# Tissue Culture Studies of *Berberis lycium* Royle

*By*

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# Tissue Culture Studies of *Berberis lycium* Royle


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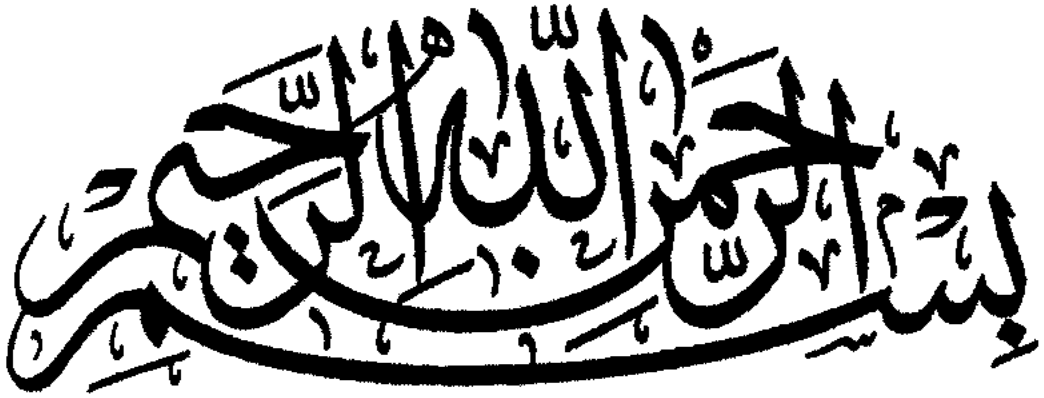
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Dated: 10/08/2015

# FINAL APPROVAL

It is certificate that we have read the thesis entitled "Tissue Culture Studies of *Berberis lycium* Royle" submitted by Ms. Ammara Saleem and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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# DEDICATION

*I would like to dedicate my thesis to my beloved parents who devoted their lives and resources for my better future.*

# DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date: 18th May, 2015.

Ammara Saleem.

# CONTENTS

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**Ammara Saleem.**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 2, 4-D | 2, 4-Dichlorophenoxyacetic acid |
| BAP | 6-Benzylaminopurine |
| Kn | Kinetin |
| NAA | Naphthalene Acetic Acid |
| IBA | Indole Butyric Acid |
| MS | Murashige and Skoog |
| $ddH_2O$ | double distilled water |
| HCl | Hydrochloric acid |
| NaOH | Sodium hydroxide |
| $FeCl_3$ | Ferric Chloride |
| CI | Callus Induction Media |
| SI | Shoot Induction Media |

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# ABSTRACT

The current study was aimed for the *in vitro* multiplication of *Berberis lycium* Royle, an important medicinal shrub by employing tissue culture technique and to optimize a protocol for callogenesis and *in vitro* regeneration. *Berberis lycium* Royle belongs from the genus Berberidaceae and is native to Pakistan, India and whole region of Himalayas. In Pakistan, it grows in Baluchistan, NWFP, Punjab and Azad Kashmir at elevation of 900 to 2900 m. The plant shows various healing activities and for this purpose different portion of the plant such as roots, leaves and fruits etc are utilized. The plant is identified to prevent renal disorders, skin infections, abdominal disorders, common cold and cough, typhoid etc. *Berberis lycium* is also known to have antidiabetic, antihyperlipidemic, hepatoprotective, antibacterial, antifungal, anticoccidial, pesticidal and wound healing properties.

There is only a little published information on the *in vitro* and cutting propagation of *Berberis lycium*. No work has been done on *in vitro* conservation of this plant in Pakistan yet. According to International Union for Conservation of Nature (IUCN) categories, *Berberis lycium* species are vulnerable. Therefore a quick clonal proliferation method and an optimized protocol for the *in vitro* regeneration of *Berberis lycium*, an important therapeutic shrub is needed.

For callus induction different concentrations that are 0.5, 1.0 and 1.5 mg/L of 2, 4-D and different explants like leaves and nodal segments were used. The plant showed best callus induction on nodal explants at the concentration of 1.0 mg/L followed by 1.5 and 0.5 mg/L. Calli were then transferred to MS medium containing cytokinins for regeneration. Different concentrations of kinetin that are 0.1, 0.5 and 1.0 mg/L were used whereas 0.8 mg/L BAP in

combination with 0.2 mg/L kinetin was also tested. Calli increased in size but showed no shoot induction on all the regeneration media combinations indicating that this media combination is only aiding in increasing the size of calli. For direct regeneration nodal explants were cultured on SI-I, SI-II, SI-III and SI-IV. SI-I showed best regeneration having 0.1 mg/L kinetin followed by SI-II having 0.5 mg/L kinetin, SI-III with 1.0 mg/L kinetin and 0.8 mg/L BAP in combination with 0.2 mg/L kinetin. To boost regeneration and shoot proliferation 0.01g/L Silver Nitrate was also added in the medium. Phytochemical analysis of the leaves and callus extracts of *Berberis lycium* were also performed. Alkaloids and phenols were found to be present in the leaf extract of the plant while a complete absence of both of the compounds was observed in the callus extract. The optimized *in vitro* protocol of *Berberis lycium* once established can be used for the large scale production of plants, they can be supplied to pharmacological industries, can be used for gene transformation and biological activities can be studied as well.

# Chapter 1

# Introduction

# 1. Introduction

## 1.1. Medicinal Plants

Medicinal plants are the plants that comprise of constituents having curative effects therefore have been employed from a long time to cure different diseases. In the recent times, due to the fact that pathogens had developed resistance against existing antibiotics, several traditional medicines are recognized as a substitute of health care which has recommenced the research area for the medicinal plants to check their biological activities (Arias *et al.*, 2004).

Nowadays markets are filled with synthetic medicines having severe side effects, huge cost and also disturbing our environment (Zain-Ullah *et al.*, 2013). Whilst, the drugs derived from the medicinal plants are inexpensive having minor side effects and are therefore well-accepted amongst the population and are also considered best for the health (Hussain *et al.*, 2010).

A survey study revealed that about 80% of population rely on these plants worldwide, because they are thought to be a healthy source for life and are also well-known for their curing abilities for numerous diseases (Shinwari and Khan, 2000).

## 1.2. Medicinal Flora of Pakistan

Medicinal plants are considered as the vital natural sources for potentially secure drugs which play an essential part in assuage human health by contributing as an herbal

medicine. In Pakistan, greater than 6000 higher species of plants are present out of which 12% are being used as medicinally (Shinwari and Qaiser, 2011). Many native plants are utilized in herbal medicine to treat diseases and heal different injuries. Such flora frequently shows a broad spectrum of biologic and pharmacologic activities, for instance they are intended to reduce inflammation, shows bactericidal and fungicidal properties (Cowan, 1999). The root, bark, seed and fruit extracts of these plants are exploit in the development of syrups and infusions in traditional medicine (Imtiaz and Manzoor, 2003).

A huge drug market system known as "Pansara" is totally reliant on the natural flora of Pakistan. Treatment of diseases in persons and animals both are achieved by the utilization of therapeutic plants (Shinwari and Qaiser, 2011).

## 1.3. Threats to the Endurance of Medicinal Plant Species in Pakistan

The main threats to the endurance of several medicinal plant species in Pakistan are:

1. Deforestation,
2. Reliance of local people and Afghan refugees on medicinal plants,
3. Traditional health care system,
4. Unsystematic collection of medicinal plants.

Due to such threats a great majority of the plant varieties particularly those comprising therapeutic significance are on disturbingly rate becoming the members of "Endangered Species Club". For instance the Ghamkol region in Kohat (Pakistan) was

once well-off in *Berberis lycium* and *Delphinium kohatense* species but then in early 1980s the Ghamkol immigrant encampment started due to which nowadays, both these species are completely vanished from that vicinity (Shinwari, 2010).

## 1.4. Genus Berberidaceae

Berbericidacease is a huge genus of therapeutic flora having almost 500 species in it (Bhattacharjee, 2001). These are semi-deciduous bushes or tiny trees dispersed in the tropical and semitropical division of Asia, Europe and America. It is mostly found in the mountainous regions of Kashmir and Northwestern Himalayan areas of Pakistan. Among several varieties of *Berberis* that are naturally growing in the Himalayan semitropical belt at elevation of 1000 - 2200 meters, the most frequently accounted ones are *Berberis asiatica, Berberis aristata, Berberis chitria, Berberis osmastonii, Berberis insignis, Berberis vulgaris, Berberis wallichinana, Berberis coriaria, Berberis floribunda, Berberis himalaica, Berberis lambertii, Berberis tinctoria, Berberis virescens, Berberis nepalensis, Berberis petiolaris* and *Berberis umbellate* (Chopra *et al.*, 1996).

## 1.5. *Berberis lycium* Royle

*Berberis lycium* Royle is a spiky plant which is the member of the genus *Berberis* of family Berberidacae. Al-Biruni named *Berberis lycium* as Ambaribis. He referred its name in Persian as Zirkash as well. It is commonly called as Indian barberry in English and Kashmal or Ishkeen in Urdu. It was illustrated by John Forbes Royle in 1837 (Sabir *et al.*, 2013).

### 1.5.1. Distribution

It is dispersed in the moderate and semitropical Asian, European and American divisions (Jafri, n.d.; Irshad *et al.,* 2013). In Pakistan it is extensively distributed in Baluchistan, NWFP, Punjab and in northerly regions like Gilgit, Baltistan, Ghizer, Astor, Diamer, Swat and Azad Kashmir at elevation of 900 to 2900 m (Ahmad *et al.,* 2009a; Ali and Khan, 1978).

### 1.5.2. Morphology

*Berberis lycium* is a vertical flowering bush plant that increases to a length of 3-4 meters, having a solid timber stem and is enclosed in a slight fragile bark. The plant has androgynous (containing both sex organs) flowers which are self pollinated but pollination occurs via insects too (Irshad *et al.*, 2013). The branches of *Berberis lycium* are light white to grayish and have thorns alternatively fixed on them. Leaves of the plant have vibrant color (Ahmad *et al.*, 2009b). The plant blooms from May to June. The flowers have a cupped shape which are arranged in racemes and are mostly pale yellow in color, and are larger than the leaves (Malik *et al.,* 2013). The fruit of this plant are the berries which are oval in shape having bright red or purplish color on ripening. In average the fruit of the plant is 7 mm in length, 4 mm in width and have weight of 227 mg. Its pulp or juice is plum purplish in color and contains approximately 2-5 seeds. Root is stiff, branched and 3-8 cm in width while its timber is leveled and intense yellow in color. Root bark could be approximately 3mm solid, from the outside fractured and inside smooth (Ahmad *et al.*, 2009b).

## 1.5.3. Bioactive Constituents

Numerous biologically significant isolated compounds are berberine, palmatine, berbamine, aromoline, oxyacanithine, umbellatine, ß-sitosterole, punjabine, balochistanamine, oxyberberine, berberine chloroform and palmatine chloroform (some of them are shown in Figure 1.1). The major biological activities are characterized to its most important element, berberine which is an alkaloid (Sabir *et al.*, 2013).

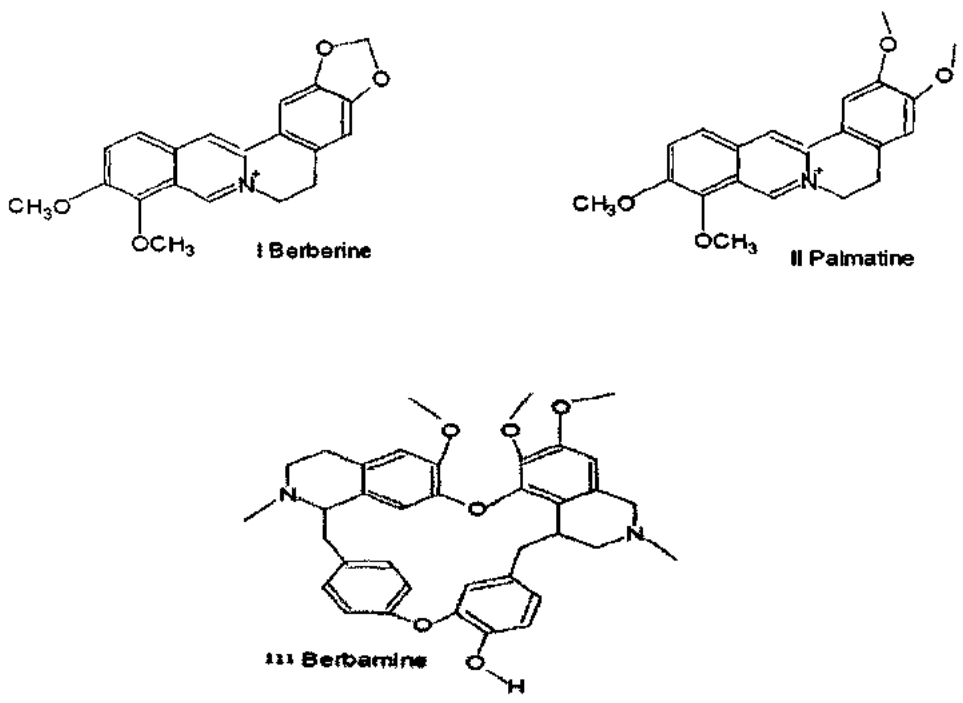



**Figure 1.1: Alkaloids of *Berberis lycium* Royle**

Berberine has a wide spectrum of pharmacological and biological properties which includes anti-inflammatory, antimicrobial properties and antidiabetic properties. Furthermore

*Berberis lycium* had shown antidepressant effects in a variety of behavioral animal models (Gulfraz *et al.*, 2008). Leng *et al.*, (2004) have illustrated the effect of berberine to lower glucose levels, and quite a lot of works have been carried out concerning about its function in the cure of diabetes mellitus, but it is still not clear that how the mechanism is triggered.

Berberine is also useful against amoebiosis, cholera and dysentery (Khan *et al.*, 2010), alleviates fever and has pain-relieving effects (Yesilada and Kupeli, 2002), and was also stated to show antiarrhythmic, anti-tumor (Yamamoto *et al.*, 1993), anti-inflammatory (Iizuka *et al.*, 2000), and rheumatic properties (Yesilada and Kupeli, 2002). There is a very little knowledge about the cellular and molecular anti-tumorous mechanisms which are activated via berberine or through the extracts of *Berberis* species (Khan *et al.*, 2010).

## 1.6. *Berberis lycium* Royal in the Traditional Folk Medicine

*Berberis lycium* is extensively utilized for medical purposes. A general method is to boil small parts of its root and bark in water which is then drained and boiled further until it forms a partially firm accumulate, known as "Rasaut". The root extract is applied for curing UTI, swelling of spleen, stomach and intestinal ulcer and liver diseases. The extract is also used for the external application of the eyelids in acute conjunctivitis, in combination with butter and alum (Sabir *et al.*, 2013). The local population utilizes the powdered form of dried root bark after combining with dissolved animal fat for bone fractures as a bandage. In Asian countries, for the treatment of renal disorders the fruit of the plant is utilized and for gum and tooth diseases the fruit juice is employed. For typhoid and common cold, fruit extract is used (Shah and Khan, 2006). Shoots of the plant are employed for the belly ache, jaundice and

loose bowels (Sabir *et al.*, 2013). The bark of the plant has wound healing activity (Asif *et al.*, 2007). The leaves of the plant are used as an alternative to the tea. The local population utilizes the entire plant for curing bulging and stinging eyes, fractured bones, internal wounds, ulcer, jaundice and rheumatism (Khan *et al.*, 2010).

## 1.7. Conservation Status of *Berberis lycium* Royle

According to International Union for Conservation of Nature (IUCN) categories, *Berberis lycium* species are vulnerable (Waseem *et al.*, 2006; Hamayun *et al.*, 2006a). Hamayun *et al.*, 2006b also stated it as one of the endangered medicinal plant species in Pakistan. Therefore a quick clonal propagation system for *Berberis lycium* (Berberidaceae) an endangered medicinal shrub is needed.

## 1.8. Conservation through Tissue Culture Technique

Medicinal plants are the essential source of medical and a lot of other pharmaceutical products. The conventionally used breeding techniques are the major means of multiplication which acquires a large period for development caused due to the small rate of fruit set or deprived germination and occasionally clonal homogeny is not sustained by seeds as well. In the recent time, because of the increase in the demand for the naturally occurring drugs, the plants are being over-utilized, menacing the endurance of numerous rate species. Furthermore, numerous medicinal plant species are vanishing at a disquieting rate because of the quick rural and urban growth, unrestrained disforestation, and unsystematic assemblage of the plants. Modern biotechnological techniques of culturing plant cells and tissues are

supposed to supply novel ways for preserving and quickly proliferating of endangered medicinal plant species (Nalawade and Tsay, 2004).

The method of cell and tissues culture *in vitro* is a great perspective to biodiversity conservation (Zaiats and Mitrofanova, 2013). The reproduction by *in vitro* culture means micropropagation which is an essential approach to obtain copious amount of identical plants in a short period of time. Through tissue culture technique, explants from different species could be micropropagated under optimized condition of culture media, temperature and photoperiod (Leal *et al.*, 2012).

## 1.9. Factors Affecting *in vitro* Regeneration

A number of factors have an effect on the plant genetic engineering involving a successful tissue culture system for plant regeneration. These factors encompass the type and structure of the explants, the donor plant's genotype, the combinations and concentrations of the media and growth regulators. Primarily the most essential amongst these factors is the genotype (Raghavendra *et al.*, 2010).

All the *in vitro* plant tissue culture procedures are done in a sealed environment in which the culture recipients have a partial or no gaseous exchange due to which ethylene (a gaseous plant hormone) is formed. Amassing of this gas in the culture hinders the embryo and shoot regeneration and development. Ethylene *in vitro* is shown to negatively affect the development of callus, shoot initiation and somatic embryogenesis. Silver nitrate is documented as one of the best inhibitor of ethylene action. It is involved in modifying the

activity or the formation process of ethylene. From years, a great amount of studies have highlighted the helpfulness of silver nitrate when added to the tissue culture medium of plants (Cristea *et al.*, 2012).

## 1.10. Objective of the Study

The present study is focused on an important medicinal plant *Berberis lycium* Royle and is aimed on the *in vitro* callogenesis and $AgNO_3$ mediated direct shoot regeneration of this medicinal shrub by employing tissue culture technique. In this regard the concentration and combinations of different media and plant growth hormones which have been thought to play an important role in callus induction and regeneration frequency was investigated and also phytochemical analysis of the plant was performed.

# Chapter 2

# Review of Literature

# 2. Review of Literature

## 2.1. *In vitro* Multiplication of *Berberis lycium* Royle

*Berberis lycium* Royle is one of the endangered medicinal plant species in Pakistan. There is only a little published information on the *in vitro* and cutting propagation of *Berberis* species (Arena *et al.*, 2000). According to the information, only two studies had been conducted on the callus induction and *in vitro* proliferation of *Berberis lycium* Royle up till now (Dhar *et al.*, 2012; Dhar *et al.*, 2013). No work has been done on the *in vitro* propagation of this plant in Pakistan yet.

In a study callus cultures were initiated from the explants (leaves, internodes and hypocotyls) of the mature *Berberis lycium* Royle plant. Continual growth of callus was found on MS agar medium. Enhancement in the weight occurred for the period of 4 week at temperature 25±2°C. The most favorable concentration of plant growth hormones for the induction and enlargement of callus were 1 mg/L 2, 4-D and 0.2 mg/L Kn (Dhar *et al.*, 2013).

A quick clonal proliferation method of *Berberis lycium* Royle a threatened therapeutic hedge plant was reported. Different quantities of plant growth regulators were examined. Cotyledonary nodal explants that were attained from *in vitro* developed seedlings shown the most excellent results for shoot initiation when 6-benzylaminopurine, GA and 3% sucrose were used. Regenerated plantlets were produced and were successfully transferred to field (Dhar *et al.*, 2012).

## 2.2. *In vitro* Micropropagation of different Species of Genus Berbridaceae

Several studies had been conducted on successful callus induction and regeneration enhancements of different species of genus Berberidacae. *Berberis chitria* is a vital Himalayan flowering shrub recognized for its various therapeutic effects. A successful *in vitro* regeneration procedure for *Berberis chitria* had been established. The seeds of *Berberis chitria* were first cultured on Woody Plant Medium which showed 78.89% of seed germination. WPM when supplemented with BAP + NAA resulted in a greatest number of shoots as per explant. The reproduction rate of the shoots was improved on the subsequent culture media but the trouble that resulted in the reduction of shoot elevation was shoot necrosis. In the shoot multiplication medium casein hydrolysate and gibberellic acid were added which improved the amount of shoots devoid of any necrosis. Microshoots were then transferred to the rooting media containing auxins. The maximum rooting proportion (100%) was noticed on WPM when supplemented with IBA for 7 days after the 8 weeks of the culture. Successfully grown plantlets were then shifted to the pots and hardened (Pandey *et al.*, 2013).

Callus regeneration of *Berberis vulgaris* (seedless barberry) was carried out. Explants that were used were leaves, buds and stems. 2, 5 and 10 mg/L of 2, 4-D, NAA, IBA, IAA and picloram were used on 1/2 and 1/4$^{th}$ strength of MS for callus induction .WPM and B5 medium were also tested. Leaf explants showed the best results for callus development. 8 g/L agar when used in MS medium, shown to have a powerful effect on callus formation. Enhancement in

callus induction was observed extensively on MS media having 2, 4-D, NAA and 10 mg/L picloram (Mohammadi *et al.*, 2011).

For maintaining the growth of *Berberis buxifolia, in vitro* cultures of the plant were developed by means of picloram (4 and 40 mM) or thidiazuron (4.5, 23 and 45 mM) as phytohormones. Initially, for the biomass production picloram or thidiazuron were utilized followed by the addition of BAP (4.4 mM). The *in vitro* grown shoots were considerably lesser than the plants that are grown into the fields. The maximum efficiency was achieved via picloram (Maria *et al.*, 2009).

*In vitro* rooting of *Berberis buxifolia* through polyamines and a mixture of polyamine inhibitors was effectively carried out, in which an innovative rooting medium was employed. Occasionally, the polyamine inhibitors are thought to enhance the rooting but a low concentration (i.e., 1 μM) of polyamine enhanced rooting when added to the nutrient media in contrast with the control medium. Results show that the greatest amount of rhizogenesis of microshoots occurred after the incorporation of 1 μM spermidine in the absence of inhibitors. Generally, the addition of polyamine in the succeeding media increases the amount and the quality of roots in *Berberis buxifolia* (Arena and Pastur, 2005).

*Berberis tinctoria* L. is a native plant to elevated hills of Nilgiris containing lots of therapeutic activities. For the preservation of this plant through mass development, efforts had been made by optimizing the tissue culture techniques. For the callus formation, 0.5 mg/L of BAP and NAA was resulted to be most favorable in the basal medium. Shoot regeneration was

found to be extremely efficient with 0.5 mg/L of BAP in the basal medium. The highest rhizogenesis was observed at 1.0 mg/L of NAA in the basal medium. The successfully grown plants were transferred to the hardening medium made up of soil and vermiculite at the ratio of 1:1 (Paulsamy *et al.*, 2004).

A Patagonian shrub *Berberis buxifolia* is an important plant in immense fiscal prospective. Clonal proliferation is probable by *in vitro* culture but on the other hand these varieties are hard to root. The internally originating polyamine (putrescine, spermidine, spermine) were thought to effect the rhizogenesis phase. Thus, the polyamine alteration, peroxidase action progression, and morphologic improvement were considered to exemplify the *in vitro* rooting of *Berberis buxifolia* microshoots. Polyamine and peroxidase levels were altered extensively throughout the rhizogenesis phase, and showed a reverse effect which was interrelated to the IBA media content directly. For the duration of the first four days, the maximum peroxidase activity and the lesser concentration of polyamine were found in a dark phase with IBA in the culture media. Following culture media can further be improved together with polyamines or extra composites and by changing the ecological circumstances for the achievement of better rhizogenesis (Arena *et al.*, 2003).

The result of different concentrations of BAP, its permanent presence in the culture medium and the incorporation of the activated charcoal for *Berberis buxifolia in vitro* shoot reproduction were tested. The incorporation of BAP supported the regeneration index in the three subcultures in contrast of BAP in the initial subculture, particularly for BAP when used in the concentration of 0.55 and 1.10 µM. The uppermost regeneration index was attained on the lowest

BAP concentrations of 0.55 to 1.10 µM, these lower concentrations were more effective for shoot regeneration as well than the higher concentration of 2.22 µM of BAP. The shooting percentage of the microshoots that were greater in length than 5 mm was highest when supplemented with activated charcoal whereas the regeneration index were found drastically inferior than the ones that were acquired with no activated charcoal. In the subsequent culture phase the regeneration index were lower than the initial culture phase. On the other hand, the shooting percentage of shoots greater than 5 mm was extensively high in the subsequent culture phase than the initial culture. At last the consequences of the study reveals that the morphological procedures tested throughout the *in vitro* shoot regeneration of *Berberis buxifolia* can be managed through the concentration changes, permanent presence of BAP and the by the incorporation of activated charcoal (Arena *et al.*, 2001).

*Berberis buxifolia* is an importance endemic hedge plant of Patagonia because of its therapeutic purposes. An efficient system for *in vitro* regeneration of *Berberis buxifolia* particularly highlighting the rhizogenesis phase was developed. The explants were cultured on MS media supplemented with BAP (0.55 pM). On the MS medium with 1/2 strength of macronutrients, microshoots along with roots were attained. The rooting percentage (80%) was achieved when on the initial stage of culture the shoots were kept in dark for 4 days on a media supplemented with IBA (1.25 pM) for 7 days which was then followed by a media with no IBA till 28$^{th}$ day. The results demonstrated that *Berberis buxifolia* can *in vitro* be proliferated (Arena *et al.*, 2000).

For the clonal proliferation of agarita (*Berberis trifoliata* M.), different tests were performed. As explants the young meristematic agarita shoots were used which were obtained from a fully-grown agarita plant. A basal medium supplemented with woody plant medium (WPM) salts, Murashige and Skoog vitamins, sucrose at 30 g/L, 0.8% Phytagar and 11.1 mM BAP was used. Subculture time, period of cultures, and different cytokinins that are benzyladenine, kinetin, and thidiazuron were examined. A subculture time of 4 weeks and WPM basal medium having 5.5 mM BAP were found to be the most favorable conditions for shoot propagation. There was no affect of culture period on shoot regeneration but the culture period proven to have an effect on rhizogenesis phase. Initial tests that were performed with 1.0 mM of NAA produced almost 100% of roots of the microshoots. The basal medium having 5.4 mM of NAA produced almost 68% of roots of the microshoots with a mean of 1.2 roots per microshoot (Molinar *et al.*, 1996).

An optimized protocol for the complete plant regeneration of *Berberis thunbergii* was developed. During the microculture procedures, 2 mg/L benzyladenine was found to be more effective than the 2 mg/L of isopentenyl adenosine or 1 mg/L of zeatine for the promotion of axillary branches. Different ways for shutting the culture tubes were tested and also 30% of the light intensity was diminished, but these changes had shown no significant result on shoot regeneration. The rhizogenesis of the microshoots was excellent on a medium lacking auxins (Karhu and Hakala, 1991).

## 2.3. Phytochemical Studies

Numerous studies that cover the phytochemical aspects of genus *Berberis* had also been conducted. The major bioactive constituents found, are berberine and berbamine in a range of species of *Berberis* (Mokhber-Dezfuli *et al.*, 2014).

The examination of the crude extract of *Berberis vulgaris* revealed the existence of alkaloids, phenolic compounds and tannins. Berbamine, palmatine and berberine were the first reported alkaloids from *Berberis vulgaris*. Further significant alkaloids that were found are oxyberberine, columbamine, isocorydine, lambertine, magniflorine and bis-benzyl isoquinolines e.g., oxycanthine. Another specie of *Berberis* that is *Berberis stolonifera*, was also examined using dried callus and suspension cultures. Results revealed the presence of five bis-benzyl isoquinoline alkaloids (Mokhber-Dezfuli *et al.*, 2014).

Another study was performed to be familiar with the bioactive components of the bark of *Berberis tinctoria* L. Methanol, acetone, and ethyl acetate extracts were used during the study. The results had shown the presence of 22 main phytocompounds (Deepak and Gopal, 2014).

Tiwari *et al.*, (2014) carried out the phytochemical screening of different extracts of the plant *Berberis aristata*. The extracts had shown the existence of alkaloids, tannins, resins, saponins and proteins in the plant.

Acetone and 70% ethanolic extracts of *Berberis aristata* were examined for the identification of phytochemicals present in it. Alkaloids, phenols tannins, , terpenoids, flavonoids

ans saponins were found to be present in the plant which specifies that the plant may work as photoprotectant and these extracts may be useful in cure of diseases caused by UV rays (Singh and Sharma, 2013).

Phytochemical analysis of roots and stem bark of *Berberis asiatica* was performed.The results reveals the existence of phytocomponents such as phenols, alkaloids, tannins, flavonoids, steroids, saponins, proteins, carbohydrates and free amino acids whereas glycosides were not present in both root and stem bark of the plant (Patni *et al.*, 2012).

The phytochemical study of water extract of *Berberis lycium* Royle was performed. The results suggested the presence of alkaloids, hydrolysable tannins, cardioactive glycosides and saponins in the water extract solution (Ahmad *et al.*, 2009a).

# Chapter 3

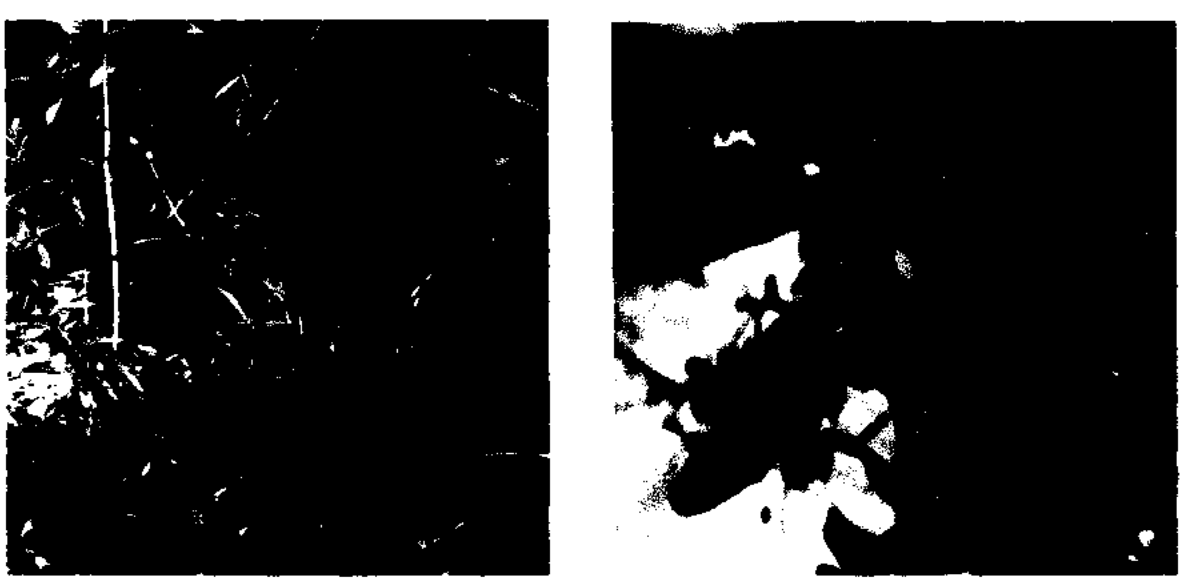
# Materials and Methods

# 3. Materials and Methods

The research work was conducted in Applied Biotechnology and Genetic Engineering Laboratory (ABGE), International Islamic University, Islamabad.

## 3.1. Plant Material

The *Berberis lycium* Royle cultivars used in the research study were obtained from the National Herbarium, NARC, Islamabad (Figure 3.1)



**(a)** **(b)**

**Figure 3.1: (a) A mature clump of *Berberis lycium* Royle obtained as a source material from the National Herbarium, NARC, Islamabad**

**(b) Close up view of clump of *Berberis lycium* Royle**

## 3.2. Apparatus

### 3.2.1. Machinery

Laminar airflow cabinet, fully automatic autoclave (Classic 1050), pH meter (Martini Instruments), Electronic balance (shimadzu), Microwave oven (Orient), Drying oven (DHG-9053A), Shaker (HY-4 speed adjusting multipurpose vibrator).

### 3.2.2. Glassware

Petri plates and beakers (Pyrex, Germany), Culture tubes (25 x 150 mm) and conical flasks (100 ml) were used as glassware for the callus induction, multiplication, and regeneration. Culture tubes and conical flasks were plugged using non-absorbent cotton wrapped in muslin cloth.

### 3.2.3. Tools

Falcon tubes, Micropipettes (Biohit, Finland), Surgical blades, Forceps and scalpels.

## 3.3. Methodology

### 3.3.1. Stock Solution and Media Preparation

### 3.3.1.1. Preparation of Plant Growth Regulators Stock

The auxin used in the present study was 2, 4-D while the cytokinins used were BAP and Kinetin. Stock solutions of all the plant growth regulators were prepared at the concentration of 0.1mg/ml using 1N concentration of NaOH, followed by making up the volumes with double distilled water, appropriately as required. However stock solution of 2, 4-D was prepared at the concentration of 1mg/ml using 1N concentration of NaOH. All plant growth regulators were stored at 4°C and used employing sterile micropipette tips as per requirement prior to autoclaving (Table 3.1).

**Table 3.1: Preparation and Storage of Stocks of Plant Growth Hormones**

| Growth Regulators | Auxins | | | Cytokinins | |
|---|---|---|---|---|---|
| | 2,4-D (1mg/ml) | IAA (0.1mg/ml) | NAA (0.1mg/ml) | Kn (0.1mg/ml) | BAP (0.1mg/ml) |
| Mol. Weight | 221.0 | 175.20 | 186.2 | 215.2 | 225.3 |
| Solvent | 1 NaOH | 1 NaOH | 1 NaOH | 1 NaOH | 1 NaOH |
| Diluvent | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ |
| Storage Temp. | 4°C | 4°C | 4°C | 4°C | 4°C |
| Storage Time | 2 months | 1 week | 1 week | 2 months | 2 months |

### 3.3.1.2. Media Preparation

Synthetic MS medium with vitamins was employed in the study (Murashige and Skoog, 1962). Sucrose was used as a sole carbon source and was added as a solid at the concentration of 3% (w/v). The ph of the media was adjusted by adding either 0.5M NaOH or 0.5M HCl, as required to get a final value in the range of 5.7-5.8. Gelrite was used as a solidifying agent at the concentration of 0.3%. The media was sterilized by autoclaving at 121°C for 15 minutes at 15 psi.

## 3.3.2. Preparation of Explants and Surface Sterilization

*Berberis lycium*, explants were collected from newly grown shoots (nodal and internodal segments). The collected shoots were cut into single nodal segments, apical shoot segments and leaf segments, depending on the requirement of the experiments.

Leaf, apical and nodal shoot segments were washed thoroughly with tap water followed by liquid detergent and shaked continuously for 1-2 minutes to remove dust from the explant surface and washed in distilled water for 5-7 times to remove the traces of the detergent to avoid the carryover effect, after this step explants were surface sterilized under aseptic conditions in Laminar Air Flow that was carried out by using 70% ethanol (v/v) 2 minutes, followed by 10% and 50% Clorox (v/v) for 15-20 minutes. This was followed by thorough washing (4-5 times) with sterile distilled water. The explants were then dried out on autoclaved filter paper (Figure 3.2).

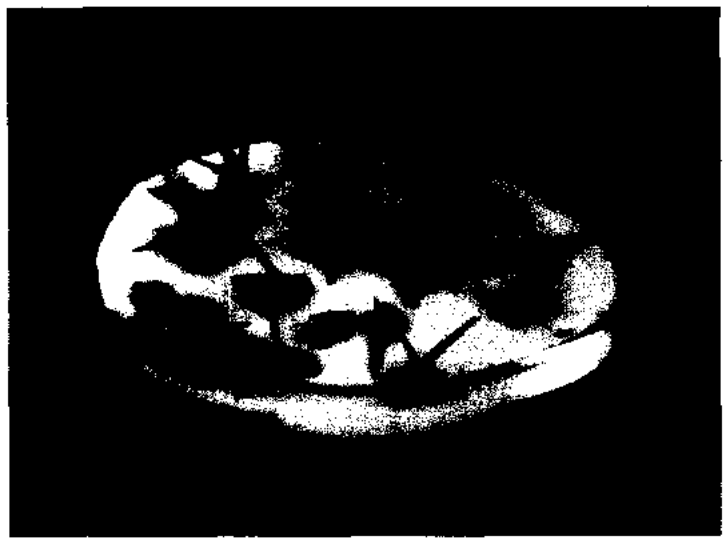


**Figure 3.2: Incised single nodal shoot and leaf segments kept on autoclaved filter paper to dry up**

### 3.3.3. Callus Induction

Different concentrations of 2, 4-D that are 0.5, 1.0 and 1.5 mg/L were used in callus induction media for the initiation of callus. The surface sterilized dried explants were then placed in callus induction media. All cultures were maintained under 16 hours light and 8 hours dark photoperiod at 25±2 ℃ temperature. The calli formed after some specific time of incubation from the explants and then transferred to MS medium having cytokinins for regeneration. The callus induction frequency was analyzed using the formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of calli obtained}}{\text{Total number of explants cultured}} \times 100$$

### 3.3.4. Regeneration

For the regeneration of calluses, different concentrations of BAP that are 0.8, 1.0 and 1.2 mg/L were used whereas 0.8 mg/L BAP in combination with 0.2 mg/L kinetin was also tested. For the direct regeneration procedure from the nodal explants different concentrations of kinetin that are 0.1, 0.5, 1.0 mg/L and 0.8 mg/L BAP along with 0.2 mg/L kinetin were used.

Additionally MS medium supplemented with and without silver nitrate was also tested. 0.01g/L Silver Nitrate was added in the medium to speed up the regeneration and shoot proliferation process. The culture tubes were labeled and were maintained under 16 hours light and 8 hours dark photoperiod at 25±2 °C temperature for four to six weeks and then the data was recorded. The regeneration frequency was calculated by using formula:

$$\text{Regeneration frequency (\%)} = \frac{\text{No. of explants regenerated}}{\text{Total number of explants cultured}} \times 100$$

### 3.3.5. Experimental Design

Factorial design was employed for each and every one of the experiment. All of the experiments were made up of 3 replications. Each replication is comprised of 10 nodes per treatment for both callus induction and regeneration.

#### 3.3.5.1. Statistical Analysis

The data recorded were analyzed statistically by two factorial Analysis of Variance (ANOVA) using Statistix software version 9.0. Significance level was considered as P values <0.05 and the means were evaluated by LSD.

### 3.3.6. Phytochemical Analysis

#### 3.3.6.1. Extract Preparation

The crude methanolic extracts of leaf and callus of *Berberis lycium* were prepared by using the method described by Wadood *et al.*, (2013) with some modifications. For the preparation of leaf extract, fresh leaves of the plant were collected from a mature *Berberis lycium* Royle plant. After that the leaves were washed with the running tap water to wipe out any dust particles and then were kept in a drying oven for some days to dry up. The dried leaves were then powdered using mortar and pestle. A total 1 g of powder was obtained which was then dissolved in 30 ml of methanol in a conical flask and covered with aluminium foil. The mixture was then shaken overnight in a shaker and a total amount of 30 ml of extract was produced.

For the preparation of callus extract, fresh calli obtained from the preliminary experiments were grinded by using mortar and pestle. A total 650 mg of the grinded accumulate of calli was obtained which was then dissolved in 20 ml of methanol in a conical flask and covered with aluminium foil. The mixture was then shaken overnight in a shaker and a total amount of 20 ml of extract was produced. Both the leaf and callus extract solutions were then

filtered with the help of Whatman filter paper No. 1 and used for further phytochemical analysis (Figure 3.3).

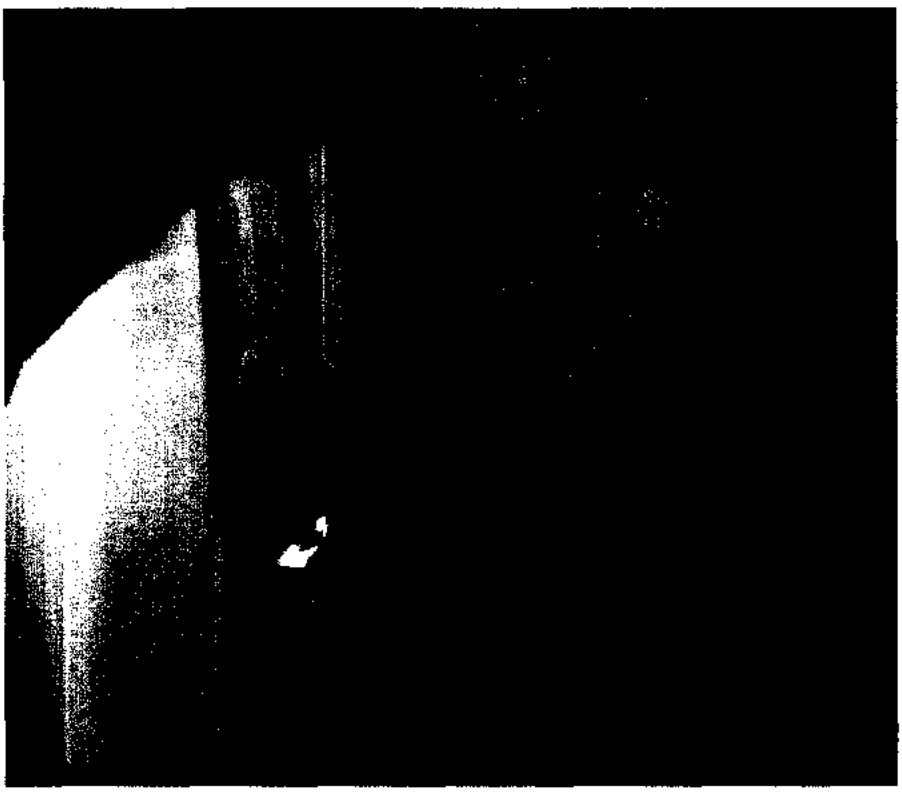


**Figure 3.3: Crude methanolic extracts prepared from the *in vitro* mediated callus and mature leaves of *Berberis lycium* Royle**

### 3.3.6.2. Test for Alkaloids

For the detection of alkaloids in the crude leaf and callus extracts of *Berberis lycium* Royle, 2 ml of each extract was added in a separate test tube and then a small amount of picric

acid was poured in both of the test tubes having extracts. Formation of orange color precipitate indicates the presence of alkaloids.

### 3.3.6.3. Test for Phenolic Compounds

For the identification of phenolic compounds in the crude leaf and callus extracts of *Berberis lycium* Royle, 2ml of each extract was mixed in a 1 ml solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols.

# Chapter 4

# Results

# 4. RESULTS

## 4.1. Effect of different Clorox Concentrations

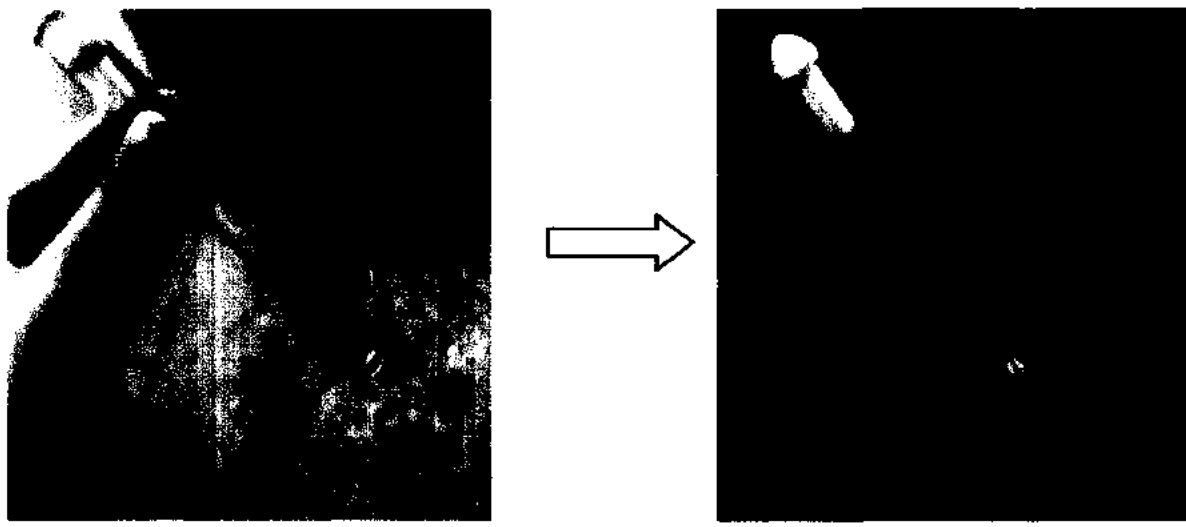
For the prevention of microbial growth in the culture medium, explants were gone through several washing steps and to boost the inhibition of most of the microorganisms, all of these washing steps were carried out very cautiously. The time for every washing step was appropriately recorded. Clorox (commercial bleach) is usually employed in the tissue culture labs for the sterilization of explants. Our results illustrate that 50% Clorox in combination with 70% ethanol and liquid detergent was sufficient to prevent contamination. The rate of contamination enhanced hastily when the explants were examined with 10% Clorox concentration (Figure 4.1). With 50% Clorox the contamination frequency was 14.44 % while with 10 % Clorox the contamination frequency increased up to 75.00 % (Table 4.1). Both of the means were found to be significantly different from one another, therefore 50% Clorox was proved to be sufficient for the eradication of microbial contamination.

## 4.2. Effect of 2, 4-D on Number of Days to Callus Induction

A total 180 explants were cultured, 90 of each explant type, 30 on all three media combinations. Both of the explants took about 10-14 days for the induction calli.

Table 4.2 illustrates that both of the CI-I and CI-III media combinations took minimum number of average days (10) for initiation of calli. Statistically it was revealed that no

significant difference ($P > 0.05$) occurred among media for number of days to calli initiation whereas significant difference ($P < 0.05$) for the number of days to calli formation was found among the explants.



(a) (b)

**Figure 4.1: (a) Nodal and leaf explants washed out with 10% of Clorox along with 70% ethanol and liquid detergent to prevent contamination and are kept in highly aseptic conditions**

**(b) Fungal contamination observed on day 15th in majority of the cultures that were washed with 10% of Clorox**

**Table 4.1: Contamination frequency at different Clorox concentrations**

| S. # | Clorox Concentration | Contamination Frequency |
|---|---|---|
| 1 | 10% | 75.00A |
| 2 | 50% | 14.44B |

LSD = 6.0305

(Data followed by capital alphabets correspond to the individual values as an average of three replicates. Each replicate consisted of 10 treatments).



## 4.3. Effect of Media Combination on Callus Induction Frequency

Optimal callus initiation was found on MS medium supplemented with 2, 4-D although explant types differ extensively in their abilities to start callus induction. Three types of media CI-I, CI-II and CI-III (MS media + 2, 4-D) were used for callus induction frequency. In all media combinations different concentrations of 2, 4-D (0.5, 1 and 1.5 mg/L respectively) were tested.

Callus initiation was observed 14 days after the cultures were inoculated on callus induction media. The plant showed best callus induction on nodal explants at the concentration of 1.0 mg/L of 2, 4-D followed by 1.5 and 0.5 mg/L (Table 4.3) (Figure 4.2). A friable yellowish callus in different sizes was produced. Higher callus induction frequency of 61.66% was

obtained for CI-II followed by 41.66% for CI-III and 30.33% for CI-I. All three media (CI-I, CI-II and CI-III) and both of the explants were found to be significantly different ($P < 0.001$) from one another for callus induction frequency.

Nodal explants showed greatest capability to produce callus whereas not enough amount of callus was produced by the leaf explants. (Krens and Jamara, 1989; Kathiravan *et al.,* 1995) also reported a difference in callus producing capability in different explant types in *Beta vulgaris* and *Morus alba.*

**Table 4.2: The effect of 2, 4-D on number of days to calli initiation**

| Explant | MS + 2,4-D (mg/L) | | | Explant Mean |
|---|---|---|---|---|
| | CI-I (0.5mg/L) | CI-II (1mg/L) | CI-III (1.5mg/L) | |
| Nodes | 13.33b | 25.00a | 15.00ab | 17.77A |
| Leaves | 6.667b | 10.00b | 5.000b | 7.22B |
| Media Mean | 10.00A | 17.50A | 10.00A | |

LSD for media = 7.9341

LSD for explant = 6.4781

(At 5% probability level means followed by same alphabets are not significantly different. Data followed by capital letters are designated to the explant and media means whereas the data

followed by small alphabets stand for the individual values as an average of three replicates. Each replicate consisted of 10 treatments).

**Table 4.3: Callus induction frequency at various concentrations of 2, 4-D**

| Explant | MS + 2,4-D (mg/L) | | | Explant Mean |
|---|---|---|---|---|
| | CI-I (0.5mg/L) | CI-II (1mg/L) | CI-III (1.5mg/L) | |
| Nodes | 30.33cd | 61.66a | 41.66b | 44.44A |
| Leaves | 18.33e | 35.00bc | 25.00de | 26.11B |
| Media Mean | 24.16C | 48.33A | 33.33B | |

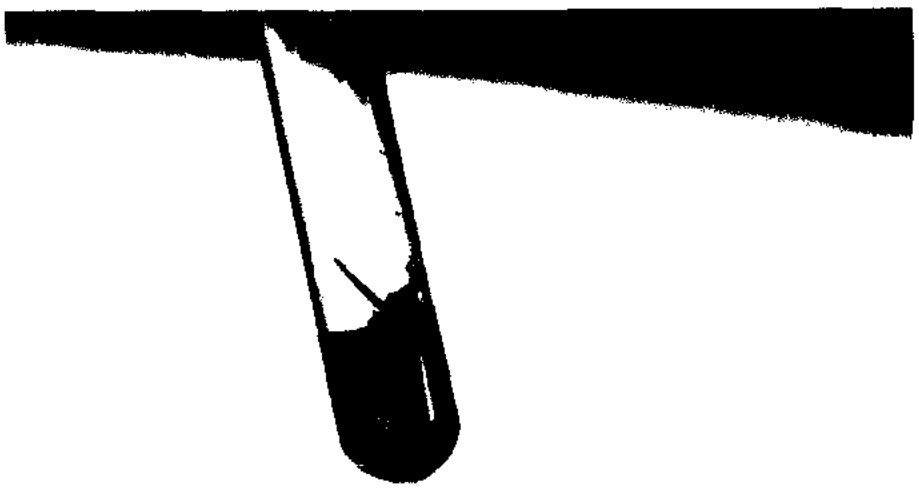
LSD for media = 5.5930

LSD for explant = 4.5667

(Data followed by capital alphabets is designated to the explant and media means whereas the data followed by small alphabets stand for the individual values as an average of three replicates. Each replicate consisted of 10 treatments).

**(a)**

**(b)**



(c)

**Figure 4.2: Callus initiation of *Berberis lycium* Royle on: (a) Callus induction media II (CI-II) supplemented with 1 mg/L 2, 4-D**

**(b) Callus induction media III (CI-III) supplemented with 1.5 mg/L 2, 4-D and**

**(c) Callus induction media I (CI-I) supplemented with 0.5 mg/L 2, 4-D**

## 4.4. Shoot Regeneration

Four different kind of media (SI-I, SI-II, SI-III and SI-IV) were tested for regeneration potential. After shifting of calli to regeneration media, calli showed a constant increase in size but exhibited no growth at all on all the regeneration media combinations indicating that this media combination is only aiding in increasing the size of calli (Figure 4.3). Dhar *et al.,* (2013)

reported no organogenesis or shoot production of *Berberis lycium* Royle from the calli as well while our results indicated a consistent growth in size.

For direct regeneration, SI-I (shoot induction media I) supplemented with 0.1 mg/L kinetin was found to be best showing the highest shoot induction frequency of 86.66% which was followed by SI-II having 0.5 mg/L kinetin with shoot induction frequency of 56.66% and SI-III having 1.0 mg/L of kinetin with shoot induction frequency of 45.00% whereas 0.2 mg/L kinetin in combination with 0.8 mg/L BAP showed 33.33% shoot induction frequency (Table 4.4) (Figure 4.4). Statistical analysis revealed that a significant difference ($P < 0.001$) existed among all of the four media combinations for regeneration efficiency.

**Table 4.4: Regeneration frequency of nodal explants on different media combinations**

| Ms + Kinetin (mg/L) | | | |
|---|---|---|---|
| **SI-I** | **SI-II** | **SI-III** | **SI-IV** |
| **(0.1 mg/L)** | **(0.5 mg/L)** | **(1 mg/L)** | **(0.8 BAP + 0.2 Kn mg/L)** |
| 86.66A | 56.66B | 45.00BC | 33.33 C |

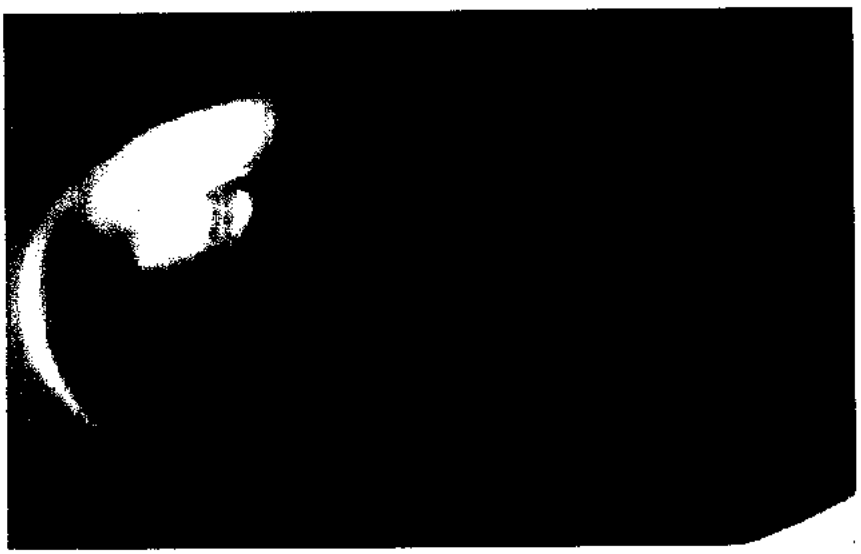
LSD = 14.122

(Data followed by capital alphabets stand for the individual values as an average of three replicates. Each replicate consisted of 10 treatments).

(a)

(b)

**Figure 4.3: An increase in size of calli on MS media supplemented with different concentrarions of (a) BAP and (b) BAP + kinetin**



**(a)**

**(b)**

(c)

(d)

**Figure 4.4: Complete shoot regeneration of *Berberis lycium* Royle on (a) Shoot induction media I (SI-I) supplemented with 0.1 mg/L kinetin**

**(b) Shoot induction media II (SI-II) supplemented with 0.5 mg/L kinetin**

**(c) Shoot induction media III (SI-III) supplemented with 1 mg/L kinetin and**

**(d) Shoot induction media IV (SI-IV) supplemented with 0.8 mg/L BAP, 0.2 mg/L kinetin. The concentration of $AgNO_3$ is similar in all the shoot induction media types.**

### 4.4.1. Effect of Silver Nitrate on Shoot Regeneration

When SI-I, SI-II, SI-III and SI-IV media with no $AgNO_3$ was tested 11.66% regeneration frequency was obtained while the addition of $AgNO_3$ to the same media combinations improved the regeneration frequency up to 75% which clearly indicates that the use of $AgNO_3$, a potent inhibitor of ethylene action, with kinetin had a synergistic effect on regeneration (Figure 4.5). Statistical analysis revealed that both media with or without $AgNO_3$ are significantly different ($P < 0.001$) from each other.

**Figure 4.5: Shoot regeneration of *Berberis lycium* Royle on MS media supplemented with $AgNO_3$**

## 4.5. Phytochemical Screening

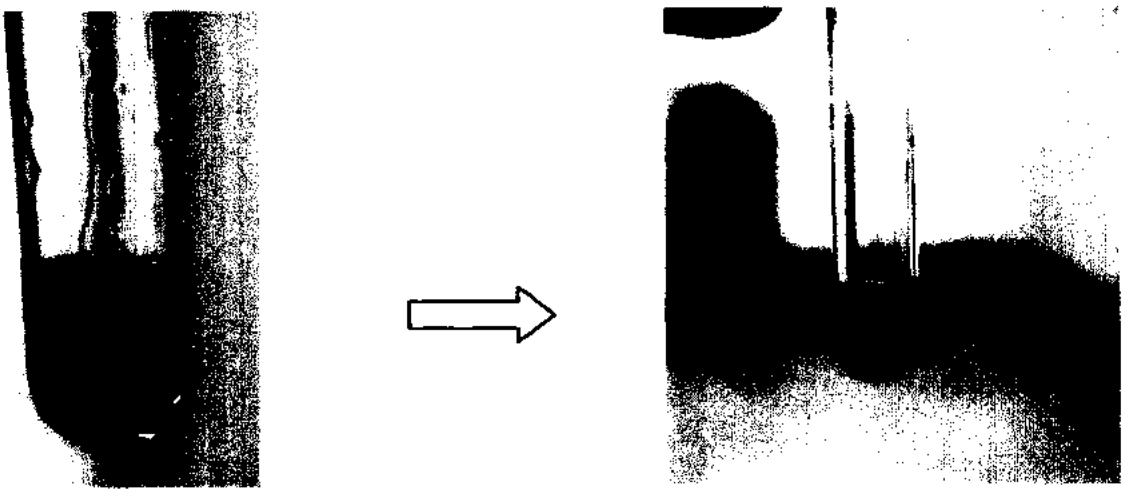
The methanolic extracts of the leaves and calli were subjected to the phytochemical tests to find out the nature of phtyoconstituents. Our results suggested the presence of both of the compounds (alkaloids and phenols) in the leaf extract of *Berberis lycium* (Figure4.6) while a complete absence of both of the compounds was shown by the callus extract (Figure 4.7) (Table 4.5).

Cultured plant cells are normally identified as complicated to produce alkaloids. The actual cause for this is not yet known but it has been recommended that the failure or decrease of alkaloid production is because of the blockage of a specific metabolic reaction step in the biosynthesis reaction. Less alkaloid constituents in the undifferentiated cultured cells of *Datura* and *Scopolia* were found which are mainly caused by the inhibition of tropic acid production (Barz *et al.*, 1977).

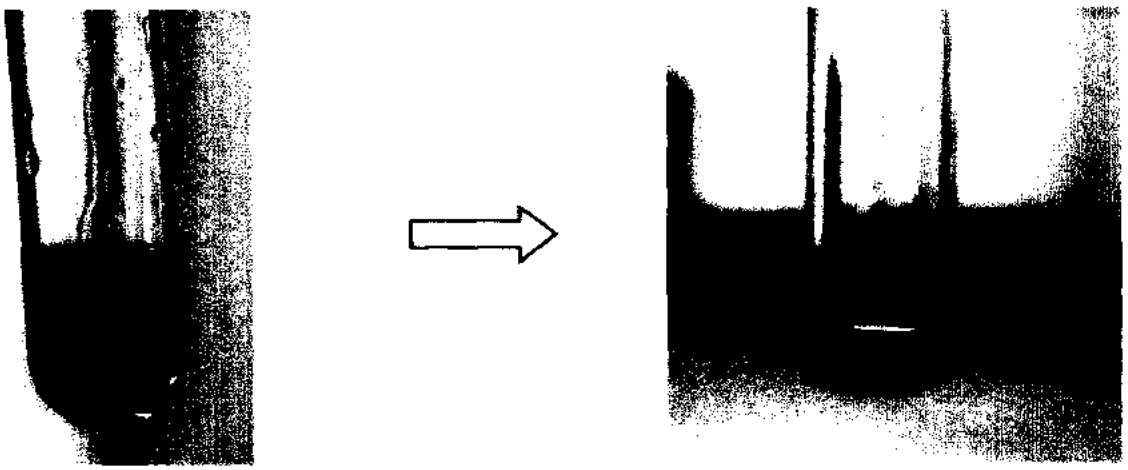
**Table 4.5: The status of secondary metabolites in leaf and callus extracts of *Berberis lycium***

| S. No | Phytochemicals | Methanolic Extracts | |
|---|---|---|---|
| | | Leaf | Callus |
| 1 | Alkaloids | + | - |
| 2 | Phenols | + | - |

+ Present, - Absent



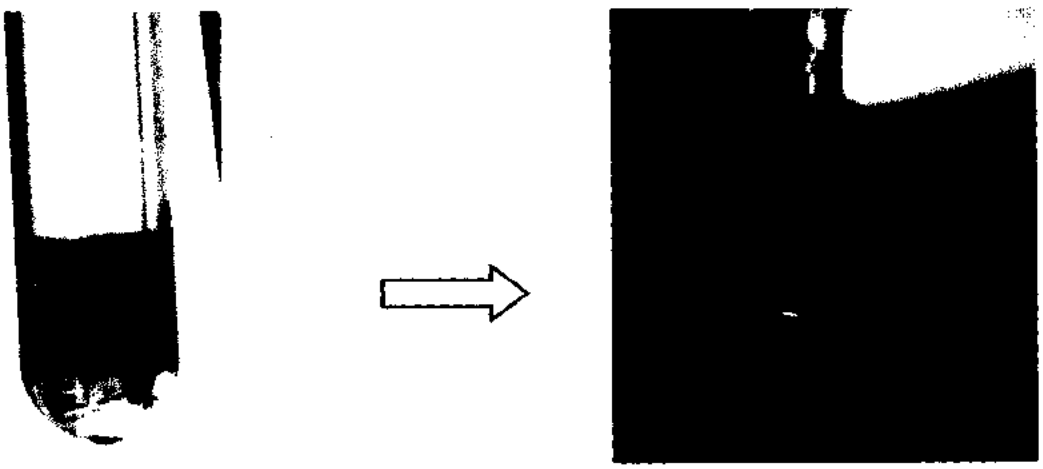
**(a)**



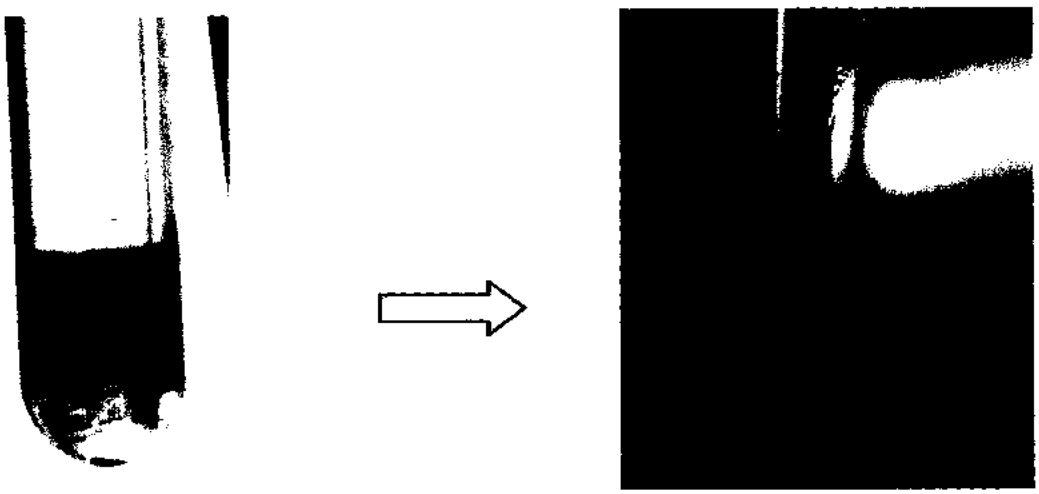
**(b)**

**Figure 4.6: Phytochemical test performed on the leaf extract of *Berberis lycium* Royle indicated the presence of (a) alkaloids and**

**(b) Phenolic compounds**



(a)



(b)

**Figure 4.7: Phytochemical test performed on the callus extract of *Berberis lycium* Royle indicated the absence of (a) Alkaloids and**

**(b) Phenolic compounds**

# Chapter 5

# Discussion

# 5. Discussion

A huge amount of people still relies on medicinal flora and materials derived from them for their daily healthcare needs on this planet. Therapeutic plants naturally contain combinations of different chemical composites that may possibly act independently, additionally or in synergism to get better physical condition. For example, only a single plant might hold astringent materials in it that encourage digestion, anti-inflammatory complex that decreases inflammation and ache, phenols that works as an antioxidant and venotropics, tannins that can work as natural antibiotics, diuretic compounds that improves the removal of waste material and body toxins and alkaloids that improves temper. According to the information one quarter of all medical prescriptions are based on plants derived compounds and as stated by the WHO, a total 80% of the world's population mostly from the developing countries are the ones that relies on medicines derived from the plants for their healthcare (Gurib-Fakim, 2006).

Due to the dependence of approximately 80% of the population globally, medicinal plant species are declining (Zain-Ullah *et al.,* 2013; Shinwari and Khan, 2000). *Berberis lycium* Royle a semi-deciduous shrub is also a threatened remedial plant species of Pakistan (Hamayun *et al.,* 2006b; Dhar *et al.,* 2013). It is mostly found in the mountainous regions of Kashmir and Northwestern Himalayan areas of Pakistan. Berberidaceae family is well-known for its therapeutic properties and is a part of British and Indian pharmacopoeias (Mashwani *et al.,* 2013). Therapeutic effects are masked in its rhizome, rhytidome and dark purplish berries. It posses spasmolytic activity and is employed for the cure of jaundice and injuries within the body

as well (Manan *et al.*, 2007). *Berberis lycium* Royle also acts as a host for stem rust fungus (*Puccinia graminis*) that mostly attacks wheat and other cereals crops (Jin, 2010).

Conservation of medicinal plants requires both worldwide awareness and prompt actions (Hamayun *et al.*, 2006b). In traditional breeding techniques most of the plants do not grow, bloom or germinate seed in harsh environments or takes an extensive phase for their reproduction and development. Therefore, tissue culture techniques are considered good for obtaining a large bulk of plants in the smallest amount of time and space (Sidhu, 2010).

According to the information, only two studies have been conducted on the callus induction and *in vitro* proliferation of *Berberis lycium* up till now (Dhar *et al.*, 2012; Dhar *et al.*, 2013). No work has been done on the *in vitro* propagation of this plant in Pakistan yet. In the current study *Berberis lycium* Royle, an important medicinal shrub was tested for its callus induction and regeneration potential. Phytochemical analysis of *Berberis lycium* Royle was also performed.

For the elimination of microorganisms, NaOCl has normally being employed for the sterilization of explants in tissue culture (Sauer and Burroughs, 1986). In the present study 10% and 50% Clorox was utilized for explant sterilization and 50% of Clorox was found sufficient for controlling the microbial growth. Our results were similar to Oyebanji *et al.*, (2009) and Yildiz and Er, (2002) who also achieved a better surface sterilization at 40% and 50% Clorox concentration.

Effect of plant growth regulators on callus induction and regeneration efficiency differs greatly depending on the types and concentrations of plant growth regulators and substances added to culture media (Somers *et al.,* 2003). In the present study, leaf and nodal shoot segments of *Berberis lycium* Royle were used for the calli production on different concentrations of 2, 4-D. The concentrations of 2, 4-D used were 0.5 mg/L, 1.0 mg/L and 1.5 mg/L. Maximum number of calli were observed on media supplemented with 1.0 mg/L of 2,4-D. Our results revealed that for callus induction of *Berberis lycium* Royle 1.0 mg/L 2, 4-D was found to be adequate, which was deviated from Dhar *et al.,* (2013) who reported that for callus induction of *Berberis lycium* Royle 1.0 mg/L 2, 4-D in combination of 0.2 mg/L Kn was found to be sufficient which reveals that the presence of 2, 4-D either alone or in combination with cytokinins proved essential for the callogenesis of *Berberis lycium* Royle.

For the regeneration of *Berberis lycium* Royle, different regeneration media were examined. The calli shifted to the regeneration media failed to regenerate, similar results were showed by Dhar *et al.,* (2013) who also reported no organogenesis from the calli of *Berberis lycium* Royle. Whereas for direct regeneration from nodal explants, SI-I (0.1 mg/L Kinetin) had shown best regeneration. Our results were deviated from Dhar *et al.,* (2012) who reported maximum regeneration of *Berberis lycium* Royle on 0.8 mg/L BAP along with 0.5 mg/L $GA_3$ on cotyledonary node explants. This may suggest that kinetin alone is sufficient for the direct regeneration of *Berberis lycium* Royle from the nodal explants whereas BAP in combination with $GA_3$ was found to be good for cotyledonary nodal explants obtained from germinated seeds.

Moreover MS medium supplemented with and without silver nitrate was also tested. An amount of 0.01 g/L silver nitrate was added in the regeneration medium which improved the regeneration frequency up to 75% from 11.66%. Ethylene is believed to suppress shoot regeneration *in vitro* where $AgNO_3$ is a strong inhibitor of ethylene action. Therefore the activity of $AgNO_3$ was tested which showed an increase in shoot regeneration frequency which was caused by the disturbance of an ethylene signal transduction pathway. The addition of $AgNO_3$ to the medium showed shoot regeneration lacking the callus phase signifying that these two procedures are inversely interrelated (Zhang *et al.*, 1998).

The optimistic result of $AgNO_3$ in plant tissue culture have been reported in a number of studies for instance in cucumber (Mohiuddin *et al.*, 1997), Chinese cabbage (Zhang *et al.*, 1998), apple (Ma *et al.*, 1998), cassava (Zhang *et al.*, 2001), pearl millet and sorghum (Oldach *et al.*, 2001), date palm (Al-Khayri and Al-Bahrany, 2001) and rapeseed (Akasaka-Kennedy *et al.*, 2005).

The phytochemical analysis revealed the presence of alkaloids similar results to the Ahmad *et al.*, (2009a) and phenolic compounds in the plant. Leaf extract showed the presence of both compounds while in callus both of the compounds were absent which may suggest that *Berberis lycium* Royle at callus stage might not be able to produce these compounds.

# Conclusion

To sum up, a rapid and an efficient method for the *in vitro* callogenesis and $AgNO_3$ mediated direct shoot regeneration of an endangered medicinal plant *Berberis lycium* Royle using nodal explants has been established. The explants were cultured on MS (Murashige and Skoog's) medium supplemented with different concentrations and combinations of plant growth regulators. The plant showed best callus induction in media having 2, 4-D at the concentration of 1.0 mg/L. High frequency direct shoot proliferation was also induced in nodal explants cultured on MS medium supplemented with different concentrations and combinations of cytokinins. Amongst the various concentrations tested, 0.1 mg/L kinetin proved to be the most effective. A proliferating shoot culture was initiated by repeatedly subculturing the original nodal explants on shoot multiplication medium. Our research can successfully be utilized for other important species of genus *Berberis* for their large scale production. The plants can be supplied to pharmacological industries and can be used for gene transformation as well. Biological activities of the plant and callus extracts of *Berberis lycium* Royle can also be studied.

# Future Recommendations

Most of the *Berberis* species are difficult to root *in vitro* and to improve this further awareness of rhizogenesis procedures are required. Therefore further studies can be conducted specially emphasizing on its *in vitro* rhizogenesis.

# Chapter 6

# References

# 6. References

Ahmad M., Alamgeer, and Sharif T., (2009a) A potential adjunct to insulin: *Berberis lycium* Royle, *Diabetologia Croatic*a, 38(1), p. 13-18.

Ahmad M., Alamgeer, Sharif T., Zabta C. H., and Akbar A., (2009b) Effect of *Berberis lycium* Royle on lipid profile in alloxan induced diabetic rabbits, *Ethnobotanical Leaflets*, 13, p. 702-708.

Akasaka-Kennedy Y., Yoshida H., and Takahata Y., (2005) Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): the influence of AgNO3 and genotype, *Plant Cell Reports*, 24, p. 649-654.

Ali M. N., and Khan A. A., (1978) Pharmacognostic studies of *Berberis lycium* Royle and its importance as a source of raw material for the manufacture of berberine in Pakistan, *Pakistan Journal of Forestry*.

Al-Khayri J. M., and Al-Bahrany A. M., (2001) Silver nitrate and 2-isopentyladenine promote somatic embryogenesis in date palm (*Phoenix dactylifera* L.), *Scientia Horticulturae*, 89, p. 291-298.

Arena M. E., and Pastur G. M., (2001) 6-Benzylaminopurine and activated charcoal affect *in vitro* shoot morphogenesis of *Berberis buxifolia* Lam, *Rev. Facultad De Agronomía*, 21 (1), p. 41-47.

Arena M. E., Pastur G. M., Benavides M. P., and Curvetto N., (2005) Polyamines and inhibitors used in successive culture media for *in vitro* rooting in *Berberis buxifolia, New Zealand Journal of Botany*, 43(2), p. 373-380.

Arena M. E., Pastur G. M., Benavides M. P., Zappacosta D., Eliasco E., and Curvetto N., (2003) Peroxidase and polyamine activity variation during the *in vitro* rooting of *Berberis buxifolia, New Zealand Journal of Botany*, 41(3), p. 475-485.

Arias M. E., Gomez J. D., Cudmani N. M., Vattuone M. A., and Isla M. I., (2004) Antibacterial activity of ethanolic and aqueous extracts of *Acacia aroma* Gill. ex Hook et Arn, *Life Sciences*, 75(2), p. 191-202.

Asif A., Kakub G., Mehmood S., Khunum R., and Gulfraz M., (2007) Wound healing activity of root extracts of *Berberis lycium* Royle in rats, *Phytotherapy Research*, 21, p. 589-591.

Barz W., Reinhard E., and Zenk M. H., (1977) Plant tissue culture and its bio-technological application, *In Plant tissue culture and its bio-technological application. Proceedings of the First International Congress on Medicinal Plant Research, Section B, held at the University of Munich, Germany, Springer-Verlag.*

Bhattacharjee S. K., (2001) Berbericidaceae. Handbook of medicinal plants, 3rd edition, *Pointer Publishers*, Jaipur India, p. 58-59.

Chopra R. N., Nayar S. L., and Chopra I. C., (1996) Glossary of Indian medicinal plants, *CSIR*, New Delhi.

Cowan M. M., (1999) Plant products as antimicrobial agents, *Clinical microbiology reviews,* 12, p. 564-582.

Cristea T. O., Leonte C., Brezeanu C., Brezeanu M., Ambarus S., Calin M., and Prisecaru M., (2012) Effect of $AgNO_3$ on androgenesis of *Brassica oleracea* L. anthers cultivated *in vitro*, *African Journal of Biotechnology*, 11(73), p. 13788-13795.

Deepak P., and Gopal G. V., (2014) Phytochemical profile of *Berberis tinctoria* Lesch Bark using GC-MS analysis, *European Journal of Experimental Biology*, 4(2), p. 419-425.

Dhar S., Sharma Y. P., and Wakhlu A. K., (2012) *In vitro* plant regeneration system for *Berberis lycium* using cotyledonary node explant, *Journal of Tropical Medicinal Plants,* 13.1, p. 51-55.

Dhar S. K., Sharma Y. P., and Wakhlu A. K., (2013) Determination of berberine in the callus of the *Berberis lycium*, *Journal of Tropical Medicinal Plants,* 14(1/2), p. 13-17.

Gulfraz M., Mehmood S., Ahmad A., Fatima N., Praveen Z., and Williamson E. M., (2008) Comparison of the antidiabetic activity of *Berberis lyceum* root extract and berberine in alloxan-induced diabetic rats, *Phytotherapy Research.*, 22, p. 1208–1212.

Gurib-Fakim A., (2006) Medicinal plants: traditions of yesterday and drugs of tomorrow, *Molecular aspects of Medicine*, 27(1), p. 1-93.

Hamayun M., Khan S. A., Kim H. Y., Na C. I., and Lee I. J., (2006b) Traditional knowledge and *ex situ* conservation of some threatened medicinal plants of Swat Kohistan Pakistan, *International Journal of Botany*, 2(2), p. 205-209.

Hamayun M, Khan S. A., Sohn E. Y., and In-jung L., (2006a) Folk medicinal knowledge and conservation status of some economically valued medicinal plants of District Swat Pakistan, *Lyonia*, 11(2), p. 101-113.

Hussain J., Ullah R., Rehman N., Khan A. L., Muhammad Z., Khan F. U., Hussain S. T., and Anwar S., (2010) Endogenous transitional metal and proximate analysis of selected medicinal plants from Pakistan, *Journal of Medicinal Plants Research*, 4(3), p. 267-270.

Iizuka N., Miyamoto K., Okita K., Tangoku A., Hayashi H., Yosino S., Abe T., Morioka T., Hazama S., and Oka M., (2000) Inhibitory effect of coptidis rhizome and berberine on proliferation of human esophagus cancer cell line, *Cancer Letters*, 148(1), p. 19–25.

Imtiaz U. H., and Manzoor H., (2003) Medicinal plants of Mansehra, *Hamdard Medicus*, 36, p. 69.

Irshad A. H., Pervaiz A. H., Abrar Y. B., Fahelboum I., and Awen B. Z. S., (2013) Antibacterial activity of *berberis lycium* root extract, *Trakia Journal of Sciences*, 11(1), p. 88-90.

Jafri S. M. H., (n.d.) Retrieved July 24, 2014, from http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=10100

Jin Y., Szabo L. J., and Carson M., (2010) Century-old mystery of *Puccinia striiformis* life history solved with the identification of *Berberis* as an alternate host, *Phytopathology*, 100(5), p. 432-435.

Karhu S. T., and Hakala K. L., (1991) Micropropagation of *Berberis thunbergii*, In *International Symposium on Plant Biotechnology and its Contribution to Plant Development, Multiplication and Improvement*, 289, p. 119-120.

Kathiravan K., Shajahan A., and Ganapathi A., (1995) Regeneration of plantlets from hypocotyle derived callus of *Morus alba*, *Israel Journal of Plant Sciences*, 43, p. 259-262.

Khan M., Giessrigl B., Vonach C., Madlener S., Prinz S., Herbaceck I., Hölzl C., Bauer S., Viola K., Mikulits W., Quereshi R. A., Knasmüller S., Grusch M., Kopp B., and Krupitza G., (2010) Berberine and a *Berberis lycium* extract inactivate Cdc25A and induce a-tubulin acetylation that correlate with HL-60 cell cycle inhibition and apoptosis, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 683, p. 123-130.

Kren F. A., and Jmara D., (1989) The role of explant source and culture conditions on callus induction and shoot regeneration in sugarbeet (*Beta vulgaris* L.), *Journal of Plant Physiology*, 134, p. 651-655.

Leal F., Matos M., Coelho A. N., and Carnide O., (2012) *In vitro* multiplication of aromatic and medicinal plants and fungicide activity, Fungicides for Plant and Animal Diseases, Dr. Dharumadurai Dhanasekaran (Ed.), ISBN: 978-953-307-804-5, InTech, Available from:

http://www.intechopen.com/books/fungicides-for-plant-and-animal-diseases/in-vitro-multiplication-of-aromaticand-medicinal-plants-and-fungicide-activity.

Leng S. H., Lu F., Xu L. J., (2004) Therapeutic effect of berberine in impaired glucose tolerance rats and its influence on insulin secretion, *Acta Pharmacologica Sinica*, 25, p. 496–502.

Manan Z., Sirajuddin, Razzaq A., Islam M., and Ikramullah., (2007) Diversity of medicinal plants in Wari subdivision District upper Dir Pakistan, *Pakistan Journal of Plant Sciences*, 13(1), p. 21-28.

Mashwani Z., Khan M. A., Irum S., and Ahmad M., (2013) Antioxidant potential of root bark of *Berberis lycium* Royle from Galliyat, Western Himalaya Pakistan, *Pakistan Journal of Botany*, 45(Si), p. 231-234.

Mohammadi M., Bagheri A., Marashi H., Moshtaghi N., and Balandari A., (2011) Investigation into seasonal effect and browning inhibitor on callus regeneration of seedless barberry (*Berberis vulgaris* var. asperma), *Plant Tissue Culture and Biotechnology*, 21(2), p. 161-168.

Mohiuddin A. K. M., Chowdhury M. K. U., Zaliha C. A., and Suhaimi N., (1997) Influence of silver nitrate (ethylene inhibitor) on cucumber *in vitro* shoot regeneration. *Plant Cell Tissue and Organ Culture*, 51, p. 75-78.

Mokhber-Dezfuli N., Saeidnia S., Gohari A. R., and Kurepaz-Mahmoodabadi M., (2014) Phytochemistry and pharmacology of berberis species, *Pharmacognosy reviews*, 8(15), p. 8.

Molinar F., Mackay W. A., Wall M. M., and Cardenas M., (1996) Micropropagation of Agarita (*Berberis trifoliata* Moric.), *HortScience*, 31(6), p. 1030-1032.

Murashige T., and Skoog F., (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture, *Physiologia Plantarum*, 15, p. 473-497.

Nalawade S M., and Tsay H. S., (2004) *In vitro* propagation of some important Chinese medicinal plants and their sustainable usage, *In Vitro Cellular and Developmental Biology-Plant*, 40(2), p. 143-154.

Oldach K. H., Morgenstern A., Rother S., Girgi M., O'Kennedy M., and Lorz H., (2001) Efficient *in vitro* plant regeneration from immature zygotic embryos of pearl millet [*Pennisetum glaucum* (L.) R.Br.] and *Sorghum bicolor* (L.) Moench, *Plant Cell Reports*, 20, p. 416-421.

Oyebanji O. B., Nweke O., Odebunmi O., Galadima N. B., Idris M. S., Nnodi U. N., Afolabi A. S., and Ogbadu G. H., (2009) Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds, *African Journal of Biotechnology*, 8, p. 395-5399.

Patni S., Sah A. N., Meena H., Pandey H. K., and Manchanda A., (2012) Physico-chemical, phyto-chemical and elemental analysis of stem bark and roots of *Berberis asiatica, Advances in Applied Science Research*, 3(6), p. 3624-3628.

Raghavendra G., Kumaraswamy G. K., Ramya B., Sandesh H. S., Yogendra K. N., Deepak N., and Gowda P. H. R., (2010) Direct multiple shoot regeneration of *indica* Rice (*Oryza sativa*)

Var. 'Rasi', *The Asian and Australian Journal of Plant Science and Biotechnology*, 4(1), p. 71-73.

Sabir S., Tahir K., Rashid N., Naz S., Masood B., Shah M.A., and Sualeh M., (2013) Phytochemical and antioxidant studies of *Berberis lycium*, *Pakistan Journal of Pharmaceutical Sciences*, 26(6), p. 1165-1172.

Sauer D. B., and Burroughs R., (1986) Disinfection of seed surfaces with sodium hypochlorite, *Phytopathology*, 76(7), p. 745-749.

Shah G. M., and Khan M. A., (2006) Common medicinal folk recipes of Siran valley Mansehra-Pakistan, *Ethnobotanical Leaflets*, 10, p. 49-62.

Shinwari M. I., and Khan M. A., (2000) Folk use of medicinal herbs of Margalla Hills National Park Islamabad, *Journal of Ethnopharmacology*, 69, p. 45-56.

Shinwari Z. K., and Qaiser M., (2011) Efforts on conservation and sustainable use of medicinal plants of Pakistan, *Pakistan Journal of Botany*, 43(Si), p. 5-10.

Shinwari Z. K., (2010) Medicinal plants research in Pakistan, *Journal of Medicinal Plants Research*, 4(3), p. 161-176. Available online at http://www.academicjournals.org/JMPR

Sidhu Y., (2010) *In vitro* micropropagation of medicinal plants by tissue culture, *The Plymouth Student Scientist*, 4(1), p. 432-449.

Singh M., and Sharma E., (2013) Preliminary phytochemical investigation of *Berberis aristata*, Acacia catechu and Ficus bengalensis- important medicinal plants for photoprotection, *International journal of Biological & Pharmaceutical Research*, *4*(9), p. 614-617.

Somers D. A., Samac D. A., and Olhoft P. M., (2003) Recent advances in legume transformation, *Plant Physiology*, 131, p. 892-899.

Tiwari A. K., Ahirwar P. K., and Chaturvedi A., (2014) Preliminary pharmacognostic and phytochemical analysis of *Berberis aristata*, *Journal of Chemical and Pharmaceutical Research*, 6(11).

Wadood A., Ghufran M., Jamal S. B., Naeem M., and Khan, A., (2013) Phytochemical analysis of medicinal plants occurring in local area of Mardan, *Biochemistry and Analytical Biochemistry*, 2(144), p. 2161-1009.

Waseem M., Shah M. A., Qureshi R. A., Iqbal M., Afza R., and Yousaf S., (2006) Ethnopharmacological survey of plants used for the treatment of stomach, diabetes, and ophthalmic diseases in Sudhan Gali, Kashmir Pakistan, *Acta Botanica Yunnanica*, 28(5), p. 535-542.

Yamamoto K., Takase H., Abe K., Saito Y., and Suzuki A., (1993) Pharmacological studies on antidiarrheal effects of a preparation containing berberine and geranii herba, *Nihon Yakurigaku Zassh*, 101(3), p. 169–175.

Yesilada E., and Küpeli E., (2002) *Berberis crataegina* DC, roots exhibits potent antiinflammatory, analgesic and febrifuge effects in mice and rats, *Journal of ethnopharmacology*, 79, p. 237–249.

Yildiz M., and Er C., (2002) The effect of sodium hypochlorite solutions on *in vitro* seedling growth and shoot regeneration of flax (*Linum usitatissimum*), *Naturwissenschaften*, 89(6), p. 259-261.

Zaiats O., and Mitrofanova I., (2013) Features of induction of morphogenesis *in vitro* in some species of genus *Potentilla* L., *Biologija*, 59(3), p. 309–318.

Zain-Ullah., Baloch M. K., Baloch I. B., and Bibi F., (2013) Proximate and nutrient analysis of selected medicinal plants of Tank and South Waziristan Area of Pakistan, *Middle-East Journal of Scientific Research*, 13(10), p. 1345-1350.

Zhang F. L., Takahata Y., and Xu J. B., (1998) Medium and genotype factors influencing shoot regeneration from cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*), *Plant Cell Reports*, 17, p. 780-786.

Zhang P., Phansiri S., and Puonti-Kaerlas J., (2001) Improvement of cassava shoot organogenesis by the use of silver nitrate *in vitro*, *Plant Cell, Tissue and Organ Culture*, 67, p. 45-54.

# Appendix

# Output of Statistix

Callus induction

**Factorial AOV Table for Frequency**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Media | 2 | 3572.22 | 1786.11 | 39.69 | 0.0000 |
| Explant | 1 | 3025.00 | 3025.00 | 67.22 | 0.0000 |
| Media*Explant | 2 | 350.00 | 175.00 | 3.89 | 0.0315 |
| Error | 30 | 1350.00 | 45.00 | | |
| Total | 35 | 8297.22 | | | |

Grand Mean 35.278
CV 19.02
Statistix 10.0 (30-day Trial) 2/16/2015, 9:57:29 AM

**LSD All-Pairwise Comparisons Test of Frequency for Media**

| Media | Mean | Homogeneous Groups |
|---|---|---|
| 1 | 48.333 | A |
| 2 | 33.333 | B |
| 3 | 24.167 | C |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 2.7386 |
| Critical T Value | 2.042 | Critical Value for Comparison | 5.5930 |

All 3 means are significantly different from one another.

**LSD All-Pairwise Comparisons Test of Frequency for Media*Explant**

| Media | Explant | Mean | Homogeneous Groups |
|---|---|---|---|
| 1 | 1 | 61.667 | A |
| 2 | 1 | 41.667 | B |
| 1 | 2 | 35.000 | BC |
| 3 | 1 | 30.000 | CD |
| 2 | 2 | 25.000 | DE |
| 3 | 2 | 18.333 | E |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 3.8730 |
| Critical T Value | 2.042 | Critical Value for Comparison | 7.9097 |

There are 5 groups (A, B, etc.) in which the means
are not significantly different from one another.

**LSD All-Pairwise Comparisons Test of Frequency for Explant**

| Explant | Mean | Homogeneous Groups |
|---|---|---|
| 1 | 44.444 | A |
| 2 | 26.111 | B |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 2.2361 |
| Critical T Value | 2.042 | Critical Value for Comparison | 4.5667 |

All 2 means are significantly different from one another.

Days for Callus induction

Statistix 10.0 (30-day Trial) 2/16/2015, 10:08:35 AM

**Factorial AOV Table for Frequency**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Media | 2 | 450.00 | 225.00 | 2.48 | 0.1003 |
| Explant | 1 | 1002.78 | 1002.78 | 11.07 | 0.0023 |
| Media*Explant | 2 | 105.56 | 52.78 | 0.58 | 0.5645 |
| Error | 30 | 2716.67 | 90.56 | | |
| Total | 35 | 4275.00 | | | |

Grand Mean 12.500
CV 76.13

Statistix 10.0 (30-day Trial) 2/16/2015, 10:09:33 AM

**LSD All-Pairwise Comparisons Test of Frequency for Media**

| Media | Mean | Homogeneous Groups |
|---|---|---|
| 1 | 17.500 | A |
| 2 | 10.000 | A |
| 3 | 10.000 | A |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 3.8849 |
| Critical T Value | 2.042 | Critical Value for Comparison | 7.9341 |

There are no significant pairwise differences among the means.

**LSD All-Pairwise Comparisons Test of Frequency for Media*Explant**

| Media | Explant | Mean | Homogeneous Groups |
|---|---|---|---|
| 1 | 1 | 25.000 | A |
| 2 | 1 | 15.000 | AB |
| 3 | 1 | 13.333 | B |
| 1 | 2 | 10.000 | B |
| 3 | 2 | 6.667 | B |
| 2 | 2 | 5.000 | B |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 5.4941 |
| Critical T Value | 2.042 | Critical Value for Comparison | 11.220 |

There are 2 groups (A and B) in which the means
are not significantly different from one another.

**LSD All-Pairwise Comparisons Test of Frequency for Explant**

| Explant | Mean | Homogeneous Groups |
|---|---|---|
| 1 | 17.778 | A |
| 2 | 7.222 | B |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 3.1720 |
| Critical T Value | 2.042 | Critical Value for Comparison | 6.4781 |

All 2 means are significantly different from one another.

Clorox concentration

Statistix 10.0 (30-day Trial) 2/16/2015, 10:11:34 AM

**Factorial AOV Table for Frequency**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| clorox | 1 | 33002.8 | 33002.8 | 416.45 | 0.0000 |
| Error | 34 | 2694.4 | 79.2 | | |
| Total | 35 | 35697.2 | | | |

| | |
|---|---|
| Grand Mean | 44.722 |
| CV | 19.91 |

Statistix 10.0 (30-day Trial) 2/16/2015, 10:12:29 AM

**LSD All-Pairwise Comparisons Test of Frequency for clorox**

| clorox | Mean | Homogeneous Groups |
|---|---|---|
| 2 | 75.000 | A |
| 1 | 14.444 | B |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 2.9674 |
| Critical T Value | 2.032 | Critical Value for Comparison | 6.0305 |

All 2 means are significantly different from one another.

Shoot induction

Statistix 10.0 (30-day Trial) 2/16/2015, 9:23:23 PM

**Factorial AOV Table for Frequency**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Media | 3 | 9445.8 | 3148.61 | 22.90 | 0.0000 |
| Error | 20 | 2750.0 | 137.50 | | |
| Total | 23 | 12195.8 | | | |

Grand Mean 55.417
CV 21.16

Statistix 10.0 (30-day Trial) 2/16/2015, 9:23:51 PM

**LSD All-Pairwise Comparisons Test of Frequency for Media**

| Media | Mean | Homogeneous Groups |
|---|---|---|
| 1 | 86.667 | A |
| 2 | 56.667 | B |
| 3 | 45.000 | BC |
| 4 | 33.333 | C |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 6.7700 |
| Critical T Value | 2.086 | Critical Value for Comparison | 14.122 |

There are 3 groups (A, B, etc.) in which the means
are not significantly different from one another.

$AgNO_3$

Statistix 10.0 (30-day Trial) 5/8/2015, 3:55:28 PM

**Factorial AOV Table for Frequency**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Agno3 | 1 | 36100.0 | 36100.0 | 454.59 | 0.0000 |
| Error | 34 | 2700.0 | 79.4 | | |
| Total | 35 | 38800.0 | | | |

Grand Mean 43.333
CV 20.56

Statistix 10.0 (30-day Trial) 5/8/2015, 3:56:46 PM

**LSD All-Pairwise Comparisons Test of Frequency for Agno3**

| Agno3 | Mean | Homogeneous Groups |
|---|---|---|
| 2 | 75.000 | A |
| 1 | 11.667 | B |

| | | | |
|---|---|---|---|
| Alpha | 0.05 | Standard Error for Comparison | 2.9704 |
| Critical T Value | 2.032 | Critical Value for Comparison | 6.0367 |

All 2 means are significantly different from one another.