CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Optimization of Conditions for Micropropogation of Spinach (*Spinacia oleracea*)

by

Fatima Tuz Zahra

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2018

This effort is dedicated to the Holy Prophet (P.B.U.H)

And

My Parents

CAPITAL UNIVERSITY OF SCIENCE & TECHNOLOGY
ISLAMABAD

CERTIFICATE OF APPROVAL

Optimization of Conditions for Micropropogation of Spinach (*Spinacia oleracea*)

by

Fatima Tuz Zahra

(MBS163005)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Examiner Name	Organization
(b)	Internal Examiner	Examiner Name	CUST, Islamabad
(c)	Supervisor	Dr Erum Dilshad	CUST, Islamabad

Dr Erum Dilshad
Thesis Supervisor
October, 2018

Dr. Sahar Fazal
Head
Dept. of Bioinformatics and Biosciences
October, 2018

Dr. Muhammad Abdul Qadir
Dean
Faculty of Health and Life Sciences
October, 2018

Author's Declaration

I, **Fatima Tuz Zahra** hereby state that my MS thesis titled "**Optimization of Conditions for Micropropogation of Spinach (*Spinacia oleracea*)**" is my own work and has not been submitted previously by me for taking any degree from Capital University of Science and Technology, Islamabad or anywhere else in the country/abroad.

At any time if my statement is found to be incorrect even after my graduation, the University has the right to withdraw my MS Degree.

(Fatima Tuz Zahra)

Registration No: MBS163005

Plagiarism Undertaking

I solemnly declare that research work presented in this thesis titled "**Optimization of Conditions for Micropropogation of Spinach (*Spinacia oleracea*)**" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been dully acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Capital University of Science and Technology towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS Degree, the University reserves the right to withdraw/revoke my MS degree and that HEC and the University have the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized work.

(Fatima Tuz Zahra)

Registration No: MBS163005

Acknowledgements

Survival of the fittest, interdependence of the economics, development of the countries, needs of the world and many other slogans and parameters have taken the quantum leap during the last 2 decade or so. The futuristic initiatives and approaches of the development keep oneself to be in an active mode all the time. The case leads us to some job oriented and task oriented goals in our life. Every human being is a story in himself. Some people are deceived by the circumstances; some meet the total loss while some retrieve its dwindling power, scattered ideas in the re-utilization by facing all sort of the music. My dwindling power to fight against all the bitter realities were innovated once again by the **ALMIGHTY ALLAH** and made me able to carry on this contribution for my personality and intellectual development. I have no suitable words to thank **ALMIGHTY ALLAH** for His blessings.

I wish to extend my greatest appreciation and thanks to my thesis supervisor, **Dr. Erum Dilshad**, Department of Biosciences, CUST Islamabad for her affectionate efforts, guidance, support and encouragement. I also pay my thanks and regards to **Dr.Sahar Fazal**, HOD, Department of Biosciences, CUST, Islamabad, for providing all the research facilities.

I must acknowledge my debt to my lab fellows and friends and Sir Shahid Hussain. No words can express my thanks to my family members, my Ami Jan **Tehmina Masood**, my Abu Jan **Dr. Muhammad Masood** and my sweet loving husband **Dr. Abdul Mateen** and last but not least my brother whom love, affection, prayers, care and support helped me not only during my studies but throughout my life.

Abstract

The spinach is scientifically known as *Spincia oleracea*. It is generally used as food and it also has medicinal significance. In the current study micropropogation of *Spincia oleracea* was studied. Two sterilizing agents including 70% ethanol and 15% bleach were used to select best sterilizing agent. Seeds showed better regeneration efficiency (97%) with regeneration response within 6 days, when treated for 1 minute. Greater exposure time resulted in decline in regeneration efficiency. After seed germination different combination of BAP and NAA were tested for callus induction and then shoot regeneration and root formation. Ms media supplemented with BAP 2.5 mg/L showed best callus induction, whereas 2 mg/L along with 0.2 mg/L of NAA showed best efficiency for shoot formation. Highest number of roots was observed when media was supplemented with 0.5 mg/L of NAA. After complete in vitro regeneration plants were shifted to pots containing mud for acclimatization. The above mentioned protocol developed for in vitro regeneration of *Spincia oleracea* is useful for further research involving micropropogation such as genetic transformation and to study impact of different elicitors on plant growth.

Contents

List of Figures

List of Tables

Abbreviations

FAO	Food and Agriculture Organization
LB	Luria Broth
Ms media	Murashige and Skoog **Medium**
NAA	Napthalene Acetic Acid
BAP	Benzyl Amino Purine
LFH	Laminar Flow Hood
CIM	Callus Induction Media
SIM	Shoot Induction Media
RIM	Root Induction Media

Chapter 1

Introduction

1.1 Edible Plants

Humans have four basic requirements in life: food, clothing, shelter, and fuel. However, an adequate the food is basic necessity of every human and they either depend directly or in directly on plants. Plants are the ultimate source of food-stuff and metabolic energy for almost all animals that cannot prepare their own food. Plants with parts that are safely edible by humans are known as edible plants. About 20,000 species are known which are edible [1]. Most common edible plants are grown all over the world both commercially and in home gardens in different regions and climates. Plants that are cultivated on large scale are usually crops such as maize, rice, wheat, oats and potatoes while those grown on small scale are vegetables like garlic, cabbage and fruit crops like oranges, apples, banana and pineapple. There are different types of vegetables that are grown for the utilization of their leafy parts. Many plant's leaves are consumed as a spice like oregano, vegetables such as cabbage, spinach, mustard greens, Swiss chard and turnip greens. Leafy vegetables refer to the plant leaves that are eaten as raw, sometimes together with petioles and shoots. Although they appeared from different types of plants, most of them share similarities with other leaf vegetables

in nutrition and for cooking method. Over 1,000 species of short-lived herbaceous plants like spinach and lettuce which are edible [2].

1.2 Natural Products

Natural products are being used by human since the down of time as antidotes of health disorders and to cure different ailments having several other uses, as narcotics, dyes and poison for warfare and hunting. Mostly the crude forms of the important metabolites were used until 19th century when isolation of pure compounds started. Some of those natural products are still in use today i.e. morphine and quinine (Fig.1.1). Morphine was isolated first time in 1803 from Papaver somniferum and P. setigru, having analgesics and narcotic effects. Quinine is another example with antimalarial effects, isolated from Cinchona tree [3]

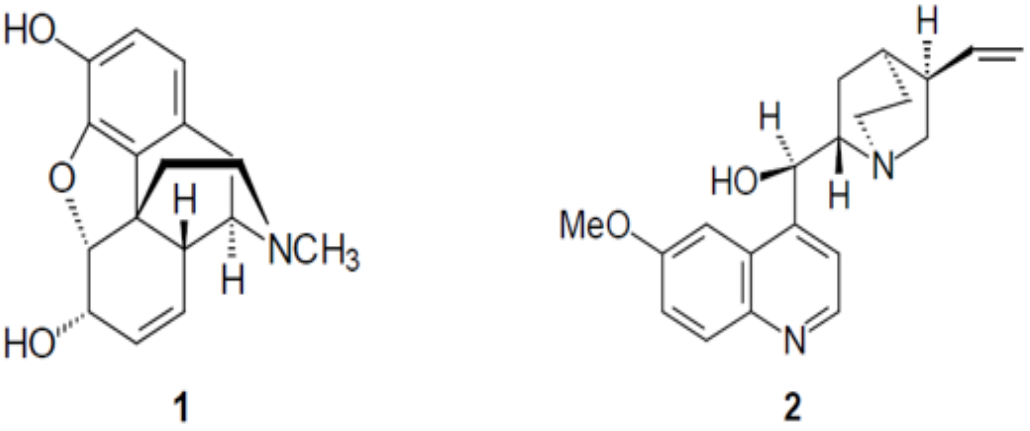


FIGURE 1.1: *Structure of morphine (1) and quinine (2)..*

1.3 Primary and Secondary metabolites

Primary metabolites refer to the all essential elements, a well-being requires for its survival such as amino acids, carbohydrates, fats, proteins and nucleic acids [4]. Apart from the primary metabolism intermediates and products, the vascular plants also contain vast array of chemical compounds playing role primarily

in their defense mechanisms, called secondary metabolites [5]. Although being nonessential to the survival of the plant, they have contribution towards their fitness and survival [4]. The pathways that lead to the secondary plant products also include the pathways other than primary metabolites, being specific to a particular plant family or genus, have been acquired through evolution. The secondary metabolites in past have been given very little attention and were considered the waste products of plant primary metabolism. Kossel was the first one who distinguished these compounds from those of primary ones which were later named as secondary metabolites. These compounds have low abundance usually less than 1% of the total carbon [6]. They have significance in protection against herbivore and microbial infection: they are also important allelopathic agents, attractants for pollinators and seed-dispersing animals. Besides they have great utility as dyes, fibres, waxes, polymers, flavouring agents and drugs [7].

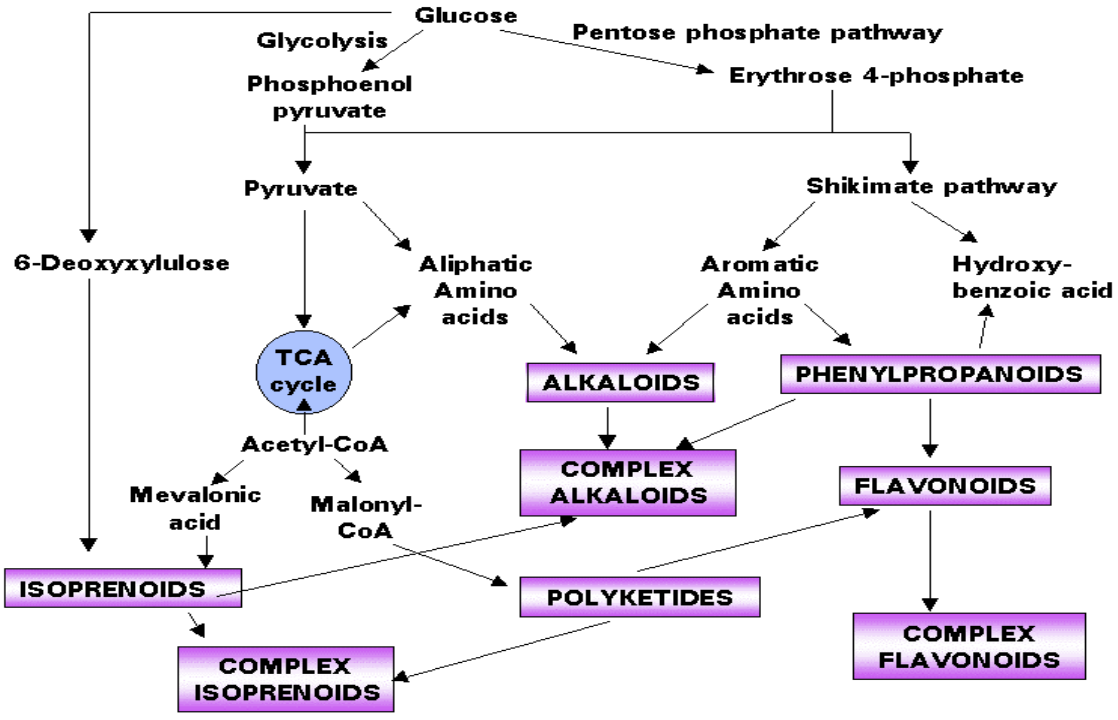


FIGURE 1.2: Schematic Diagram Showing Different Classes of Secondary Metabolites Along with their Biosynthesis [8].

1.4 Classes of Secondary Metabolites

Owing to the chemical composition, secondary plant compounds can be generally separated into two main groups: nitrogenous compounds (alkaloids) and non-nitrogenous compounds (phenolics and terpenoids: Fig. 1.3) [8].

1.5 Nitrogenous Compounds the Alkaloids

Alkaloid is a varied group of secondary plant compounds with a range of structure forms, biosynthetic pathways and pharmacological potential. The compounds of this class account for about 12,000 different molecules. Pelletier defined alkaloids as cyclic compounds which have limited distribution in the living organisms and they contain nitrogen in negative oxidation state [9]. Nitrogen can be either in the heterocyclic ring or it can be extracyclic bond. The sources of alkaloids other than plants include insects, microorganisms, marine invertebrates and animals. Alkaloids have been used as medicines since hundreds of years and are prominent drugs even today [10]. An example of naturally occurring alkaloids are morphine and quinine discussed before. The classification of alkaloids based on the structure is given in Fig. 1.3.

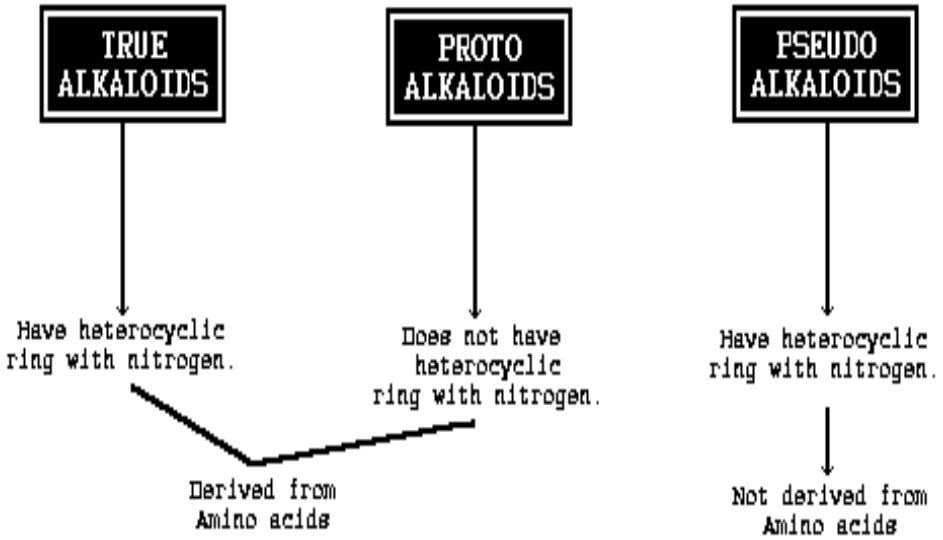


FIGURE 1.3: Structural Classification of Alkaloids.

1.6 Phenolics

One of the main classes of secondary metabolites is phenolics having unique position among natural products owing to ubiquitous distribution in plant kingdom. They are aromatic organic compounds in which a benzene ring is attached with at least one hydroxyl group. They are of variable structure and function. Besides contributing to the nutritional qualities of plant derived food they are also responsible for their major organoleptic properties including taste and color. They are protective against a broad range of ailments including coronary heart diseases, stroke and various types of cancer. They also possess the antimicrobial, insecticidal, algicidal, estrogenic and keratolytic activities. The hydroxyl groups of phenolics donate H thus scavenging reactive oxygen species and stopping the cycle of generation of new free radicals. They are able to inhibit free radical mediated oxidative damage to the bio macromolecules including lipids, proteins and DNA. They also inhibit the enzymes involved in the free radical generation [11]. Different classes of phenolics are given in Fig. 1.5.

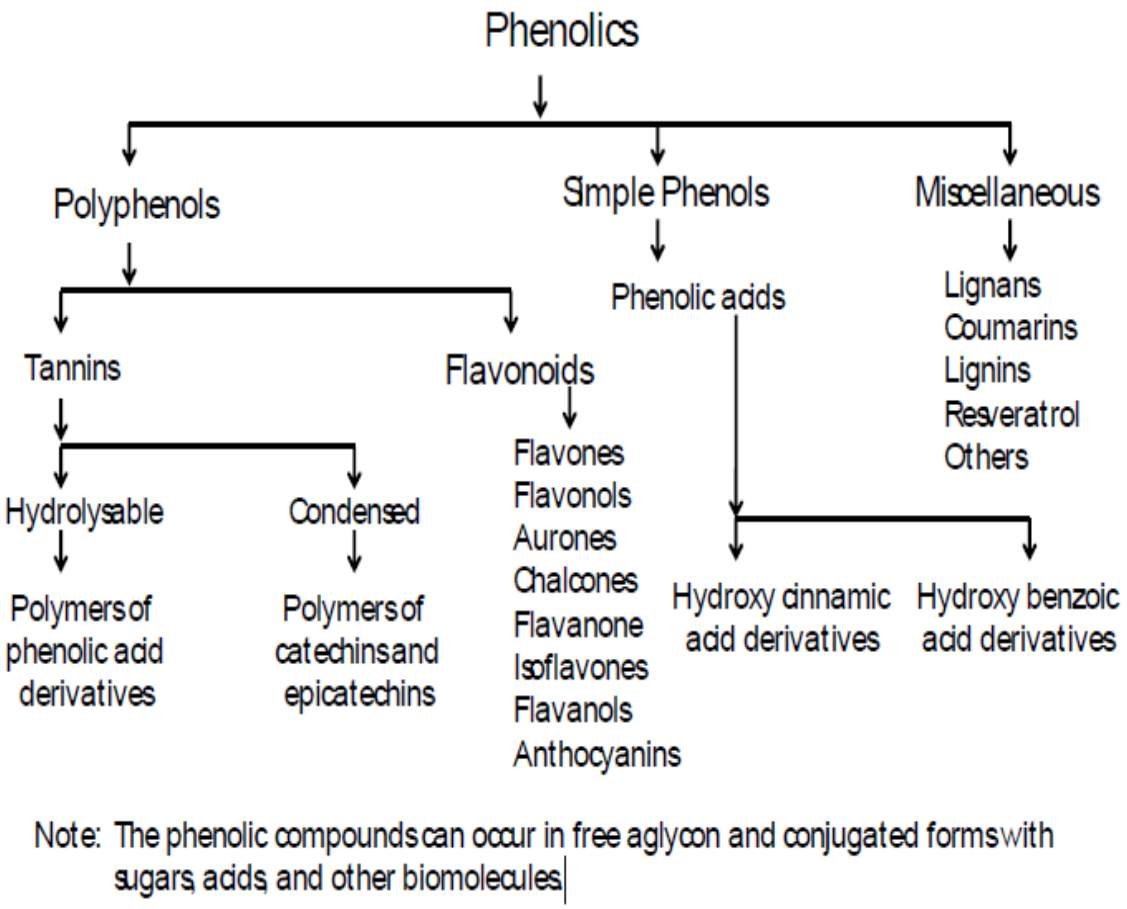
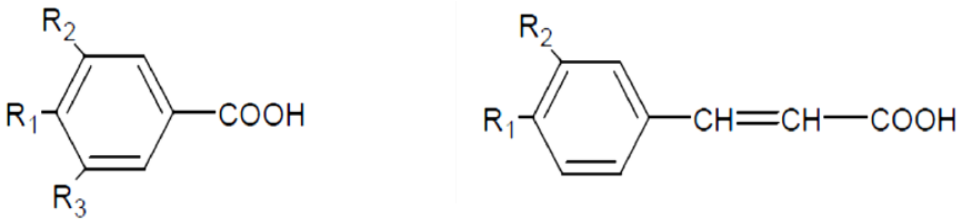


FIGURE 1.4: Classification of Phenolics [12].

1.7 Flavonoids

Flavonoids are known as hydroxylated polyphenols sharing a common three ring chemical structure (C6–C3–C6). Their amount varies depending on plant species, developmental stage and growth conditions of the plant [13]. They are ubiquitously present in plants mainly synthesized by phenylpropanoid pathway in response to microbial infection. They are diverse in function having structure dependent activities. The antiooxidant effect of flavonoids is mediated by the hydroxyl group attached, which act as the scavengers of free radicals and metal chelators [14]. Regarding chemical structure of flavonoids, there is a backbone of fifteen-carbon skeleton having two benzene rings (A and B as shown in Fig. 1.7) linked via a heterocyclic pyrane ring (C).



Phenolic acids:

Benzoic:	R_1=R_2=R_3=H	Cinnamic:	R_1=R_2=H
p-hydroxy-benzoic:	R_1=OH; R_2=R_3=H	Caffeic:	R_1=R_2=OH
p-bromo-benzoic:	R_1=Br; R_2=R_3=H		
p-cyano-benzoic:	R_1=CN; R_2=R_3=H		
p-chloro-benzoic:	R_1=Cl; R_2=R_3=H		
Vanillic:	R_1=OH; R_2=CH_3O; R_3=H		
Gallic:	R_1=R_2=R_3=OH		

FIGURE 1.5: Structure of Phenolic Acids.

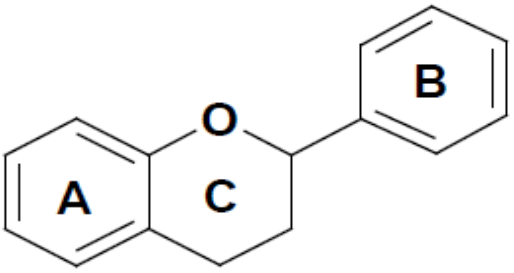


FIGURE 1.6: Flavonoid Basic Skeleton

1.8 *Spinacia Oleracea*

Spinach (*Spinacia oleracea*) is used as vegetable it also bear flowers. They are included in family Amaranthaceae they are found in central and western Asia. It is used as a vegetable.

It is a biennial plant which is 30 *cm* in length. Spinach gets by in winter in calm areas. The leaves are basic, ovalate and are triangular and are 20-300 *mm* long and 10-150 *mm* wide, with large and small leaves. The flowers are yellow-green, with the diameter of 3-4 *mm* developing into a "lumpy fruit cluster" that contain 5-10 *mm* seeds. spinach, was considered in family Chenopodiaceae in old times, but recently in 2003, this family got merged into the Amaranthaceae family in order

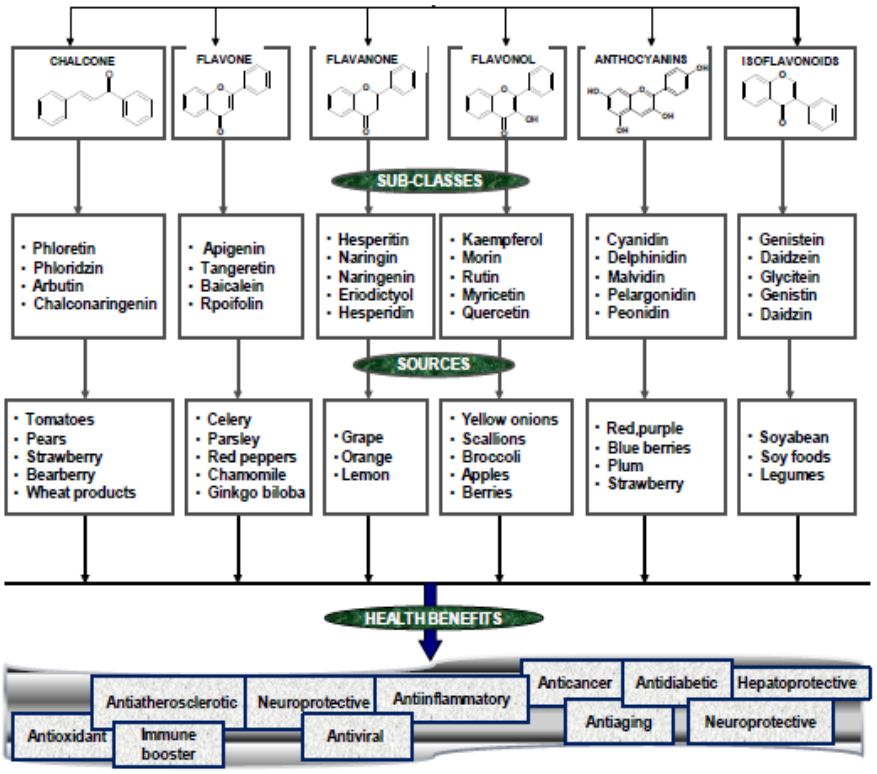


FIGURE 1.7: Flavonoids Classification, Sources and Health Benefits

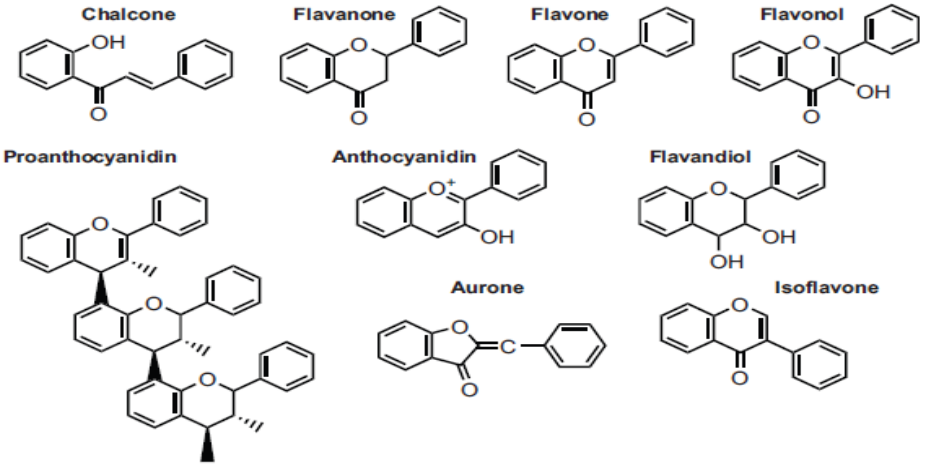


FIGURE 1.8: Structure of Different Classes of Flavonoids.

Caryophyllales. Within the family Amaranthaceae *sensulato*, Chenopodioideae is the subfamily of spinach [15].

TABLE 1.1: Taxonomic Classification [16].

Kingdom	**Plantae**
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Caryophyllales
Family	Amaranthaceae
Genus	Spinacia
Specie	*s. oleraceae*

1.9 Morphology

The leaves of spinach are present in different sizes and texture; its width is 7 cm and 15cm is its length on average. Spinach leaves are simple and occur in turn repeatedly, small leaves of spinach are present on the top of the stem flower and the big leaves appear on the base of the stem of blossom. It additionally has detached blooms which develop into a little clumpy mass, that is dry and hard and its seeds are minor and sparkly dark. The blooms are swinger. The blossoming stem of a spinach; that has branches become upward in single point, is normally delicate, round, and holds a great deal of water and becomes upward to around 1m in tallness. The spinach is a verdant vegetable with wide, fresh, dull green leaves and is the most well known all things considered. It is a fast developing, cool season yearly yield and is developed for its green takes off [2].

The stem of spinach is composed of nodes and inter-nodes. Stem main function is to transport water and other nutrients essential for plant to all parts of plant.it also support the plant and make it firm and support other parts of plant as well. Green pigment chlorophyll is present in them that is essential for photosynthesis [17].

FIGURE 1.9: *Spinacia oleracea*.

1.10 Distribution

Spinach (*Spinacia oleracea*) belongs to the Amaranthaceae plant family spinach is recorded in South western and Central Asia. Spinach bear flowers and is edible plant that grows 30 *cm* long. It survives in winter. spinach is available in early spring and mostly in early winter [16].

1.11 World Distribution of Spinach

The origin of the spinach is centered in the Southwest of Asia. It was introduced by the Arabs in Spain. The data of the FAO publication from 2000 indicate that the world-wide production is 7,755,161 t, distributed by continents in the following way(Figure 1.2).

The continent with larger production of spinach in the world is Asia. The main producing countries are shown in figure 1.3.

TABLE 1.2: World Distribution of Spinach [18]

Continent	**Tons**
Africa	85,000
Asia	6,872,577
Europe	512,675
Oceania	7,200
South America	10,819
North and Central America	266,890
Total	**7,755,161**

TABLE 1.3: Spinach Producing Countries [19]

Country	**Tons**
Pakistan	80,212
Italy	85,367
France	130,000
Turkey	190,000
Japan	329,100
China	6,012,000
Indonesia	61,124

1.12 Nutrition

Spinach is an eatable and easily available vegetable although it can either be eaten in cooked or uncooked form. It is also available either fresh, frozen, or in canned form. It is likewise essential for bone, skin, hair wellbeing and give minerals, iron, vitamins and protein that are useful for skin and bones. It is wealthy in flavonoids-a phytonutrient with hostile to growth properties.it is compelling in backing off the cell division in human stomach and skin malignancy cells [20]. Raw spinach include 27 calories, protein content (0.86 grams), calcium (30 milligrams), Iron (0.81 grams), magnesium (24 milligrams), potassium (167 milligrams), Vitamin A (2813 IU_s)and folate (58 micrograms).

1.13 Medicinal Significance

1.13.1 Diabetes Management

Spinach contains alpha-lipoic corrosive which has property of cell reinforcement, it is used to cut down glucose levels, increase insulin affectability, and balance oxidative weight incited changes in patients with diabetes.

1.13.2 Cancer Prevention

Spinach additionally has property of obstructing the cancer-causing impacts of heterocyclic amines, since spinach and every single green vegetable contain chlorophyll and these cancer-causing impacts are created when barbecuing substances at a high temperature [21].

1.13.3 Prevent Asthma

The dangers for having asthma is diminished in individuals who expend a high measure of specific supplements. spinach is magnificent wellspring of β-carotene and its different sources are vegetables, for example, broccoli, carrots, melon, pumpkin and apricots are likewise rich wellsprings of beta-carotene [22].

1.13.4 Lowering Blood Pressure

High blood pressure people are recommended to take spinach in their diet; because of its high potassium it reduces the effects of Na (sodium) in the body. Intake of low K (potassium) can result high blood pressure and spinach is source for potassium [2].

1.13.5 Bone Health

Low admissions of vitamin K increment danger of bone crack. Vitamin K is wealthy in spinach. Its sufficient utilization is imperative for good wellbeing, it goes about as a modifier of bone grid proteins, enhances calcium ingestion, and may diminish urinary discharge of calcium [23].

1.13.6 Tooth Disorders

Spinach juice is good for gums and also prevents dental cavities. Chewing raw spinach leaves cures pyorrhea, when carrot and spinach juice taken together in the morning, can cure dental issues like bleeding and ulcerated gums [24]. Spinach and tomato contain enough measure of vitamin C to anticipate and fix scurvy. Spinach juice is especially strengthening to the teeth and gums because of its high concentration of alkaline minerals. However, because it is rich in oxalic acid (an acid that require exercise to be metabolized), spinach juice is best used in moderate amounts, in combination with other juices, once or twice weekly [22].

1.13.7 Pregnancy and Lactation

Spinach is useful during pregnancy and also important for lactating mother. Megaloblasticanaemia of pregnancy occurs because the mother is not having enough folic acid or not taking enrich folic acid diet , as folic acid is essential for fetus growth and for proper development. Regular intake of spinach in diet during pregnancy prevent the deficiency of folic acid. Spinach is also good source of nutrition for feeding mothers and also help in improvement of their milk [25].

1.14 Plant Tissue Culture

Plant cell or tissue culture can be defined as the capability of any plant part to regenerate into a complete plant, tissue or organ under sterilized and controlled

environmental conditions. Tissue culture is also called clonal propagation, micro-propagation, rapid propagation or *in vitro* propagation. The term "*in vitro*" refers to "in glass" or in an artificial condition [26]. It is an important tool for both basic and applied studies and has many practical applications in industries, agriculture and is a prerequisite for plant genetic engineering [27]. In 1898 German botanist Gottlieb Haberland called as the father of plant tissue culture, for the first time separated and cultured the plant cells on Knop's salt solution [14]. RJ Gautheret [28] was the first to obtain true plant tissue cultures from cambial tissue of *Acer pseudoplatanus* [28].

In conventional method of cultivation many plants under certain climatic conditions produce seeds and some of them are even unable to germinate seeds and produce flower. They also require long time for growth and development. On the other hand plant tissue culture techniques have many advantages over conventional methodology [29]. These techniques require less time and space and are also less expensive than conventional methods. It is also helpful in the production of plants from seeds that otherwise are unable to germinate for example, orchids and *Nepenthes* (Pitcher plant) [27].

Plant tissue culture is based on the idea of totipotency i.e. the capability of plant cell or tissue to develop into a complete plant [30]. It is one of the most incredible properties of plant cells. This property of totipotency fascinated researchers to carry out various studies on plants and thus succeeded in establishing efficient methods of growing plant cells and tissues under controlled environment [31]. As the degree of differentiation and specialization of one cell vary from the other, therefore different cells have different totipotency [22].

There are three different strategies of tissue culture that are used for the regeneration of plants which are (a) using apical meristem (shoot tips or nodes) (b) organogenesis (direct or indirect by using callus) and (c) somatic embryogenesis. The pathway through which cells and tissues are recovered into finish plants is partitioned into three steps (1) shoot initiation and multiplication, (2) shoot elongation and (3) *in vitro* rooting from the shoots to from stably growing plantlets.

The success of plant tissue culture mainly depends on the source of the explant i.e. age, size, type and position of that explant [22]. All plant cells have different ability to regenerate into a new plant [32]. Shoots, stems, leaves, roots, flowers and callus (undifferentiated cells) can be used as an explant but shoot tips, nodal buds and root tips are the most commonly used explants in tissue culture. The rate of cell division in these tissues is high and has the ability to produce large amount of growth harmones such as auxins and cytokinins. Small size explants sometimes show less growth while using large explants enhance the chances of contamination [33].

Plant tissue culture technique is widely used for large scale multiplication of plants. Apart from research tool, its importance in the area of disease elimination, plant improvement, plant propagation and production of secondary metabolites is increasing day by day. Using plant tissue culture technique, a single explant can be multiplied into thousands of plants in relatively very short period of time and space under sterile and controlled conditions, irrespective of the weather and season on a year round basis [34].

Beside various advantages, tissue culture is also facing some serious challenges. This technique requires keen and close attention of the researches in order to overcome the infections that may proceed through generations. One of the limitations of tissue culture is the unavailability of a standard protocol for all plants because the regeneration strongly depends on the genotype [35]. In plant cell and tissue culture, somaclonalvariations are ubiquitous. According to Israeli *et al.* [35] depending on the genotype, the rates of semi clonal variations resulted from shoot tip culture of Musa species, vary from 0-70%. However this genetic instability may be by chance as it may also provide us novel items and foods in large quantity and less time [36].

1.15 Plant Growth Hormones

1.15.1 Auxins

Auxins is a solid development hormone that is delivered normally by plants. They happen in shoot and root tips and furthermore assume part in cell division, stem and root development. They can in like manner certainly impact plant acquaintance by hoisting cell division with the opposite side of the plant in light of sunshine and gravity [37]. Auxins four important role are:

1. **Stimulating shoot elongation:** Auxin and gibberelin both when combined promote cell elongation and increases in plant length. Both these harmones increase the distance between nodes, spacing the branch points from each other.

2. **Controlling seedling orientation:** Charles Darwin and his child Francis watched that seeds twist toward the light. another shoot develops into the dirt or towards light relies upon where auxins are found and how they impact cells inside the plant. Auxins move descending against gravity, far from light. Cells develop more in territories where auxin fixation is more.

3. **Stimulating root branching:** When auxin is used for cutting stem instead that place it form root.

4. **Promoting fruit development:** Auxins in the blossom advance development of the ovary divider and advance strides in the full improvement of the natural product.

1.15.2 Cytokinin

Cytokinins are the synthetic compounds that induce cell proliferation and trigger callus separation to shoot when connected with auxins, cytokinins additionally assume part in numerous parts of plant development and advancement, including

embryogenesis, support of root and shoot meristems, and vascular improvement. They likewise regulate root extension, sidelong root number, knob arrangement, and apical strength in light of ecological jolts. Cytokinins are essential flagging atoms for direction, development and for improvement of the plant.

1.16 Aims and Objectives

The aims and objectives of the present study were;

- To select the best sterilizing agent to optimize the germination efficiency of seeds of *Spinaciaoleracea*
- To optimize regeneration of shoots of *Spinaciaoleracea* by using different concentrations of plant growth hormones (auxins and cytokinin).
- To optimize regeneration of rooted invitro plants of *Spinaciaoleracea* by using different concentration of auxins.
- To acclimatize the invitro grown plants to the external environment.

Chapter 2

Materials and Methods

The target of the present study was to optimize conditions for seed germination, callus induction and shoot regeneration of *Spinacia oleracea*. The adopted methodology is given below.

2.1 Working Precautions

To avoid the process of contamination sterile conditions were maintained by utilizing autoclave, disinfectants, and UV treatments.

2.2 Culture Medium

In the present experimental work, tissue culturing of *Spinacia oleracea* was conducted using Murashige and Skoog (MS) medium (1962). The chemical composition of the above media is given in table 2.1.

TABLE 2.1: Chemical Composition of Tissue Culturing Media

S/No.	Medium	Components	Conc./litre	pH
01	$\frac{1}{2}$ MS	MS basal salts	2.2 g	5.8
		Sucrose	30 g	
		Agar	10 g	
02	MS	MS basal salts	4.4 g	5.8
		Sucrose	30 g	
		Agar	10 g	

2.3 Seed Germination

The seeds of *Spinacia oleracea* purchased commercially and stored at dry place with temperature less than 20°C and relative humidity not exceeding 30% till further use.

2.3.1 Preparation of Media

Seeds were grown on 1/2 MS media. After mixing the required chemicals of each medium in 1 liter de-ionized distilled water, the pH was adjusted at 5.8 by adding 0.1 N HCl or N_aOH and solidified with 1% agar. The flask was then plugged with cotton, wrapped with aluminum foil and finally autoclaved for 20 minutes at 121°C and 15 psi. Once the sterilization was completed by autoclaving, the media was left to cool in a LFH. Roughly 40-45 *ml* of the medium was dispensed in each 9 *cm* sterilized petri plate and permitted to solidify.

2.3.2 Seeds Sterlization and Innoculation

The surface sterilization of seeds was performed prior to germination. Seeds were dipped in 70% ethanol and 15% bleach for varying durations time i.e. 1 minute, 3 minutes and 5 minutes by continuous shaking with sterile forceps in a LFH. Then the seeds were rinsed thrice with autoclaved distilled water. After sterilization and

drying on autoclaved filter papers, seeds were inoculated in petri plates containing half strength MS medium under aseptic conditions. The plates were sealed with parafilm and given chill treatment at 4°C for three days then finally shifted to growth room for 10-14 days at 25±2°C under light.

TABLE 2.2: Seed Sterilization Treatment

S.No	Sterilizing agent	Time	Time	Time
1	70% ethanol	1 min	3 min	5 min
2	15% bleach	1 min	3 min	5 min

2.4 Seed Germination Efficiency

In order to check which sterilization condition suits best for the germination, seeds were grown on two different sterilizing agents (Table 2.2) and their germination efficiency was evaluated by using following formula.

$$Seed\ germination\ efficiency\ (\%) = \frac{No.\ of\ seeds\ germinated}{Total\ no.\ of\ seeds\ grown} \times 100 \quad (2.1)$$

2.5 Plant Growth Hormones for Regeneration of Plant Tissue

In our experiment of callus induction and shoot regeneration from callus, two different types of Phytohormones or growth regulators were used. These plant growth hormones were Naphthalene acetic acid (NAA) and Benzylaminopurine (BAP). They were prepared under sterile conditions in 1.5 *ml* aliquots at −70°C. These hormones were added to the respective medium in varied concentrations.

The working concentration of each growth regulator and medium containing different hormonal combinations is shown in table 2.3 and table 2.4.

2.6 Callus Induction

2.6.1 Callus Induction Medium

Four different callus induction media (CIM) were used in this experiment. After mixing the required chemicals of each medium in 1 liter de-ionized distilled water, the pH was adjusted at 5.8 by adding 0.1 N HCl or N_aOH and solidified with 1% agar. The medium was then autoclaved in flask for 20 minutes at 121°C and 15 psi. After autoclaving, the medium was left to cool in a LFH. When the temperature dropped around 50°C, Filter sterilized BAP (using $0.2\mu m$ syringe filter) in different concentrations (Table 2.3) was added to the respective medium. Roughly 40-45 ml of the medium was dispensed in each 9 cm sterilized petri plate and permitted to solidify. Finally, the petri plates were sealed with parafilm and kept at 25±2°C in growth room until use.

TABLE 2.3: Callus Induction Media

S/No.	**Medium**	**Concentration of BAP** mg/L
01	CIM1	2.5
02	CIM2	2
03	CIM3	1.5
04	CIM4	1

2.7 Preparation of Explants

Callus induction experiment was conducted by using two types of explants i.e. cotyledons and nodes. Fresh, viable and green cotyledons from 10-15 days old *in*

vitro grown spinach seedlings were cut into small pieces of 2×4 *mm* under aseptic conditions and used as explants for callus induction. Furthermore, the nodal part of the seedlings was excised from young seedlings and also used as an explant. Both of these explants did not require surface disinfection as they were taken from the plant grown under sterile conditions.

2.8 Explants Inoculation and Callus Induction Efficiency

Sterilized cotyledons and nodal explants were then inoculated on the plates containing callus induction media added with diverse concentrations of BAP. The plates were sealed with parafilm and kept in growth room at 25±2°C under light. The experiment was performed in triplicates with 3 plates in each batch containing 5 explants. Visual observations were taken after every 2nd day. After every two weeks, induced calli were sub-cultured on the respective medium. The efficiency of callus induction was calculated by the formula given below.

$$Callus\ induction\ efficiency(\%) = \frac{No.\ of\ calli\ produced}{Total\ no.\ of\ explants\ inoculated} \times 100 \tag{2.2}$$

2.9 Shoot Regeneration

An experiment was performed to study the organogenesis of shoot from callus formed earlier on four regeneration media. In continuation of the prior experiment, the calli induced from two different explants of *Spinacia oleracea* were further cultured on the same media containing combination of different growth regulators such as BAP and NAA (Table 2.4). The media were prepared as described previously in section 2.3.1.

TABLE 2.4: Shoot Regeneration Media

S/No.	Medium	Combination 1	Concentration of NAA mg/l	Concentration of BAP Mg/L
01	SIM1	NAA+ BAP	0.25	2.5
02	SIM2	NAA+ BAP	0.2	2
03	SIM3	NAA+ BAP	0.5	1.5
04	SIM4	NAA+ BAP	0.1	1
05	SIM5	NAA+ BAP	0.05	0.5

2.10 Explants Inoculation and Shoot Regeneration Efficiency

Calli obtained from cotyledons and nodal explants were then inoculated on the respective medium added with combination of different growth regulators. Total 5 explants per plate were inoculated. The plates were then sealed as described in section 2.3.1 and kept in growth room at 25±2°C under light. After every two weeks, the explants were shifted to fresh culturing medium. Shoot regeneration efficiency of each medium was calculated after four weeks of inoculation of calli. The regeneration efficiency of shoot was evaluated by the formula given below.

$$Shoot\ regeneration\ efficiency\ (\%) = \frac{No.\ of\ shoot\ regenerated}{Total\ no.\ of\ calli\ inoculated} \times 100 \quad (2.3)$$

2.11 Rooting Response of the Regenerated Shoots

Regenerated shoots were then shifted to the MS media containing different concentrations of the NAA (Table 2.5) and where they were maintained and subcultured after every 3rd week. The rooting response was observed and best rooting concentration of NAA was selected by calculating rooting efficiency of the plants on all

concentrations of NAA tested. The rooting response was evaluated by the given formula.

$$Shoot\ regeneration\ efficiency\ (\%) = \frac{No.\ of\ shoot\ regenerated}{Total\ no.\ of\ calli\ inoculated} \times 100 \quad (2.4)$$

Table 2.5: Media Containing Different Concentrations of NAA for Rooting Response

S/No.	Medium	Concentration of NAA mg/L
01	RIM1	0.25
02	RIM2	0.5
03	RIM3	1
04	RIM4	1.5
05	RIM5	2

2.12 Hardening and Shifting to pots (Acclimatization)

After complete in vitro regeneration plants were shifted to small pots that contain soil. The soil composition was clay, sand and peat that were taken in equal amount. Small holes were made in the bases of pots so that excess water should be drained in order to avoid roots from damaging. Plants were covered with polythene bags so that moisture could be maintained. This process continued for one month in growth room 25±2°C, 16h of photoperiod, illumination of 45 $uEm^{-2}\ s^{-1}$ and 60% relative humidity. When plants were hardened in the soil then they were transferred to green house. They were under special care till plants reach stage of maturity.

Chapter 3

Results

In the present study, different conditions for seed germination, callus induction, shoot regeneration and root formation were optimized. Moreover, plants were also acclimatized to the external environment by shifting to the pots with clay and mud.

3.1 Seed Germination

3.1.1 Surface Sterilization and Inoculation of Seeds

The present research work was carried out using seeds of *Spinacia oleracea* that were purchased from Awan Seeds Store, Rawalpindi, Pakistan.Surface sterilization of seeds with different treatments of 70% ethanol and 15% sodium hypochlorite (bleach) solution was performed i.e. seeds were exposed to sterilizing agent for varying durations of time in order to select the best treatment. Seed germination was observed within a week (Figure 3.3).

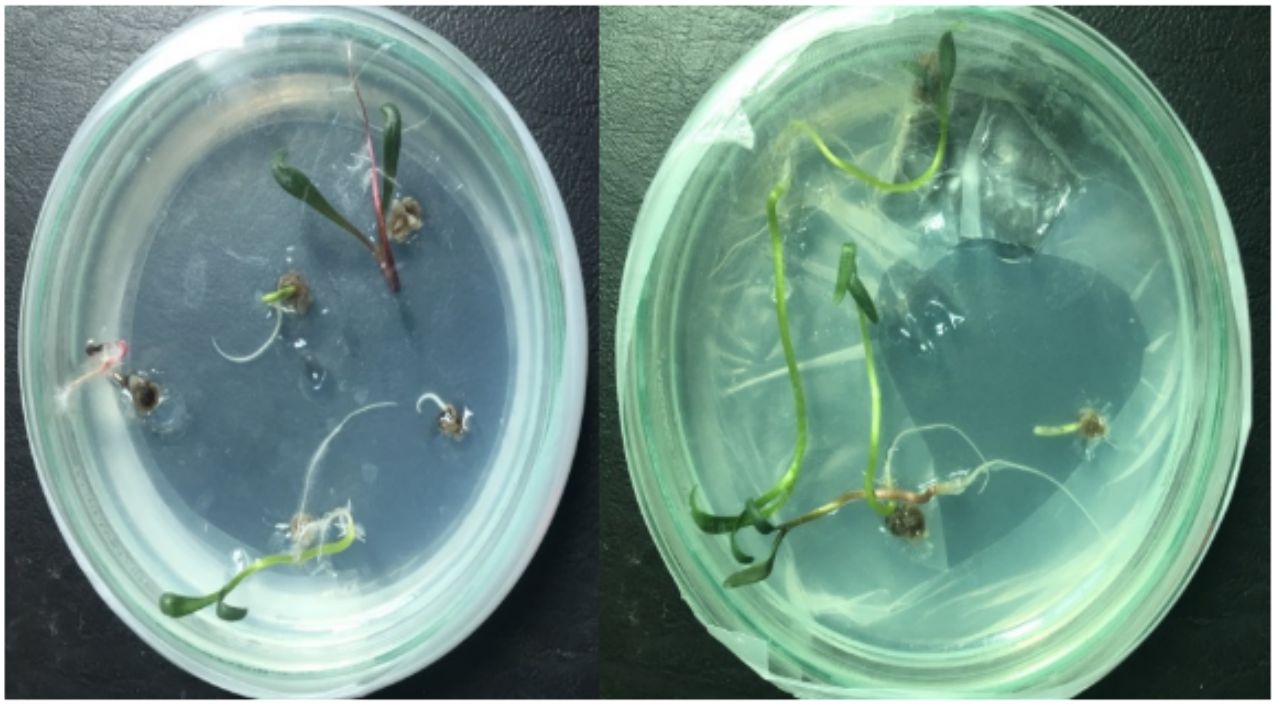


FIGURE 3.1: Seed Germination on 15% Bleach

FIGURE 3.2: (a) Seed Germination on 15% Bleach

3.2 Seed Germination Efficiency

For the germination of *Spinacia oleracea* seeds, two different sterilizing agents were used which were (i), 70% ethanol (ii) 15% bleach. The chemical composition of these media has been described in chapter 2 and section 2.2. Sterilized seeds were

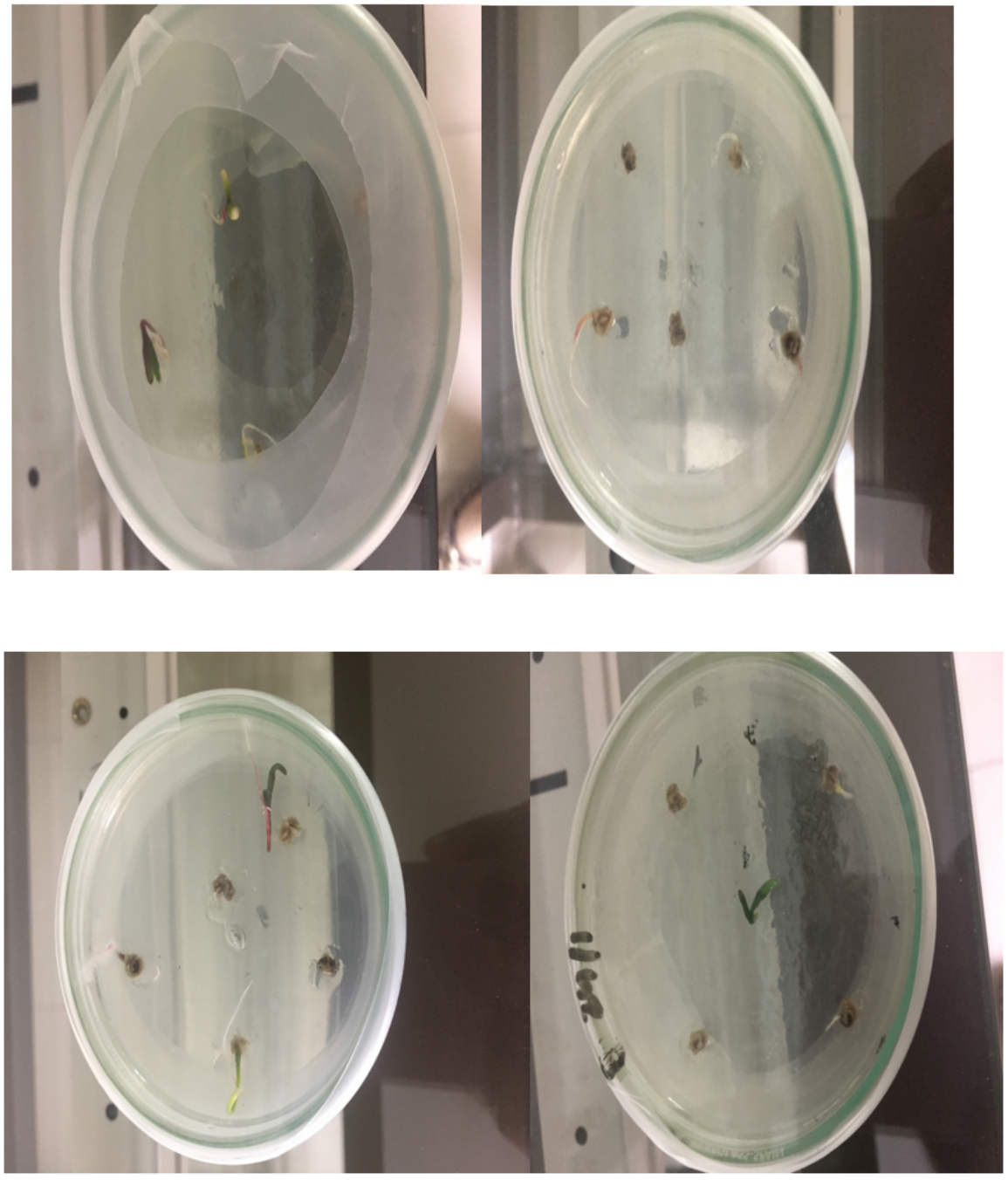


FIGURE 3.3: (b) Seed germination on 70% ethanol

inoculated under aseptic conditions in the media that resulted in the formation of hypocotyls and cotyledons within a week (Fig 3.3). Best results were obtained when seeds were sterilized with 15% bleach for one minute followed by 2 minutes treatment and then 3 minutes treatment as compared to 70% ethanol as shown in the Figure 3.4. However, longer duration of 15% sodium hypochlorite(bleach) treatment resulted in the reduction of seed germination (Table 3.1).

Seed germination is one of the most important steps in the life cycle of plants. For in vitrogermination of seeds, proper sterilization procedure is very essential. Sterilizing agents like ethanol, sodium hypochlorite, Tween 20 and mercuric chloride are used [38].Sodium hypochlorite is one of the commonly used surface disinfectants. It releases oxygen gas as a by-product that enhances oxidative respiration which in turn promotes seed germination [17] According to Talei *et al.* [27] ethanol is a strong sterilizing and highly phytotoxic chemical; therefore seeds should be exposed to it for a short period of time. For efficient sterilization of seeds, ethanol is normally used prior to the treatment with other sterilizing agents like bleach and Tween 20 [26]. Surface sterilizing agents such as 70% ethanol and 15% Sodium hypochlorite plays a very important role in the germination of seeds. In the present study, seed sterilization for 1 minute in 15% Sodium hypochlorite followed by washing thrice with sterile distilled water showed 100% germination efficiency of spinach. However, longer duration of 10% Sodium hypochlorite treatment resulted in the reduction of seed germination. According to Drew and Brocklehurst (1984), surface sterilization of spinach seeds with 10% sodium hypochlorite solution for short period of time, increased the rate of germination. Seed germination efficiency decreased when the duration of exposure of 10% sodium hypochlorite was increased.

TABLE 3.1: Seed Germination on Different Sterilizing Agents

S.No	**Sterilizing Agent**	**Time Duration (minutes)**	**Seeds Inoculated**	**Seeds Germinated**	**Germination Efficiency**	**Days of Germination**
1	Ethanol 70%	1	45	44	97.7777778	6
2	Ethanol 70%	2	45	36	80	8
3	Ethanol 70%	3	45	25	55.5555556	10
4	Bleach 15%	1	45	45	100	5
5	Bleach 15%	2	45	40	88.8888889	7
6	Bleach 15%	3	45	31	68.8888889	10

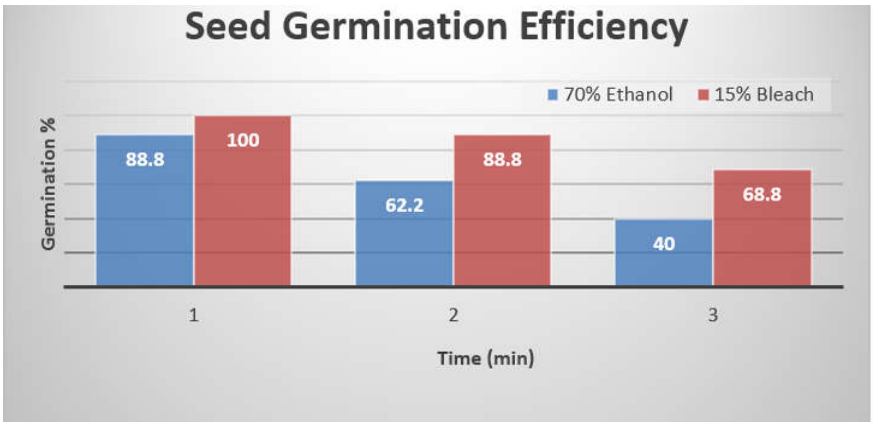


FIGURE 3.4: Seed Germination Efficiency on Different Sterilizing Agents

3.3 Callus Induction Efficiency

Four different callus induction media supplemented with combination of BAP (Table 2.3) were used for callogenesis. The response of callus induction from two different types of explants (cotyledons and nodes) was noticed in all the four media used. Callus production was observed in all media and started within a week of culture initiation (Fig 3.5). After great deal of experimentation it was found that the potential of callus formation varied with the type of explant and BAP concentration, as shown in Table 3.2.Cotyledons and nodal explants from in vitro grown 7 days old seedlings were used for callus induction, as reported by Liu *et al.* [16]. It was observed in all media and started within a week of culture initiation. Callus induction media 1 (CIM 1) with BAP 2.5 mg/L showed best callus induction efficiency. In the present study when the callus induction efficiency for each type of explant on all the four media was calculated, it was shown that cotyledons had higher potential for callogenesis as compared to nodal explants. A similar response has been reported by (Ronquist *et al.* [39]).

3.4 Effect of Media and Explant Type

The callus formation efficiency for each type of explant was calculated as an average value of all the callus formation efficiencieson four different callus induction media. It was shown that cotyledons had a higher potential for callogenesis as

compared to nodal explants on all the media tested. Callus induction media 1 (CIM1)(2.5 mg/L) of BAP showed the highest callus induction efficiency (Fig 3.5). The average callus induction frequency of cotyledon explants was 58.5% whereas nodal explants showed 47.7% callogenesis as shown in Fig 3.6.

TABLE 3.2: Callus Formation Efficiency of Different Explant on Different Media

S/No.	Media	Cotyledons			Nodes		
		No. of explants inocula-ted	No. of calli induced	Callus induction efficiency	No. of explants inocula-ted	No. of calli induce	Callus induction efficiency
01	CIM1	45	34	75.5%	45	28	62.2%
02	CIM2	45	31	68.8%	45	25	55.5%
03	CIM3	45	24	53.3%	45	18	40%
04	CIM4	45	17	37.7%	45	15	33.33%

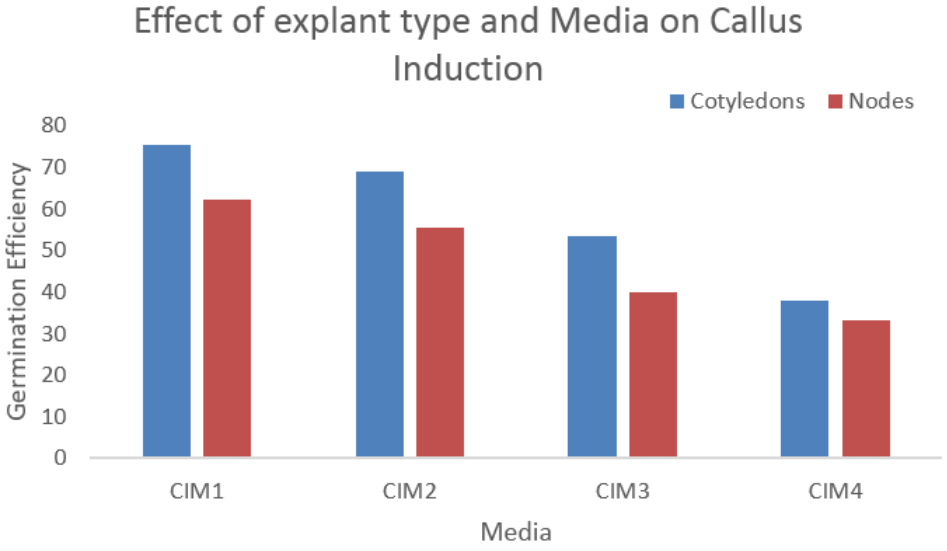


FIGURE 3.5: Effect of Media on Callogenesis

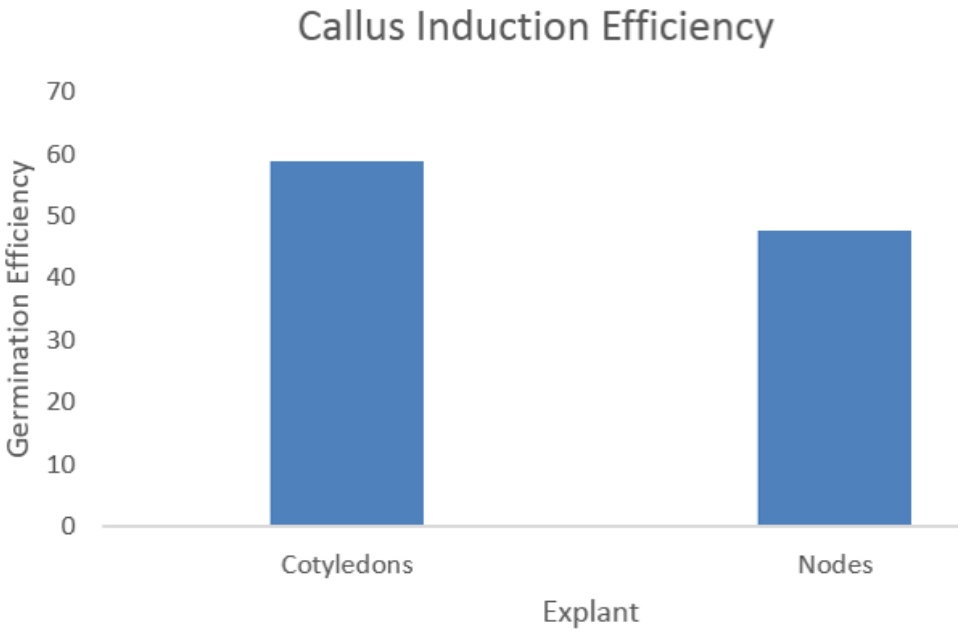


FIGURE 3.6: Effect of Explant Type on Callogenesis

3.5 Shoot Regeneration

Experiments were carried out to examine the ability of calli from different types of explants (cotyledons and nodes) to form shoots. Four different types of regeneration media (Table 2.4) supplemented with two different types of phytohormones i.e. NAA and BAP were tested. Keeping in view the days to shoot initiation and frequency of shoot regeneration, one simple but efficient shoot regeneration medium was finally selected from all the four media for final experiments as shown in (Table 3.3). The previous study also has demonstrated that growth and morphological components of spinach were significantly affected by different concentrations of growth regulators when applied. According to our findings, NAA and BAP in combination have a synergetic effect. The best shoot regeneration media was SIM 2 (NAA 0.2 mg/L and BAP 2 mg/L). It was revealed that the shoot regeneration efficiency, from calli previously formed on callus induction media, varied with type of explant and hormonal combination. As BAP promotes cell division and NAA helps in cell elongation so combination of both in a particular combination results in better shooting response in invitro culture of plants. These results agree with

those published by [40]. Another group studied the effect of plant growth regulators on the soybean [41]. Enhanced leaf size, was observed for NAA and BAP when applied in combination.

TABLE 3.3: Shoot Regeneration Efficiency on Different Media

S.No.	**Media**	**BAP**	**NAA**	**Total no. of explants**	**No. of regenerated explants**	**Regeneration efficiency**
01	SIM1	2.5	0.25	30	20	66.6%
02	SIM2	2	0.2	30	22	73.33%
03	SIM3	1.5	0.5	30	19	63.3%
04	SIM4	1	0.1	30	14	46.6%

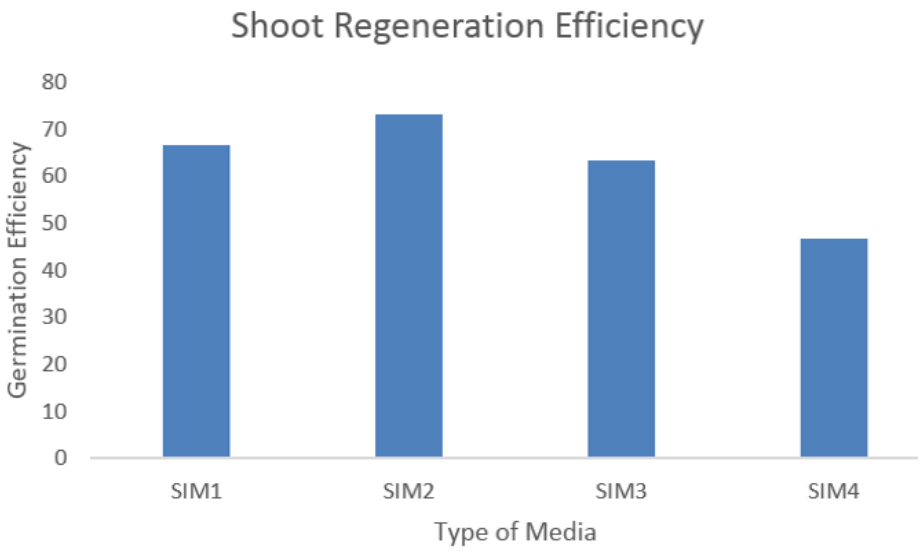


FIGURE 3.7: Effects of Media on Shoot Regeneration Efficiency

FIGURE 3.8: Shoot Regeneration from Callus

3.6 Rooting Response

All the regenerated shoots were shifted to root induction media Table 2.5. Different concentration of NAA was tested to check the best rooting response. Root formation was observed after 2 weeks of placement on root induction media. Best rooting response was shown by RIM2 having NAA 0.5 mg/l. Our results have indicated that NAA has a positive effect on vegetative growth of spinach plants giving significant root growth supported by previous reports [37]. The treatments in which NAA was applied individually yielded better results in root formation. Similar observations were reported by [42].The root regeneration efficiency is given in Table 3.4.

TABLE 3.4: Rooting Efficiency of Regenerated Shoots

S.No.	**Medium**	**Concentration of NAA mg/l**	**Total no. of Shoots**	**No. of Roots Formed**	**Rooting Efficiency**
01	RIM1	0.25	25	10	40
02	RIM2	0.5	25	15	60
03	RIM3	1	25	8	32
04	RIM4	1.5	25	9	36
05	RIM5	2	25	8	32

3.7 Acclimatization

After significant no of rooted plants were obtained, they were shifted to the pots containing mud and clay. Initially plants were kept covered with the polythene bags to maintain the humidity. Afterwards bags were gradually removed. Plants showed survival responses when shifted to the pots, although fungal infection was the limiting factor for their survival. The plants were very delicate and unresponsive to get acclimatized quickly. They took more than one month to get

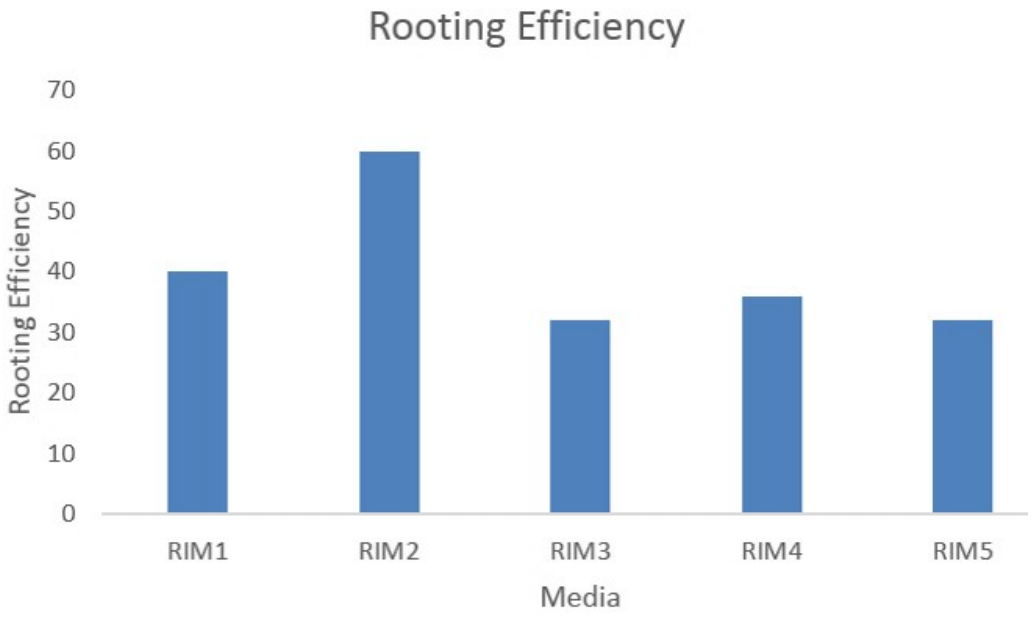


FIGURE 3.9: Effect of Media on Rooting Efficiency of Regenerated Shoots

acclimatized to the environment in the growth room. Gradually they were hardened and got the maturity to be shifted to the green house after few months (Fig 3.10). Successful aclimitization requires several features such as optimum temperature, humidity and biohardening of tissue cultured plants and also contamination control. When these parameters maintained, acclimatization is carried easily (S Chandra, R Bandopadhyay, V Kumar,2010).

FIGURE 3.10: Acclimitization of *Spinacia oleracea*

Chapter 4

Conclusion and Future Prospects

Finally, we can conclude that seeds of spinach can be germinated best on 15% bleach when treated for one minute. After germination cotyledon explants when grown on 2.5 mg/l BAP gave highest number of calli. The best shooting of spinach from callus was obtained at BAP 2 mg/l and NAA 0.2 mg/l. Whereas NAA 0.5 mg/l gave best rooting response. The above findings can be utilized for following areas of research in future,

1. Above optimized micro propagation can be utilized to study genetic transformation of spinach with different genes in future.
2. These findings can also be utilized to study impact of different elicitors on nutritious content of the spinach plant.

Bibliography

[1] B. Santos Filho, A. Lobato, R. Silva, D. Schimidt, R. Costa, G. Alves, and C. O. Neto, "Growth of lettuce (lactuca sativa l.) in protected cultivation and open field," *Journal of Applied Sciences Research*, vol. 5, no. 5, pp. 529–533, 2009.

[2] J. D. Feuerstein, G. Ketwaroo, S. K. Tewani, A. Cheesman, J. Trivella, V. Raptopoulos, and D. A. Leffler, "Localizing acute lower gastrointestinal hemorrhage: Ct angiography versus tagged rbc scintigraphy," *American Journal of Roentgenology*, vol. 207, no. 3, pp. 578–584, 2016.

[3] G. K. E. . J. C. G. Gray, C. G., "Theory of molecular fluids," *Oxford University Press.*, vol. Applications, no. (Vol. 10), (2011).

[4] K. Torssell, *Natural Product Chemistry: A mechanistic, biosynthetic and ecological approach*. Taylor & Francis, 1997. [Online]. Available: https://books.google.com.pk/books?id=23x3QgAACAAJ

[5] R. N. BENNETT and R. M. WALLSGROVE, "Secondary metabolites in plant defence mechanisms," *New Phytologist*, vol. 127, no. 4, pp. 617–633, aug 1994. [Online]. Available: http://doi.wiley.com/10.1111/j.1469-8137.1994.tb02968.x

[6] F. Bourgaud, A. Gravot, S. Milesi, and E. Gontier, "Production of plant secondary metabolites: a historical perspective," *Plant Science*, vol. 161, no. 5, pp. 839–851, oct 2001. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S0168945201004903

[7] L. N. Croteau R, Kutchan T, "Natural products (secondary metabolites). In: Buchanan, B. Gruissem, W. Jones, R, eds. Biochemistry and molecular biology of plants. Rockville, MD:," *American Society of Plant Physiol*, pp. 1250–1318, 2000.

[8] Y. L. Patra B, Schluttenhofer C, Wu Y, Pattanaik S, "Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochimica et Biophysica Acta (BBA)-Gene Regul Mechan," no. 1829(11), pp. 1236–1247, 2013.

[9] P. SW, "The nature and identification of an alkaloid, in: Alkaloid; Chemical and biological perspective," *Willy, New york, pp.*, vol. vol 1(S.W., pp. 1–31, 1993.

[10] W. M. Roberts MF, "Alkaloids: Biochem istry, Ecology and Medicinal Applications. Plenum Press,," *New York*, 1998.

[11] T. F. Castellano, G, Tena, J, "Classification of phenolic compounds by chemical structural indicators and its relation to antioxidant properties of posidonia oceanica (L)," *Delile Enviro*, vol. 67., pp. :231–250, 2012.

[12] H. A. Ferreira JFS, Luthria DL, Sasaki T, "Flavonoids from Artemisia annua L. as Antioxidants and Their Potential Synergism with Artemisinin against Malaria and Cancer," *Molecu*, vol. 15, pp. 3135–3170, 2010.

[13] K. M. Debeaujon I, Peeters AJM, Leon-Kloosterziel KM, "The TRANSPARENT TESTA12 gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium," *Plant Cell*, vol. 13, pp. 853–871, 2001.

[14] A. Krikorian and D. L. Berquam, "Plant cell and tissue cultures: the role of haberlandt," *The Botanical Review*, vol. 35, no. 1, pp. 59–67, 1969.

[15] J. Rolland and C. Sherman, *The Food Encyclopedia: Over 8,000 Ingredients, Tools, Techniques and People*. Robert Rose, 2006.

[16] Z. Liu, H. Yan, K. Wang, T. Kuang, J. Zhang, L. Gui, X. An, and W. Chang, "Crystal structure of spinach major light-harvesting complex at 2.72 å resolution," *Nature*, vol. 428, no. 6980, p. 287, 2004.

[17] G. Lopez-Velasco, G. E. Welbaum, J. O. Falkinham III, and M. A. Ponder, "Phyllopshere bacterial community structure of spinach (spinacia oleracea) as affected by cultivar and environmental conditions at time of harvest," *Diversity*, vol. 3, no. 4, pp. 721–738, 2011.

[18] "World distribution of spinach," http://www.fao.org/faostat/en/#home, accessed: 2018-07-10.

[19] "Spinach producing countries," http://www.fao.org/faostat/en/#home, accessed: 2018-07-10.

[20] A. Cassells, P. Gahan, and S. C. Debnath, "Book review-//dictionary of plant tissue culture," *Hortscience*, vol. 42, no. 3, p. 748, 2007.

[21] F. Levcik and J. Stankovsky, *Industrial Cooperation Between East and West*. Routledge, 2017.

[22] H. Carrer and P. A. L. Barboza, "Sugarcane genetic transformation—advances and perspectives," in *Compendium of Bioenergy Plants*. CRC Press, 2016, pp. 79–104.

[23] A. Barnett, A. Dixon, S. Bellary, M. Hanif, J. O'hare, N. Raymond, and S. Kumar, "Type 2 diabetes and cardiovascular risk in the uk south asian community," *Diabetologia*, vol. 49, no. 10, pp. 2234–2246, 2006.

[24] L. Iskandar, "Effect of spinach (amaranthus hibridus l.) leaves extract solution and milk to level of tooth discoloration due to coffee."

[25] L. S. G. S. S. K. S. S. B. L. I. B. M. H. O. von Berg Andrea Wichmann H-Erich Heinrich Joachim joachim. heinrich@ gsf. de, "Maternal diet during pregnancy in relation to eczema and allergic sensitization in the offspring at 2 y of age–," *The American journal of clinical nutrition*, vol. 85, no. 2, pp. 530–537, 2007.

[26] S. Thiart, "Manipulation of growth by using tissue culture techniques," in *Comb. Proc. Int. Plant Prop. Soc*, vol. 53, 2004, pp. 61–67.

[27] D. Talei, M. S. Saad, M. K. Yusop, M. A. Kadir, and A. Valdiani, "Effects of different surface sterilizers on seed germination and contamination of king of bitters (< i> andrographis paniculata</i> nees.)." *American-Eurasian Journal of Agricultural and Environmental Sciences*, vol. 10, no. 4, 2011.

[28] R. Gautheret, "Culture du tissu cambial," *CR Hebd. Seances Acad. Sc*, vol. 198, pp. 2195–2196, 1934.

[29] S. Prakash and J. Van Staden, "Micropropagation of hoslundia opposita vahl—a valuable medicinal plant," *South African Journal of Botany*, vol. 73, no. 1, pp. 60–63, 2007.

[30] E. Kristkova, I. Dolezalova, A. Lebeda, V. Vinter, and A. Novotná, "Description of morphological characters of lettuce (lactuca sativa l.) genetic resources," *Hortic Sci*, vol. 35, no. 3, pp. 113–129, 2008.

[31] J. M. Zale, S. Agarwal, S. Loar, and C. Steber, "Evidence for stable transformation of wheat by floral dip in agrobacterium tumefaciens," *Plant cell reports*, vol. 28, no. 6, pp. 903–913, 2009.

[32] Y. Sidhu, "In vitro micropropagation of medicinal plants by tissue culture," *The Plymouth Student Scientist*, vol. 4, no. 1, pp. 432–449, 2011.

[33] Á. Máthé, F. Hassan, and A. A. Kader, "In vitro micropropagation of medicinal and aromatic plants," in *Medicinal and Aromatic Plants of the World*. Springer, 2015, pp. 305–336.

[34] C. Brown, J. Lucas, I. Crute, D. Walkey, and J. Power, "An assessment of genetic variability in somacloned lettuce plants (lactuca sativa) and their offspring," *Annals of applied biology*, vol. 109, no. 2, pp. 391–407, 1986.

[35] Y. Israeli, E. Lahav, and E. Reuveni, "In vitro culture of bananas. in 'bananas and plantains'.(ed sr gowen) pp. 147–178," 1995.

[36] S. Valimareanu, "Leaf disk transformation of lactuca sativa using agrobacterium tumefaciens," *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, vol. 38, no. 3, pp. 181–186, 2010.

[37] D. A. Awan, F. Ahmad, and S. Ashraf, "Naphthalene acetic acid and benzylaminopurine enhance growth and improve quality of organic spinach in kitchen gardens," *Journal of Bioresource Management*, vol. 2, no. 3, p. 4, 2015.

[38] O. Oyebanji, O. Nweke, O. Odebunmi, N. Galadima, M. Idris, U. Nnodi, A. Afolabi, and G. Ogbadu, "Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds," *African Journal of Biotechnology*, vol. 8, no. 20, 2009.

[39] F. Ronquist, M. Teslenko, P. Van Der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck, "Mrbayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space," *Systematic biology*, vol. 61, no. 3, pp. 539–542, 2012.

[40] N. Matvieieva, A. Shakhovskij, and M. Kuchuk, "Features of lettuce transgenic plants with the ifn-α2b gene regenerated after agrobacterium rhizogenes-mediated transformation," *Cytology and Genetics*, vol. 46, no. 3, pp. 150–154, 2012.

[41] R. Kalpana and J. Krishnarajan, "Effect of combined application of nutrients and hormones on soybean yield," *Legume Res*, vol. 26, no. 2, pp. 151–152, 2003.

[42] P. Ulvskov, T. H. Nielsen, P. Seiden, and J. Marcussen, "Cytokinins and leaf development in sweet pepper (capsicum annuum l.)," *Planta*, vol. 188, no. 1, pp. 70–77, 1992.