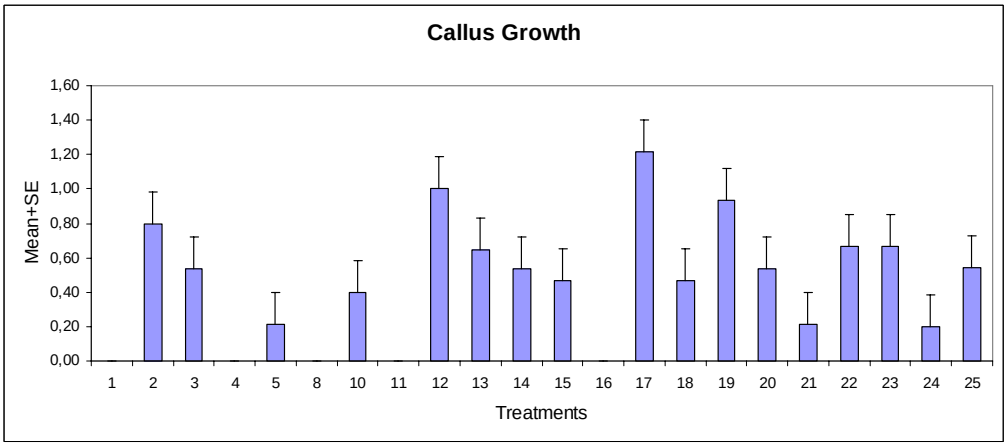

Figure 3.4 Treatment effects on callus growth (vertical lines on the bar graphs indicate the standard errors)

According to these graphs, treatment 12 (0.25 mg/L 2,4-D and 0.5 mg/L BAP) and treatment 17 (0.25 mg/L 2,4-D and 1 mg/L BAP) yielded the best responses with respect to both callus initiation and callus growth. However, treatment 17

appeared to be the best media among the applied ones for indirect organogenesis experiments.

From Table 3.3, it is evident that some treatments contained either auxin (2,3,4, and 5) or cytokinin alone (6,11,16 and 21). Therefore, ANOVA was also performed to determine the effects of auxin, cytokinin and auxin-cytokinin interaction on traits assessed in indirect organogenesis experiments and the results are presented in Table 3.5.

Table 3.5 ANOVA for the traits studied in indirect organogenesis with respect to auxin and cytokinin levels (DF: degrees of freedom)

Traits assessed for Indirect Organogenesis		Replication	Cytokinin	Auxin	Interaction (Aux x Cyt)	Error
		(DF : 2)	(DF : 4)	(DF : 4)	(DF : 13)	(DF : 256)
Callus Initiation	Mean Square	0.0685	1.3704^{ns}	5.2227**	0.7819^{ns}	0.4705
Callus Growth		0.2981	1.1607^{ns}	4.7496**	0.6121^{ns}	0.4334

** Significant at $p< 0.01$

According to the results of these experiments, auxin levels had significant effects (at $p<0.01$) on callus initiation and callus growth, but there were no significant effects of cytokinin or auxin-cytokinin interaction on both traits. The minimum, maximum, mean and standard deviation (SD) values of cytokinin effects were given in Appendix B1. To understand which auxin concentrations were more effective than the others, the mean values were plotted and the results are presented in Figures 3.5 and 3.6 for callus initiation and callus growth, respectively.

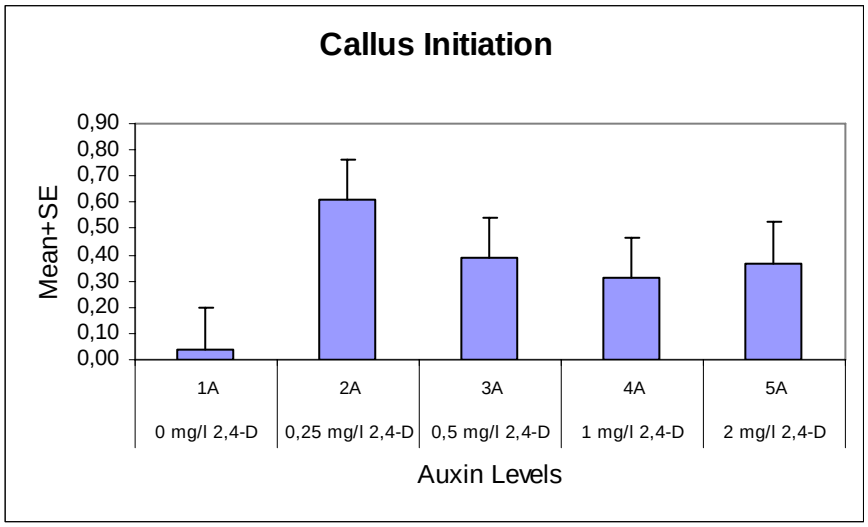

Figure 3.5 Effect of auxin levels on callus initiation (vertical lines on the bar graphs indicate the standard errors)

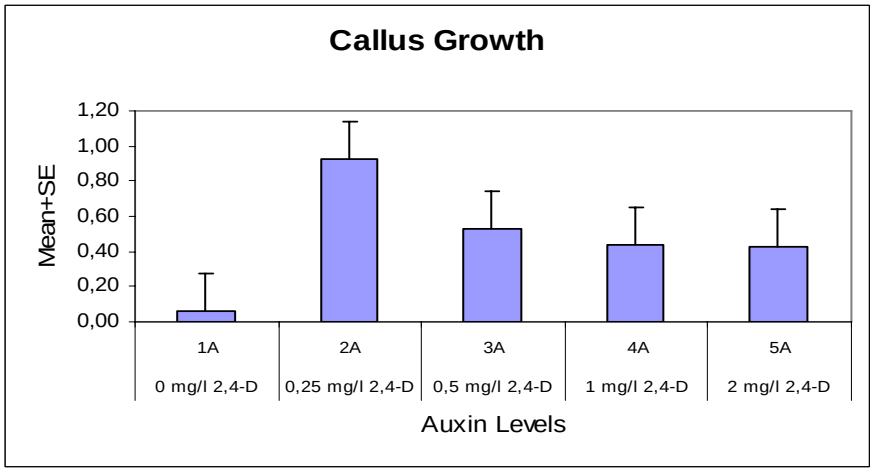

Figure 3.6 Effect of auxin levels on callus growth (vertical lines on the bar graphs indicate the standard errors)

In accordance with the results given in Figures 3.3 and 3.4, these findings are also supporting the positive effects of lower concentrations of 2.4-D (0.25 mg/L) on callus initiation and callus growth.

Nevertheless, as a result, only 35% of all explants used in indirect organogenesis experiments produced callus (Figure 3.7). As suggested by Ilahi *et al*. (1986), that callus could be differentiated into shoots with subculturing, these explants were not discarded and they were kept on the same medium for a few months. Consequently, 5% shoot production was achieved in indirect organogenesis experiments. As will be explain later, some of these shoots were used as explant sources in next experiments.

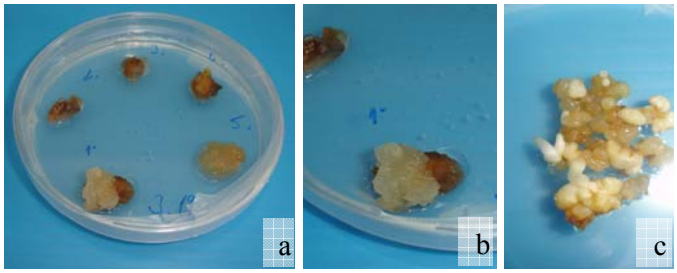

Figure 3.7 Callus and shoot primordia formation in indirect organogenesis experiments
a., b. Callus development; c. Shoot primordia formation

The importance of 2,4-D and BAP combination on indirect organogenesis of saffron was also reported in the literature (Ilahi *et al*., 1986). In their study, corms of saffron were cultured on half strength MS medium including different combinations of growth regulators. Callus was induced in a medium supplemented with 0.5 mg/L each of 2,4-D and BAP. The induced callus then differentiated into buds with subculturing on the same medium. Chen *et al*.

(2003) also reported the production of crocin using *Crocus sativus* callus by two-stage culture system. They tested various different media types and plant growth regulators. But only dissected corms cultured on MS medium supplemented with 2 mg/L 2,4-D and 0.25 mg/L BAP at 21±0.3 ºC in dark promoted callus initiation which contradicted our results.

3.2.2 Direct Organogenesis Experiments

Shoot initiation, shoot growth, callus, meristemoid and root initiation data were recorded for direct organogenesis experiments. However, only shoot initiation and shoot growth were achieved. Therefore, the direct organogenesis capacity of saffron was based on these traits.

As explained in the previous sections, fifteen different treatments including three levels of auxin and five levels of cytokinin were applied in direct organogenesis experiment (Table3.6). In order to find out the effects of auxin and cytokinin combinations, ANOVA was performed and results were shown in Table 3.7.

Table 3.6 Treatments applied in direct organogenesis (Numbers are given to treatments for easing the evaluation of ANOVA)

Treatments	Cytokinin 1 (0 mg/L BAP)	Cytokinin 2 (0.5 mg/L BAP)	Cytokinin 3 (1.0 mg/L BAP)	Cytokinin 4 (3.0 mg/L BAP)	Cytokinin 5 (5.0 mg/L BAP)
Auxin 1 (0 mg/L 2,4-D)	1	2	3*	4	5
Auxin 2 (0.1 mg/L 2,4-D)	6*	7	8	9	10
Auxin 3 (1.0 mg/L 2,4-D)	11	12	13	14	15

* These treatments were not represented with bars in Figure 3.8 due to lack of any response

Table 3.7 ANOVA for the traits studied in direct organogenesis with respect to treatments levels (DF: degrees of freedom)

Traits assessed for Direct Organogenesis		Replication	Treatment	Error
		(DF : 4)	(DF : 14)	(DF : 55)
Shoot Initiation	Mean Square	0.3723	0.4072^{ns}	0.5629
Shoot Growth		0.6738	0.5565^{ns}	0.7983

According to ANOVA results, there were no significant effects of treatments on adventitious shoot initiation and shoot growth. Nevertheless, the mean values were calculated for each treatment and they were given in graphical forms (Figure 3.8).

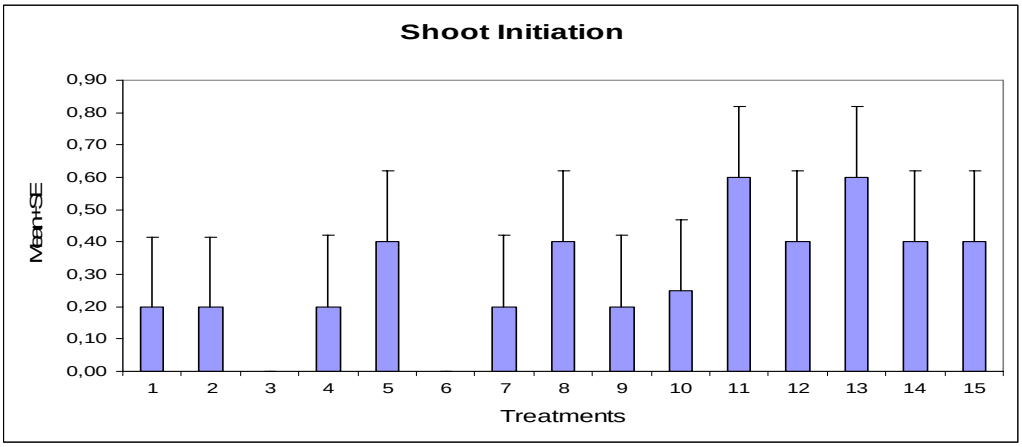

Figure 3.8 Treatment effects on shoot initiation rate (vertical lines on the bar graphs indicate the standard errors)

According to these graphs, it was observed that treatment 11 (1 mg/L 2,4-D and 0 mg/L BAP) and treatment 13 (1 mg/L 2,4-D and 1 mg/L BAP) yielded the best responses on shoot initiation. The only difference between these two treatments was the level of BAP. It was interesting that 1 mg/L 2,4-D was also effective for shoot initiation. Although there was no significant effects with respect to treatments, the combination of 1 mg/L 2,4-D and 1 mg/L BAP was more preferable for subsequent shoot development. The minimum, maximum, mean and standard deviation (SD) values of treatments are also given in Appendix B.2.

As evident from the Table 3.6, some treatments contain either cytokinin (2,3,4 and 5) or auxin alone (6 and11). Therefore, ANOVA was also performed to determine the effects of auxin, cytokinin and auxin-cytokinin interaction on traits assessed in direct organogenesis experiments and the results are shown in Table 3.8.

Table 3.8 ANOVA for the traits studied in direct organogenesis with respect to cytokinin and auxin levels (DF: degrees of freedom)

Traits assessed for Direct Organogenesis		Replication	Cytokinin	Auxin	Interaction (Aux x Cyt)	Error
		(DF : 4)	(DF : 4)	(DF : 2)	(DF : 8)	(DF : 55)
Shoot Initiation	Mean Square	0.3723	0.0605^{ns}	1.5482*	0.2920^{ns}	0.5629
Shoot Growth		0.6738	0.1780^{ns}	1.7175^{ns}	0.4523^{ns}	0.7983

*Significant at $p<0.05$

According to the ANOVA results (Table 3.8), there were no significant effects of cytokinin and auxin-cytokinin interaction on adventitious shoot initiation and

shoot growth. The minimum, maximum, mean and standard deviation (SD) values of cytokinin levels were given in Appendix B.3. However, auxin levels had a significant effect (at $p<0.05$) on shoot initiation trait. To understand which auxin concentrations were more effective than the others, the mean values for each auxin level were calculated and presented in graphical forms (Figure 3.9).

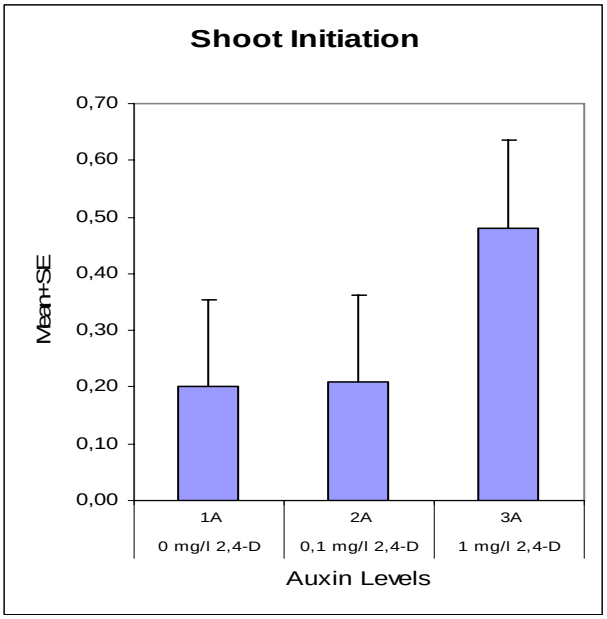

Figure 3.9 Effect of auxin levels on shoot initiation (vertical lines on the bar graphs indicate the standard errors)

These findings are also supporting the Figure 3.8 that higher auxin concentration was more favorable for shoot initiation than lower concentrations. It was found that auxin level 3 (1 mg/L 2,4-D) was the most effective one on shoot initiation trait.

As a result, adventitious shoot production was achieved with 29.73% of explants in this experiment. The importance of 2,4-D and BAP on direct organogenesis of saffron was also reported in the literature (Plessner *et al*., 1990). Corms smaller than 1 cm in diameter and isolated apical buds from larger corms were used as an explant and the nutrient medium was supplemented with 1 mg/L 2,4-D, 3-12 mg/L kinetin and 3 mg/L zeatin. Plessner *et al*., (1990) reported that 2,4-D was essential for regular bud development *in vitro*. It was also found in our study that although the lower levels were not effective, 1 mg/L 2,4-D was the most effective one for shoot initiation. The requirement of BAP as cytokinin for direct organogenesis of *C. sativus* was also reported by Himeno and Sano (1987), Sarma *et al*. (1990) and Loskutov *et al*. (1999).

In the current study, however, subsequent shoot development was inhibited by 2,4-D applications. This situation was also observed by Bhagyalakshmi (1999), who indicated that the presence of 2,4-D appeared to be the least effective for further growth of shoots. MS medium was supplemented with several combinations of different auxins and cytokinins for direct shoot regeneration of saffron. Low levels of 2,4-D (0.5-2.3 μM) and BAP (0.44-4.4 μM) produced whitish green or whitish yellow shoots at statistically insignificant levels. Consequently, 2,4-D as an auxin could be excluded from the nutrient media to stimulate the further shoot growth.

Therefore, to improve the adventitious shoot induction (direct organogenesis) further, new growth regulator combinations were generated based on the results obtained in the previous 15 treatments. Two low levels of 2,4-D (0 mg/L and 0.1 mg/L) and four levels of BAP (0.5, 1, 1.5 and 2 mg/L) were tested. They were applied to the adventitious shoot explants originating from both direct and indirect organogenesis (Figure 3.10).

In agreement with earlier studies, further effect of 2,4-D as an auxin type plant growth regulator was not observed during the improvement of direct organogenesis experiments. However, 1 mg/L BAP was effective for subsequent

shoot development. It was clear from this experiment and the one from literature that auxin type plant growth regulator could be excluded to enable the further shoot growth. As a result, overall 71.95% of explants yielded adventitious shoots in this experiment.

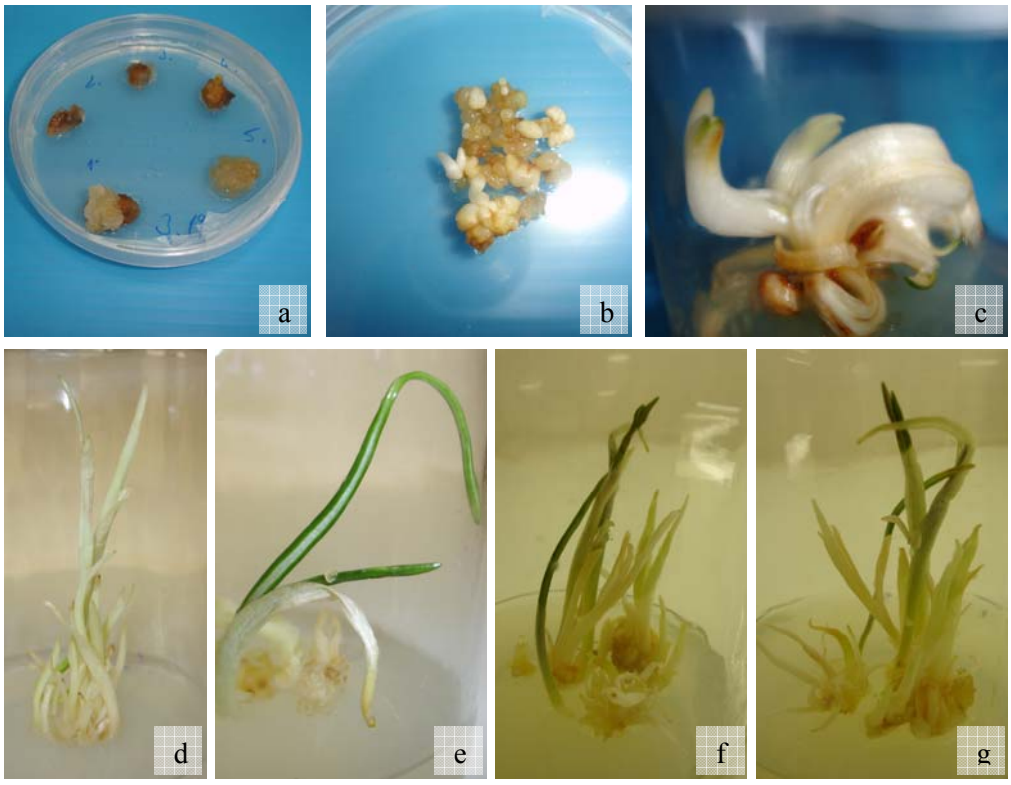

Figure 3.10 Shoots obtained from indirect organogenesis experiments

a. Five explants were put into each petri dish containing MS medium supplemented with different combinations of 2,4-D and BAP in indirect organogenesis; b. Callus and shoot primordia; c. Shoots differentiated from callus tissue which were not discarded; d., e., f. and g. Further growth of shoots originated from indirect organogenesis experiments.

3.2.3 Adventitious Corm and Root Induction Experiments

Although shoot and callus inductions were achieved in previous experiments, root formation did not occur. Thus, auxin "pulse" experiment was performed firstly to achieve this goal. Explants were treated with NAA about one week and then they were transferred to the new medium supplemented with 1 mg/L BAP as explained in the section 2.2.2.7.1.

Following the "pulse" experiment, excluding the auxin from nutrient media resulted in an increase in subsequent shoot development rather than root development. As a result, 98.59% of explants used in this experiment produced shoots without any subsequent roots.

Therefore, new combinations of plant growth regulators were designed to induce adventitious corm and root formation *in vitro*. Explants from auxin "pulse" experiments were transferred to the medium supplemented with 1 mg/L BAP and either 0.1 mg/L NAA or 0.5 mg/L NAA continuously. The shoot growth, corm initiation and adventitious root formation were recorded to evaluate adventitious corm and root induction experiments.

In this experiment, although root and corm induction were targeted, further shoot development and just a few corm formations were observed (Figure 3.11). The combination of 0.1 mg/L NAA and 1 mg/L BAP was more effective on these structures. As a result, 100% of explants yielded adventitious shoot formation and 3% of them produced adventitious corm development.

In agreement with our finding, it was indicated by Bhagyalakshmi (1999) that NAA and BAP are a suitable combination of essential plant growth regulators for supporting organogenesis in *C. sativus*. In this study, several different combinations of auxins and cytokinins were tested. On the whole, the best response towards shoot growth, both in terms of leaf length and number, was on the medium supplemented with 0.54 μM NAA and 2.22 μM BAP which was

statistically significant when compared with other combinations. Regenerated shoots then produced normal photosynthetic leaves and corms. Sarma *et al*. (1990) reported the significance of NAA and BAP on the production of stigma-like structures in tissue cultures from stigma explants of *C. sativus* under defined conditions. MS medium supplemented with 10 mg/dm^3 NAA and 1 mg/dm^3 BAP induced the optimum response through direct organogenesis. NAA was found to be an important addendum to achieve a good response.

Figure 3.11 Shoot and corm formation in adventitious corm and root induction experiments

a. Buds were cultured to the MS medium containing different combinations of 2,4-D and BAP in direct organogenesis experiments; b. and c. White and green leaves formed directly from explants; d. Developed shoots transferred to test tubes; e. and f. Shoots further developed; g.Corm formation.

Saffron is a difficult geophyte species, and recalcitrant towards adventitious roots induction under *in vitro* conditions. Since shoot induction was achieved with ease, the development of roots remained to be the most critical trait that is targeted in this study. Very limited studies are present in the literature concerning the *in vitro* root induction of *C. sativus*. One such study by Ilahi *et al*. (1986) reported morphogenesis with saffron tissue culture. They cultured the corms of saffron on half strength MS medium supplemented with 0.5 mg/L each of 2,4-D and BAP. Although they obtained root formation when the buds were inoculated on a medium containing 2 mg/L NAA for 24 h, further growth of those roots were slow when reinoculated on half strength MS medium containing 0.1 mg/L each of 2,4-D and BAP. They also performed another set of application in their experiment. MS medium containing 0.5 mg/L NAA and 0.1 mg/L of either BAP or kinetin were used. The nodules gave rise to roots after 4 weeks of culture with subsequent suppression of shoot development.

Although we have tried NAA either in "pulse" or "continuous" treatment in combination with BAP, only limited corm production without roots was achieved. Therefore we have considered changing PGR to IBA as the potential auxin candidate for root induction since this growth regulator is usually included in commercial powder preparations. In addition to IBA, 5% sucrose was also included in the growth media as used previously by Ilahi *et al*. (1986).

In adventitious corm and root induction experiment, three different IBA levels were used as shown in Table3.9. Shoot growth, shoot numbers, corm initiation, corm numbers, root initiation and root numbers were recorded to evaluate this experiments.

Table 3.9 Treatments applied in adventitious corm and root induction experiments

Treatments	Descriptions
1	1 mg/L IBA
2	2 mg/L IBA
3	0 mg/L IBA (Hormone Free)

ANOVA was performed to determine the effects of treatments, genotypes and treatment-genotype interactions (Table 3.10). Genotypes were important evaluation criteria in this experiment as explained in section 2.2.2.7.3. Throughout the study, explants from thirty different genotypes were used to determine the effect of genotypes on *in vitro* regeneration capacity of saffron. Unfortunately a large number of explants were lost due to contamination at previous experiments and there were not enough replicates for comparing the genotype effects. However, explants from limited number of genotypes were available with adequate repetition in this experiment and a total of twelve different genotypes could be used.

Table 3.10 ANOVA for the traits studied in adventitious corm and root induction experiments (DF: degrees of freedom)

Traits assessed for Adventitious Corm and Root Induction		Replication	Genotype	Treatment	Interaction (Gen x Trt)	Error
		(DF : 2)	(DF : 11)	(DF : 2)	(DF : 22)	(DF : 70)
Shoot Growth	Mean Square	3.5833	11.3232**	0.8611^{ns}	0.9722^{ns}	1.4405
Shoot Number		33.0833	48.9318**	16.3333^{ns}	12.0303^{ns}	18.9500
Corm Initiation		1.3936	1.8859**	1.1195^{ns}	0.4465^{ns}	0.3597
Corm Number		2.2593	3.8274**	2.9259^{ns}	0.9461*	0.4783
Root Initiation		0.0228	1.2628*	0.6397^{ns}	0.5151^{ns}	0.5398
Root Number		0.5278	45.3157^{ns}	58.5278^{ns}	29.0934^{ns}	22.9754

* Significant at $p<0.05$

** Significant at $p<0.01$

According to ANOVA results, there were no significant effects of treatments on adventitious corm initiation and corm number. The minimum, maximum, mean and standard deviation (SD) values of treatments were given in Appendix B.4. These findings showed that Treatment 1 (1 mg/L IBA) yielded better responses on corm initiation. It was not significantly different from the other treatments, since standart errors of estimates were high.

However, genotypes had significant effects (at $p<0.01$) on corm initiation and corm number. The treatment-genotype interaction also caused significant effects (at $p<0.05$) on corm number. The mean values were calculated for genotypes to understand which ones were more effective than the others and they were given in graphical forms (Figures 3.12 and 3.13).

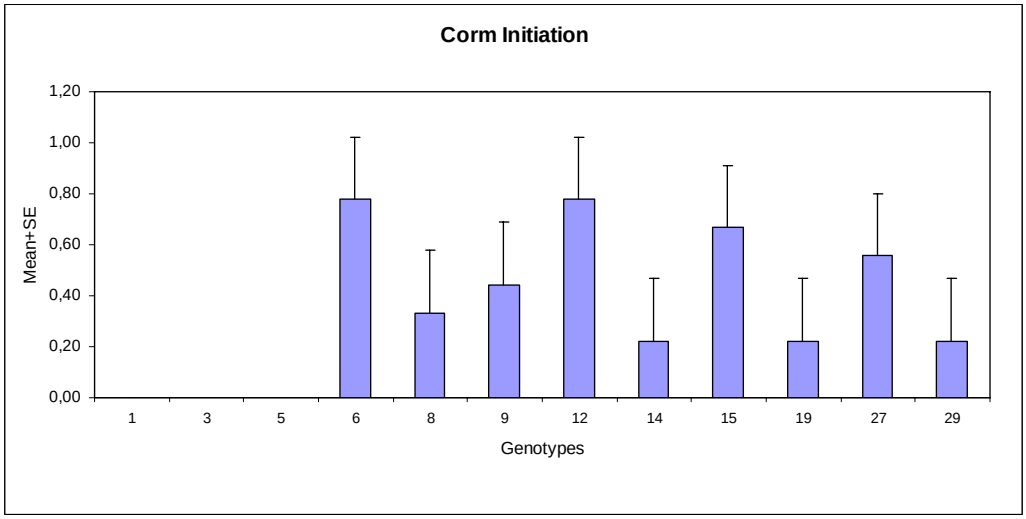

Figure 3.12 Genotype effects on corm initiation (vertical lines on the bar graphs indicate the standard errors)

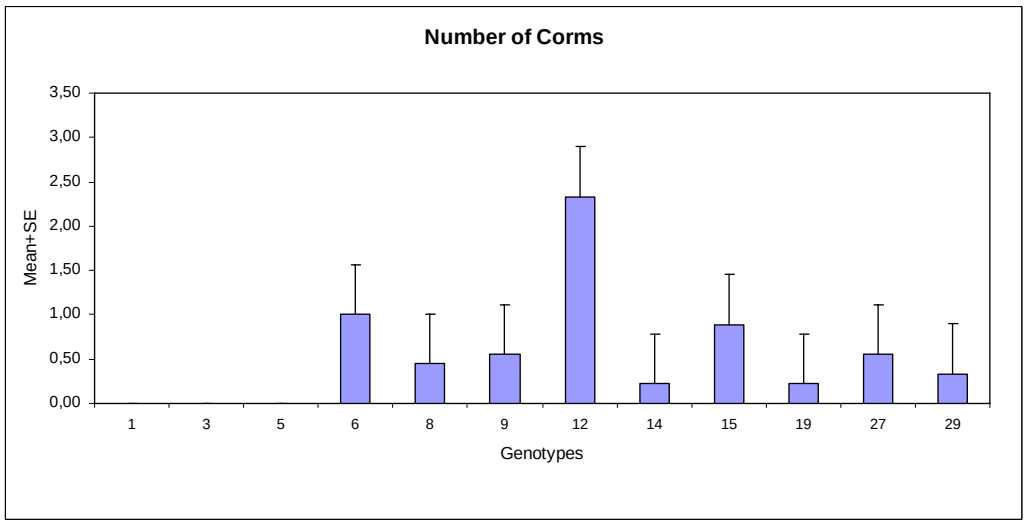

Figure 3.13 Genotype effects on corm number (vertical lines on the bar graphs indicate the standard errors)

According to these graphs, genotypes 6 and 12 were the most responsive ones on shoot initiation. However, the increase of corm number was remarkably achieved by only use of genotype 12 (Figure 3.13).

According to ANOVA results (Table 3.10), there were no significant effects of treatments on adventitious root initiation and root number. The minimum, maximum, mean and standard deviation (SD) values of treatments were given in Appendix B.5. Although there were no significant differences between treatments, explants yielded better responses to treatment 1 (1 mg/L IBA).

As in adventitious corm induction results, genotypes had significant effects (at $p<0.05$) on root initiation. The mean values were calculated for each genotype to determine the more effective ones and they were given in graphical forms (Figure 3.14).

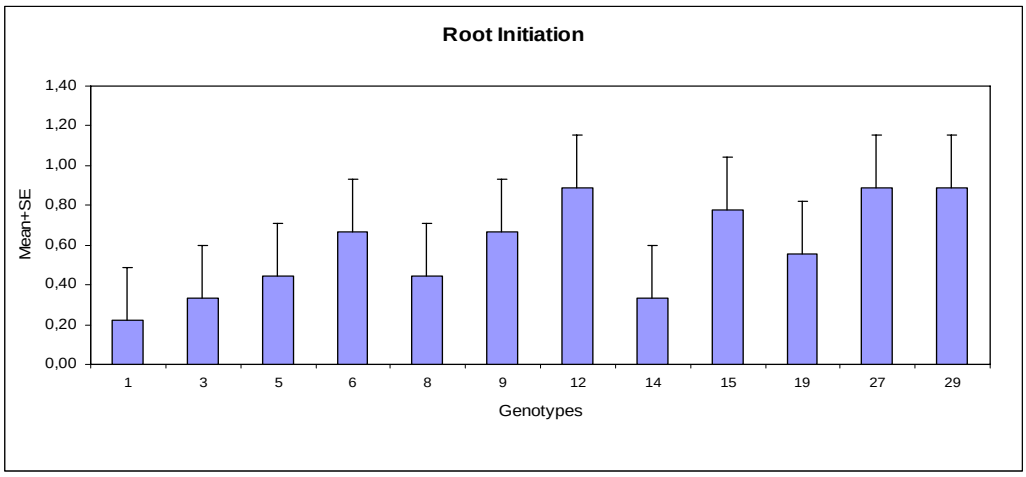

Figure 3.14 Genotype effects on root initiation (vertical lines on the bar graphs indicated the standard error)

According to this graph, genotype 12, 27 and 29 were more responsive to treatments for adventitious root initiation than the other genotypes.

In summary, 35.19% of explants produced corm formation while 59.26% of explants yielded adventitious contractile root in these experiments and results are shown in Figure 3.15. As explained in section 1.1.2, saffron corms produce both fibrous roots and contractile roots. Just contractile roots were achieved with tissue culture methods in this study. The contractile root has the appearance of a tuber organ, very large and whitish in color. This type of roots enable corms to move into the ground, so corms rest in optimum depth and position in the soil (Chio-Sang, 1996).

Obviously, genotype 12 had significant effects on both corm induction and root induction experiments. The actual reason might be explant source. As explained in section 2.2.2.3, apical and lateral buds were used for direct organogenesis and the other parts of the corms sliced into small sections were used for indirect organogenesis. The apical buds of all genotypes were used, but lots of them were lost due to contamination previously. However, the apical bud explant of genotype 12 was retained and it yielded the best responses in this experiment.

The results of this study showed that IBA and increased sucrose level were critical factors to initiate root formation. Our findings were consistent with the earlier studies. Ilahi et al. (1986) used 5% sucrose as the lone carbohydrate source to regenerate saffron plantlets. In the study conducted with Gladiolus hybridus, Kumar et al. (1999) indicated the importance of sucrose levels on root formation. Gladiolus plants are important geophytes with corms and very close to the Crocus genus. Differentiated shoots or those excised from the sprouted cormels were subcultured on MS medium containing varying concentrations of sucrose (0.058-0.348 M). For rooting of shoots, the medium was supplemented only with 1.0, 5.0 or 10.0 IBA.

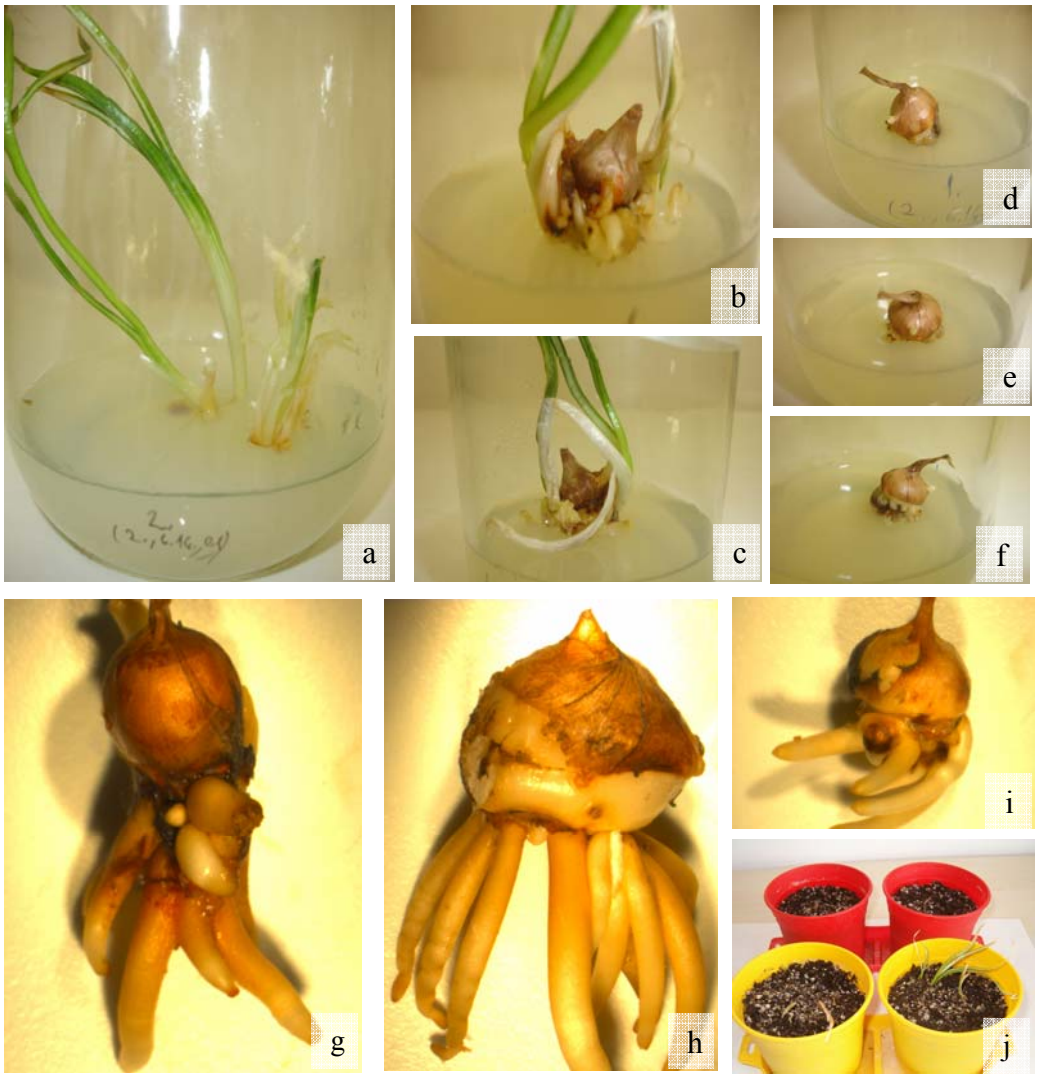

Figure 3.15 Adventitious corm and root formation

a. Shoot formation directly from the buds; b. and c. Corm development with shoots; d., e. and f. Subcultured corms separated from shoots; g., h. and i. The contractile root formation; j. Transfer of some plantlets to the pots for further studies.

It was found that the sucrose concentration had a direct bearing both on the rooting response and on the quality of roots formed. Halevy (1986) also obtained *ex vitro* contractile roots in *Gladiolus grandiflorus*. Various growth substances

applied to the leaves or corms did not induce contractile roots in dark-grown plants, but roots were induced by IBA in both small and large corms grown at constant temperatures and light

.

CHAPTER IV

CONCLUSION

In vitro micropropagation of saffron (*Crocus sativus* L.) by using direct and indirect organogenesis was the main goal of this study. Further, the effect of plant growth regulators on growth parameters, such as corm production, sprouting time and germination ratio were investigated *ex vitro* in this study.

- In *ex vitro* studies of saffron, corms were treated with 50 mg/L IAA and 50 mg/L kinetin in first year and 200 mg/L GA_3, 50 mg/L IAA and 50 mg/L kinetin were used in second year experiment. Although corm production increased in first year, the effects of plant growth regulators were not as effective as expected. In second year, the applications caused to decrease the yield in corm production, sprouting time and germination ratio at all.

- For *in vitro* regeneration of saffron, the importance of 2,4-D and BAP combinations was determined initially. Although 0,25 mg/L 2,4-D and 1 mg/L BAP combination was favorable for indirect organogenesis, 1 mg/L 2,4-D and 1 mg/L BAP combination was good for direct organogenesis. During the improvement of direct organogenesis, the presence of 2,4-D appeared least effective for further shoot development. However, the BAP (1 mg/L) as a cytokinin without auxin stimulated further adventitious shoot development.

- To obtain corm and root formation, auxin "pulse" experiment was performed firstly. Then "continuous" auxin treatments were also prepared by keeping NAA constantly in the medium in combination with BAP. Although the combination of 0.1 mg/L NAA and 1 mg/L BAP stimulated the further shoot development and a few corm formation, root initiation was not observed. It was found that NAA was more efficient than 2,4-D for subsequent shoot development.

- The effects of IBA and increased sucrose level on corm and root formation were examined finally. IBA as auxin type plant growth regulator and 5% sucrose were applied to the explants. Contractile root and corm formation were achieved successfully with 1 mg/L IBA. However, these applications cause inhibitory effect on further shoot development probably due to the cytokinin deficiency. As a result, the overall efficiency was calculated as 59.26% for contractile root formation, 35.19% for corm formation and 100% for shoot development.

- In conclusion, high efficiency in the plantlet regeneration complete with adventitious shoots, corms and contractile roots were obtained *in vitro*. However, further studies will be useful to improve the adaptation of plantlets to the field condition.

REFERENCES

1. Abdullaev, F.I. and Espinosa-Aguirre, J.J., 2004. Biomedical properties of saffron and its potential use in cancer therapy and chemoprevention trials. Cancer Detection and Prevention, v. 28: 426-432.
2. Abdullaev, F., 2003. *Crocus sativus* against cancer. Archives of Medical Research, v. 34 (4): 354.
3. Abdullaev, F.I., 2002. Cancer Chemopreventive and Tumoricidal Properties of Saffron (*Crocus sativus* L.). Exp. Biol. Med., v. 227 (1): 20-25.
4. Abe K. and Saito, H., 2000. Effects of saffron extract and its constituent crocin on learning behaviour and long-term potentiation. Phytother Res., v. 14 (3):49-52.
5. Ahuja, A., Koul, S., Ram, G. and Kaul, B.L., 1994. Somatic embryogenesis and regeneration of plantlets in saffron, *Crocus sativus* L. Indian Journal of Experimental Biology, v. 32: 135-140.
6. Arora, J.S., Singh, K., Grewal, N.S. and Singh, K., 1992. Effects of GA_3 on cormel growth in gladiolus. Indian J. Plant Physiol., v. 35: 202-206.
7. Aslanyants, L.K., Marshavina, Z.V. and Kazaryan, A.G., 1988. Productivity of a cell culture of *Iris sibirica* L. grown on a simplified culture medium. Rastitel'Nye Resursy., v. 24 (1): 107-110.
8. Azizbekova, N.Sh., Milyaeva E.L. and Chailakhyan, M.Kh., 1982. Effect of gibberellin on functional activity of dormant saffron buds. Fiziologiya Rastenii, v. 29 (6): 1164-1169.
9. Basker, D. and Negbi, M., 1983. Uses of saffron. Econ. Bot., v. 3: 228-236.
10. Bhagyalakshmi, N., 1999. Factors influencing direct shoot regeneration from ovary explants of saffron. Plant Cell Tissue and Organ Culture, v. 58: 205-211.

11. Blázquez, S., Piqueras, A., Serna, M.D. and Casas, J.L., 2004. Somatic embryogenesis in saffron: Optimisation through temporary immersion and polyamine metabolism. Acta Hort, v. 650: 269-276.
12. Brighton, C.A., 1977. Cytology of *Crocus sativus* and its allies (Iridaceae). Plant Syst. Evol., v. 128: 137-157.
13. Chen, S., Zhao, B., Wang, X., Yuan, X. and Wang, Y., 2004. Promotion of the growth of *Crocus sativus* cells and the production of crocin by rare earth elements. Biotechnology Letters, v. 26: 27-30.
14. Chen, S., Wang, X., Zhao, B., Yuan, X. and Wang, Y., 2003. Production of crocin using *Crocus sativus* callus by two-stage culture system. Biotechnology Letters, v. 25: 1235-1238.
15. Chichiricco, G., 1989. Microsporogenesis and pollen development in *Crocus sativus* L. Caryologia, v. 42: 249-257.
16. Chichiricco, G., 1984. Karyotype and meiotic behaviour of the triploid *Crocus sativus* L. Caryologia, v. 37: 233-239.
17. Chio-Sang, T., 1996. Effect of planting depth and existence of tunic on growth and flowering in Freesia forcing. J. Korean Hort. Sci., v. 37: 577-581.
18. Davis, P.H., 1984. Flora of Turkey and the East Aegean Islands. Edinburgh, University Press, v. 8: 413-438.
19. Escribano, J., Alonso, G.L., Coca-Prados, M. and Fernández. J.A., 1996. Crocin, safranal and picrocrocin from saffron (*Crocus sativus* L.) inhibit the growth of human cancer cells in vitro. Cancer Lett., v. 100: 23-30.
20. Fakhrai, F. and Evans, P.K., 1990. Mophogenic potential of cultured floral explants of *Crocus sativus* L. for the in vitro production of saffron. J. Exp. Bot., v. 41: 47-52.
21. Fatehi, M., Rashidabady, T. and Fatehi-Hassanabad, Z., 2003. Effects of Crocus sativus petals' extract on rat blood pressure and on responses induced by electrical field stimulation in the rat isolated vas deferens and guinea-pig ileum. Journal of Ethnopharmacology, v. 84 (2): 203-205.
22. Fernández, J.A., 2004. Biology, biotechnology and biomedicine of saffron. Recent Res. Devel. Plant. Sci., v. 2: 127-159.

23. Ghaffari, S.M., 1986. Cytogenetic studies of cultivated *Crocus sativus* (Iridaceae). Plant Syst. Evol., v. 153: 199-204.
24. Giaccio, M., 1990. Components and features of saffron. Proceedings of the International Conference on Saffron, L'Aquila, pp. 135-148.
25. Goo, D.H., Park, I.S. and Kim K.W., 1998. Effects of low temperature and growth regulators on dormancy breaking of gladiolus cormlets produced in vitro. J. Korean Soc. Hort. Sci., v. 39: 479-482.
26. Grilli Caiola, M., 2004. Saffron reproductive biology. Acta Hort., v. 650: 25-37.
27. Haggag, E.G., Abou-Moustafa, M.A., Boucher, W. and Theoharides, T.C., 2003. The effect of a herbal water extract on histamine release from mast cells and on allergic asthma. J. Herb. Pharmcother., v. 3, 41-54.
28. Halevy, A.H., 1986. The induction of contractile roots in *Gladiolus grandiflorus*. Planta, v. 167: 94-100.
29. Hartwell, J.L., 1982. Plants used against cancer. A survey, Quaterman Publications, Lawrence, 284 p.
30. Hill, T., 2004. The Contemporary Encyclopedia of Herbs and Spices: Seasonings for the Global Kitchen, Wiley, 272 p. (Web page of Saffron, available online at http://en.wikipedia.org/wiki/Saffron, last access date: 16.08.2007).
31. Himeno, H. and Sano, K., 1987. Synthesis of crocin, picrocrocin ans safranal by saffron stigma-like structures proliferated in vitro. Agric. Biol. Chem., v. 51: 2395-2400.
32. Hosoki, T. and Kubara, T., 1989. Breaking corm dormancy and accelerated flowering of gladiolus with methyl disulfide. Hort Science, v. 24: 968-971.
33. Hosseinzadeh, H. and Khosravan, V., 2002. Anticonvulsant effects of aqueous and ethanolic extracts of *Crocus sativus* L. stigmas in mice. Arch. Irn. Med., v. 5: 44-47.
34. Hosseinzadeh H. and Younesi H.M., 2002. Antinociceptive and anti-inflammatory effects of Crocus sativus L. stigma and petal extracts in mice. BMC Pharmacology, v. 2: 7.

35. Ilahi, I., Jabeen, M. and Firdous, N., 1986. Morphogenesis with saffron tissue culture. J. Plant Physiol., v. 128: 227-232.
36. Jia, YJ., Chen, F., Lin, H.H,Cao, Y.L., Li, Y. and Wang, S., 1996. Induction of style-stigma-like structure and regeneration of plantlets from corm of *Crocus sativus* in vitro. J. Sichuan University (Natural Science Edition), v. 33: 747-750.
37. Judd, W.S., Campbell, C.S., Kellogg, E.A., Stevens, P.F. and Donoghue, M.J., 2002. Plant systematics, a phylogenetic approach, pp. 241-267, Sinaver Associates, Inc. Publishers, second edition.
38. Karamian, R., 2004. Plantlet regeneration via somatic embryogenesis in four species of *Crocus*. Acta Hort., v. 650: 253-259.
39. Karimi, G., Hosseinzadeh, H., and Khaleghpanah P., 2001. Study of antidepressant effect of aqueous and ethanolic of Crocus sativus in mice. Iranian J. Basic. Med. Sci., v. 4: 11–15.
40. Koyama, A., Ohmori, Y., Fujioka, N., Miyagawa, H., Yamasaki, K. and Kohda, H., 1988. Formation of stigma-like structure and pigment in cultured tissues of *Crocus sativus*. Planta Med., v. 54: 375-376.
41. Kumar, A., Sood, A., Palni, L.M.S. and Gupta, A.K., 1999. *In vitro* propagation of *Gladiolus hybridus* Hort.: Synergistic effect of heat shock and sucrose on morphogenesis. Plant Cell, Tissue and Organ Culture, v. 57: 105-112.
42. Loskutov, A.V., Beninger, C.W., Ball, T.M., Hosfield G.L., Nair, M. and Sink, K.C., 1999. Optimization of in vitro conditions for stigma-like structure production from half-ovary explants of *Crocus sativus* L. in vitro. Cell. Dev. Biol. Plant, v. 35: 200-205.
43. Lu, W.L., Tong, X.R., Zhang, Q. and Gao, W.W., 1992. Study on in vitro regeneration of style-stigma-like structure in *Crocus sativus* L. Acta Bot. Sin., v. 34: 251-256.
44. Mathew, B., 1999. Botany, taxonomy and cytology of *C. sativus* L. and its allies. In: M. Negbi (ed.), saffron. Harward Academic Publishers, Amsterdam.
45. Mathew, B., 1982. The *Crocuses*. B.T. Batsford, London.

46. Mathew, B., 1977. *Crocus sativus* and its allies (Iridaceae). Plant Syst. Evol., v. 128: 89-103.
47. Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Planta., v. 15: 473-497.
48. Nair, S.C., Panikkar, B. and Panikkar, K.R., 1991. Antitumour activity of saffron (*Crocus sativus*).Cancer Lett., v. 57: 109-14.
49. Ochiai, T., Ohno, S., Soeda, S., Tanaka, H., Shoyama, Y. and Shimeno, H., 2004. Crocin prevents the death of rat pheochromyctoma (PC-12) cells by its antioxidant effects stronger than those of α-tocopherol. Neurosci. Lett., v. 362: 61-64.
50. Plessner, O., Ziv, M. and Negbi, M., 1990. In vitro corm production in the saffron crocus (*Crocus sativus* L.). Plant Cell, Tissue and Organ Culture, v. 20: 89-94.
51. Plessner, O., Negbi, M., Ziv, M. and Basker, D., 1989. Effects of temperature on the flowering of the saffron crocus (*Crocus sativus* L.): Induction of hysteranthy. Israel Journal of Botany, v. 38: 1-7.
52. Premkumar, K., Kavitha, S., Santhiya, S.T.; Ramesh, A.R. and Suwanteerangkul, J., 2004. Interactive effects of saffron with garlic and curcumin against cyclophosphamide induced genotoxicity in mice. Asia Pac. J. Clin. Nutr., v. 13 (3): 292-294.
53. Premkumar, K., Abraham, S. K., Santhiya, S. T. and Ramesh, A., 2003. Protective effects of saffron (*Crocus sativus* Linn.) ongenotoxins-induced oxidative stress in Swiss albino mice. Phytother Res., v. 17 (6): 614 - 617.
54. Premkumar, K., Abraham, S.K., Santhiya, S.T., Gopinath, P.M. and Ramesh, A., 2001. Inhibition of genotoxicity by saffron (*Crocus sativus* L.) in mice. Drug Chem. Toxicol., v. 24: 421-428.
55. Ram, R., Mukherjee, D. and Manuja, S., 2002. Plant growth regulators affect the development of both corms and cormels in gladiolus. Hort Science, v. 37 (2): 343-344.

56. Sano, K. and Himeno, H., 1987. In vitro proliferation of saffron (*Crocus sativus* L.) stigma. Plant Cell Tiss. Org. Cult., v. 11: 159-166.
57. Sarma, K.S., Maesato, K., Hara, T., and Sonoda, Y., 1990. In vitro production of stigma-like structures from stigma explants of *Crocus sativus* L. J. Exp. Bot., v. 41: 745-748.
58. SAS Inst. Inc (2002-2003) SAS/STAT User's Guide. Release 9.1. Edition, Cary, NC, USA.
59. Schenk, R.U. and Hildebrandt, A.C., 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., v. 50: 199-204.
60. Sugiura, M., Saito, H. and Abe, K., 1995. Ethanol extract of *Crocus sativus L*. antagonizes the inhibitory action of ethanol on hippocampal long-term potentiation in vivo. Phytother Res, v. 9:100-104.
61. Suh, J.K., 1989. Corm formation in gladiolus as influenced by dormancy breaking methods and cormel planting and corm harvesting dates. Proc. Korean Soc. Hort. Sci., v. 7: 158-159.
62. Sutter, E.G., 1986. Micropropagation of Ixia and Gladiolus hybrid. Scientia Hortic., v. 29: 181-189.
63. Visvanath, S., Ravishankar, G.A. and Venkataraman, L.V., 1990. Induction of crocin, crocetin, picrocrocin and safranal synthesis in callus calture of saffron- *Crocus sativus* L. Biotech. Appl. Biochem., v. 12: 336-340.
64. Vurdu, H. and Güney, K., 2004. Safran Kırmızı Altın. Gazi Üniversitesi Kastamonu Orman Fakültesi Yayınları, 36 p (in Turkish).
65. Vurdu, H., 1993. Soğanlı bitkiler ve yasaklı koruma. Fidan, v. 60: 2-3 (in Turkish).
66. Warburg, E.F., 1957. Crocuses. Endeavour, v. 16: 209-216.
67. Web page of European Saffron (available online at http://www.europeansaffron.eu/ftp/raccolta.jpg and http://www.europeansaffron.eu/ftp/donne_al_lavoro.jpg, last access date: 16.08.07).

68. Web page of Index of Zukan (available online at http://aquiya.skr.jp/zukan/, last access date: 16.08.2007).
69. Web page of Saffron (available online at http://en.wikipedia.org/wiki/Saffron, last access date: 16.08.2007).
70. Web page of Saffron Spain (available online at www.saffron-spain.com/ingles/azafran.html, last access date: 16.08.2007).
71. Web page of Seeds and Seed Germination (available online at http://plantphys.info/plants_human/seedgerm.html, last access date: 16.08.2007).
72. Web page of Webshots Home&Garden, pictures and albums about Saffron published in home-and-garden (available online at http://www.webshots.com/explains/home-and-garden/saffron.html, last access date: 16.08.07).
73. Winterhalter, P. and Straubinger, M., 2000. Saffron-renewed interest in an ancient spice. Food Rev. Int., v. 16:39-59.
74. Zeng, Y., Yan, F., Tang, L. and Chen, F., 2003. Increased crocin production and induction frequency of stigma-like-structure from floral organs of *Crocus sativus* by precursor feeding. Plant Cell, Tissue and Organ Culture, v. 72: 185-191.
75. Zhang, Y., Shoyama, Y., Sugiura, M. and Saito, H., 1994. Effects of *Crocus sativus* L. on the ethanol-induced impairment of passive avoidance performances in mice. Biol. Pharm. Bull., v. 17: 217-221.
76. Zhao, J., Chen, F., Yan, F., Tang, L. and Xu Y., 2001. In vitro regeneration of style-stigma-like structure from stamens of *Crocus sativus.* Acta Bot. Sin., v. 43: 475-479.

APPENDIX A

COMPOSITION OF MURASHIGE & SKOOG (MS) MEDIUM

Table A.1 Composition of MS basal medium (microelements, macroelements and vitamins)

COMPONENTS	mg/L
Microelements	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Macroelements	
$CaCI_2$	332.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Vitamins	
Glycine	2.00
Myo-inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCI	0.50
Thiamine HCI	0.10
TOTAL	**4405.19**

APPENDIX B

MINIMUM, MAXIMUM, MEAN AND STANDARD DEVIATION VALUES

Table B.1 Minimum, maximum, mean and standard deviation values of cytokinin levels in indirect organogenesis

Traits	Cytokinin Level	Minimum	Maximum	Mean±SD	Sample Size (N)
Callus initiation	1	0	1	0.19 ± 0.39	69
	2	0	1	0.29 ± 0.47	14
	3	0	1	0.42 ± 0.50	69
	4	0	1	0.45 ± 0.50	69
	5	0	1	0.36 ± 0.48	59
Callus growth	1	0	2	0.28 ± 0.62	69
	2	0	1	0.29 ± 0.47	14
	3	0	2	0.57 ± 0.74	69
	4	0	2	0.67 ± 0.82	69
	5	0	2	0.46 ± 0.68	59

Table B.2 Minimum, maximum, mean and standard deviation values of treatment levels in direct organogenesis

Traits	Treatments	Minimum	Maximum	Mean±SD	Sample Size (N)
Shoot initiation	1	0	1	0.20 ± 0.45	5
	2	0	1	0.20 ± 0.45	5
	3	0	0	0.00 ± 0.00	5
	4	0	1	0.20 ± 0.45	5
	5	0	1	0.40 ± 0.55	5
	6	0	0	0.00 ± 0.00	5
	7	0	1	0.20 ± 0.45	5
	8	0	1	0.40 ± 0.55	5
	9	0	1	0.20 ± 0.45	5
	10	0	1	0.25 ± 0.50	4
	11	0	1	0.60 ± 0.55	5
	12	0	1	0.40 ± 0.55	5
	13	0	1	0.60 ± 0.55	5
	14	0	1	0.40 ± 0.55	5
	15	0	1	0.40 ± 0.55	5
Shoot growth	1	0	2	0.40 ± 0.90	5
	2	0	2	0.40 ± 0.90	5
	3	0	0	0.00 ± 0.00	5
	4	0	2	0.40 ± 0.90	5
	5	0	2	0.60 ± 0.90	5
	6	0	0	0.00 ± 0.00	5
	7	0	2	0.40 ± 0.90	5
	8	0	2	0.80 ± 1.10	5
	9	0	2	0.40 ± 0.90	5
	10	0	2	0.50 ± 1.00	4
	11	0	2	1.00 ± 1.00	5
	12	0	2	0.80 ± 1.10	5
	13	0	2	1.20 ± 1.10	5
	14	0	1	0.40 ± 0.55	5
	15	0	2	0.80 ± 1.10	5

Table B.3 Minimum, maximum, mean and standard deviation values of cytokinin levels in direct organogenesis

Traits	Cytokinin Level	Minimum	Maximum	Mean±SD	Sample Size (N)
Shoot initiation	1	0	1	0.27 ± 0.46	15
	2	0	1	0.27 ± 0.46	15
	3	0	1	0.33 ± 0.49	15
	4	0	1	0.27 ± 0.46	15
	5	0	1	0.36 ± 0.50	14
Shoot growth	1	0	2	0.47 ± 0.83	15
	2	0	2	0.53 ± 0.92	15
	3	0	2	0.67 ± 0.98	15
	4	0	2	0.40 ± 0.74	15
	5	0	2	0.64 ± 0.93	14

Table B.4 Minimum, maximum, mean and standard deviation values of treatment levels in corm induction experiments

Traits	Treatments	Minimum	Maximum	Mean±SD	Sample Size (N)
Corm initiaiton	1	0	1	0.47 ± 0.51	36
	2	0	1	0.33 ± 0.48	36
	3	0	1	0.25 ± 0.44	36
Corm number	1	0	7	0.86 ± 1.40	36
	2	0	3	0.47 ± 0.77	36
	3	0	2	0.31 ± 0.58	36

Table B.5 Minimum, maximum, mean and standard deviation values of treatment levels in root induction experiments

Traits	Treatments	Minimum	Maximum	Mean±SD	Sample Size (N)
Root initiation	1	0	1	0.67 ± 0.48	36
	2	0	1	0.61 ± 0.49	36
	3	0	1	0.50 ± 0.51	36
Root number	1	0	22	4.56 ± 5.28	36
	2	0	21	4.53 ± 5.98	36
	3	0	18	2.33 ± 3.85	36