Optimization of Hydroponic system for Propagating and Hardening of in vitro grown medicinal plants

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DECLARATION

I hereby declare that the project work entitled "**Optimization of Hydroponic system for Propagating and Hardening of in vitro grown medicinal plants**" submitted to the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan is a record of an original work done by me and this project work is submitted as a part of fulfilment of award of the degree of Bachelor of Technology under supervision of **Dr. Hemant Sood.**

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SUPERVISOR'S CERTIFICATE

This is to certify that the work titled **"Optimization of Hydroponic system for Propagating and Hardening of in vitro grown medicinal plants"** submitted by **Kritika Thakur** during her end semester in May 2019 in fulfilment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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Signature of the student

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List of abbreviations

IBA	Indole-3-butyric acid
KN	Kinetin
P-I	Picroside-I
P-II	Picroside-II
HPLC	High Performance Liquid Chromatography
P. kurroa	*Picrorhiza kurroa*
S. chirayita	*Swertia chirayita*
W. somnifera	*Withania somnifera*
MS	Murashige and Skoog
EC	Electrical conductivity

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Abstract

For successful establishment of tissue cultured plants in the field conditions, hardening is the significant procedure which counts for the maximum percentage of survival . It is therefore a bottleneck ,while transferring the tissue cultured plants to the natural environment. In order to overcome this , various techniques utilizing different types of potting mixtures, hardening systems and variable physical conditions which includes different temperature and humidity regimes has already been carried out. In this study we have developed an indigenous static , active , fully immersed hydroponics system which was partially covered by thermocol sheet. The plants used for hardening are tissue cultured *Picrorhiza kurroa , Swertia chirata* , *Gentiana kurroo* and *Withania somnifera* which were immersed in media using hydroponic cups filled with coco peat. These plants were hardened using different hydroponic setups containing MS media with varied sucrose concentration which were supplemented with growth hormones like IBA and Kinetin .The system was kept in greenhouse conditions at 25^{0}±C temperature and humidity ranging from 70-80%.Therefore, we were able to achieve some exceptional results in which the plants were hardened in a week within the hydropnic system itself followed by their transplantation to the soil containing potting mixture vermiculite, perlite and cocopeat in ratio of 1:1:1 respectively.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Tissue cultured plants are most desirable as they produce the exact copies of the plant with the desirable trait .Moreover, this technique produces plants more rapidly as in comparison to the conventional technique of growing plants and similarly multiple plants can be obtained by from tissue culture even in the absence of seeds or plant pollinators. For successful propagation of plants they need to be acclimatized in an ex-vitro condition also known as hardening, which is the major bottleneck of this technique. Therefore, to overcome this impediment we have used Hydroponics for hardening procedures.

1.1 Hydroponics- It is the growing of plants without the use of soil in liquid medium that contains nutrient solution. Plants derive nutrients from soil which also anchors its roots. The hydroponic medium is enriched with nutrients and growth hormones that promote plant growth. The undesirable nutrient concentration can be avoided with the elimination of soil. Nutrient toxicity and its deficiency both are important to plant growth and can be easily manipulated with use of hydroponics. It has been noted that the hydroponically grown plants grow faster as in comparison to the soil grown plants as the plants are directly supplemented with the nutrients and they need not spend any energy for finding nutrients.

1.2 Growing media- Growing media are materials that plants grow in. Growing media is specifically designed to support plant growth and can either be a solid or a liquid. Different components are blended to create home-made and commercial growing media. Different types of growing media are used to cultivate various plants. Growing media may also be known as grow media, culture medium, or substrate. Hardening of plants require a media that can either be a mixture of components or a single component. Hardening of plants require a substrate that can either be a mixture of the below components or a single component.

1. **MS media**- Murashige and Skoog medium (or MSO or MS0 (**MS**-zero)) is a plant growth medium used in the laboratories for cultivation of plant cell culture.

2. **Coir peat**- coir which is also known as coco peat, are the leftovers of the coconut's outer most shell after the removal of its fibers. The coir contains the Trichoderma fungi that protect roots and promote root growth.

3. **Perlite**- Light weighted glass pebbles that have been expanded by superheating the volcanic rocks. Retains less water and large amount of air

4. **Vermiculite**- Retains more water than perlite.

Table1.2.1 - The table represents the composition of stock solutions of MS media

Stock solution and components	*for MS medium g/L*	*for DKW medium g/L*
Stock solution A (nitrogen):		
Ammonia nitrate	82.5	98.0
Potassium nitrate	95.0	—
Calcium nitrate	—	98.0
Stock solution B (sulfates):		
Magnesium sulfate heptahydrate	18.5	37.0
Potassium sulfate	—	78.0
Stock solution C:		
Calcium chloride dihydrate	22.0	7.35
Potassium phosphate	8.5	13.0
Stock solution D (chelated iron):		
Ferric sulfate heptahydrate	1.39	1.65
Sodium ethylene dinitrotetraacetic acid	1.88	2.25
Stock solution E (micronutrients):		
Manganese sulfate monohydrate	1.110	1.700
Zinc sulfate heptahydrate	0.430	—
Zinc nitrate hexahydrate	—	0.850
Boric acid	0.310	0.250
Potassium iodide	0.042	—
Sodium molybdate dehydrate	0.013	0.020
Cupric sulfate pentahydrate	0.0013	0.0125
Stock solution F (organics):		
Myo-inositol	5.00	5.00
Glycine	0.10	0.10
Pyridoxine hydrochloride	0.025	—
Nicotinic acid	0.025	0.05
Thiamine	0.005	0.10

1.3 Types of Hydroponic systems:

There are five different types of hydroponics

1. **NFT (Nutrient Film Technique)** - The solution is forced into the channels that retains a variable amount of plants. The assembly is slightly tilted , the solution runs through the channel touching the roots and then back in the hygroponics tray.

2. **DWC (Deep Water Culture -**It is a hydroponic growing method that retains plants roots in a well-oxygenated solution full of nutrients and water 24/7.

3. **Wick System-** A fully passive system that can wholly work without the use of electricity. The plants are kept in a coco peat along with a wick attached to it that is immersed in the solution tank.

4. **Drip Hydroponics-** The solution is forced through pipes directly to the base of the plant. It can be circulating or non- circulating. The later system drips slowly so as to provide nutrients to the plant constantly. In the circulating system the excess of the solution moves back in the tank.

5. **Aeroponics-** The plants are hung in the air and the roots are provided with the nutrients through spraying.

1.4 Factors affecting plant growth in hydroponics:

a) Electrical conductivity:

Electrical conductivity of any media used for the growth and development of plants determines the availability of nutrients or the amount of nutrients present in that particular media. This parameter is measured with the help of an instrument known as EC meter. Range of the electrical conductance varies from plant to plant. More are the salts in the media more is the EC of the media. Generally the range of EC varies from 1.0-2.5 for the optimum growth of the plants. Therefore to enhance the electrical conductivity of the media used addition of fertilizers can be used and to lower the value distilled water can be used so as to make media compatible for the plant growth.

b) PH:

Plants for their growth in hydroponic system requires optimum range of PH of their medium in which they are grown . This also affect the availability of nutrients to the plants .It ranges from 5.7-6.5 , therefore generally plants grows in the neutral range of the PH. PH of the media can be optimized using acid (HCl) or base (NaOH). This can be measured using PH meter. If medium in which plants are grown does not have the optimum range of the PH , they may loose the ability to absorb nutrients.

c) Temperature:

Temperature is another essential factor which affects the growth of the plants. Higher temperature leads to the moisture loss and whereas lower temperature contributes to the degradation of the plant growth. Optimum range varies from 18°-25°C . Excessive or very less temperature may cause stress to the plants.

d) Dissolved oxygen:

Dissolved oxygen is the total amount of gaseous oxygen present in the medium. If the plants in hydroponics are deprived of the oxygen it will lead to the less or no uptake of the minerals by the roots of the plants which will eventually leads to the death of the plants hence to maintain the sustainability of the plants dissolved oxygen is required. For the maintenance of dissolved oxygen in the media oxygen is supplied with the help of the oxygen pump and therefore helps in plant growth.

1.5Hardening:

Tissue culture is an advanced technology that can be utilized for enormous production of high quality planting material for most crops. This technology comprises of five major steps 1)Initiation 2) Multiplication 3)Shooting and rooting 4)Primary hardening in green house 5)Secondary hardening in shade net houses. Hardening is the process used for the successful transplantation of in vitro grown plants to outside environment by acclimatizing them to the harsh environmental condition step by step. This is done by exposing them to variable temperature, humidity and light conditions. It is an important step for the successful survival of plants. Research shows that number of plants die during this step due to rapid water loss and improper stomata functioning. The leaves and stock in the culture are tender due to large amount of water present in medium, when shifted to natural environment suffers high water loss and desiccation. Sudden change in nutrients availability also results in plant death, as they are not readily available in ex-vitro conditions. The transfer of the cultured plants from lab to the ex vitro conditions is an intense process so as to convert them into true autotrophs from hetrotrophs. Preliminary to hardening, there should be an equal ratio of roots and shoots in cultured plant that will aid one another. The plants can now be shifted to the outside conditions.For successful transplantation adjusting humidity is also an important aspect of acclimatization.

Therefore to overcome above problems a lot of researchers have worked on different techniques of hardening to increase the survival rate of tissue grown plants such as hardening of tissue cultured raised tea plants through the use of rhizosphere bacteria(Anita Pandey et al, 2000) , hardening of tissue cultured plants by maintaining plants in highly reduced level of MS solution (1\10th strength) and by the use of no plant growth regulators(C.R Deb et al, 2010) . The process of hardening is being carried out in multiple varities of in vitro grown plants like in *Curculigo orchioides* which used Bavistin (0.01%) along with 1\4th strength of MS(5-6ml per pot) in pots containing mixture of soil and vermiculite (1:1) ,high humidity was maintained by spraying of distilled water hourly. S.K Tiwari carried out hardening of *Tectona grandis* by transferring the pants to potting mixture containing soil:vermiculite(1:1) that is enriched by 1\4th strength of MS media,further the plants were covered with polythene bags.Shailesh R. Vasani carried out secondary hardening in tissue cultured Banana plantlets using Azotobacter(for nitrogen fixation) and Aspergillus (phosphate solubilization) in potting mixture that contained pressed mud cake, saw dust, rice husk, coir pith,organic manure and poultry manure.According to A.H. Halevy there are certain factors that affect the hardened plants like water status(water and relative humidity),agar and sucrose concentrartion and decrease in relative humidity. Some scientist have carried out biological techniques for hardening of micropropagated plants like the research carried out in Life Sciences Department of Jawaharlal University,New Delhi. They used

Piriformospora indica which is a novel plant growth promoting root endophyte. The research used regenerated plantlets of tobacco subjected to two different biological hardening techniques showed greater survival rate when inoculated with *P.indica* as in compasrison to uninoculated controls.The fungus has the capacity to provide protection to the cultured plantlets and aid them to escape the 'transient transplant shock'.There are morpho-physiological changes observed during acclimatization of micropropagated plants of *Leucaena leucocephala* like their leaves had little epicuticular wax and lacked starch grains in their cells. However, during hardening starch grain appeared following the normalization of mesophyll cells.After hardening plants can be used for the further analysis

1.6 *Picrorhiza kurroa*-

It is a perpetual herb and it belongs to the family Scrophulariaceae. It is also called as Kutki , Kadu in Gujrati, Katui in Sanskrit.It is one of the ancient plant that is rich in its medicinal properties.Present in areas of Kashmir and extends to Sikkim at an altitude of 2700-4400 m. Majorly present at an altitude of 3500-4800 m.Also found in India, Pakistan, Bhutan, China and Tibet. In India present in areas of Himachal Pradesh, Uttarakhand. The plant is bitter in flavor and has stretched out rhizomes of 10-15 cm in length. *Picrorhiza* has tiny, frail and hairy stem,10-15cm elongated leaves that are woody in nature. The flowers are small , bluish purple in color , shape is spiky and pointed. The stamens are much extended to outwards. The fruits are spherical capsules and 1-1.3 cm in length and the seeds are whitish in color. It is a medicinal herb that is traditionally used to cure liver and respiratory illness, bronchial asthma, constipation and jaundice. The plant retains a number of medicinal properties which includes antimicrobial, hepatoprotective , antioxidant, antimalarial, antidiabetic and antiallergic.

Fig.1.6.1-*Picrorhiza kurroa*

1.7 *Swertia chirayita:*

It belongs to the family **Gentianaceae** and a large group of annual and perennial herbs are also included in this genus.*Swertia* was first reported by Roxburgh by the name of *Gentiana chyrata* .Also known by different names like Chirata , Nelavemu ,Chirayita,Chiretta. It is critically endangered plant species due to its excessive exploitation for its medicinal properties.The plant is distributed all over the Himalayan region at an altitude of 1200 m - 3000 m.Found in parts of China and Nepal and also grown in parts of north-eastern Himalayas.Swertia has an upright annual herb about 0.5-1.5 m in length.The stem is robust and brownish in color and length extends from 1-2ft.The leaves are five nerved with sharp tips.The flowers are yellowish green in color with a purplish tint on it, numerous petals are present with leafy panicles.The fruits are capsule shaped and are minuscule with acute tips.The seeds are smooth and dark brown in color.The plant is medically important as it is used in treatment of diabetes , piles, ulcers and certain skin infections.It has multiple curative properties such as anti-fungal anticancer anti-pyretic anti-oxidant and anti-bacterial.

Fig 1.7.1-*Swertia chirayita*

1.8 ***Gentiana kurroo:***

Gentiana kurroo commonly known as ' kutki' is an endangered plant of Himalayas, however in some region of Kashmir is known as 'Neelkanth'.The plant is known for it's antimicrobial, antidiabitic and antioxidant

Properties. This plant belongs to Gentianaceae family. Rhizome and roots of this plant is utilised for the medicinal purpose . Due to its expoitation plant had become intensly endangered species. nearly 360 species have been reported till now.These annual and perrenial herbs are also found in temperate regions of

India , Nepal and Pakistan. At an altitude of 1850-2000 m from pahalgam area of Kashmir plant was found

for the first time. New plant species were also spotted in the north western regions like Mussooree

Budraj and Shimla. Plant generally have Dark blue to purple flowers with fruits of 0.6 to 0.75 inc in diameter. Height of plant varies from 0.5 to 1 foot. Parts used for various purposes - Roots containing kaloids, gentioflavine,xanthones,gentiopicrin. Leucoderma and Syphillus can be cured using the roots of plant. Plant also contributes in treatment of fever and also acts as blood purifier.

Fig1.8.1-*Gentiana kurroo*

1.9 *Whitania somnifera:*

Whitania somnifera which belongs to the family solanaceace is also known as **Ashvagandha,** which derived from the Sanskrit word Ashva meaning horse and Gandha meaning smell ,that is horse like odor from its roots.Plants is generally found in the region of Nepal,China and Yemen. Height of the plant varies from 10-15 cm in height and produces fruit of orange colour. Plant contains withanolides along with alkaloids. This plant holds great medicinal values and is useful in many ways. Plant helps in treatment and prevention of several diseases like arthritis, impotence, amnesia,anxiety,cancer, neurodegenerative and cardiovascular diseases. Commonly known by the name of Ashwgandha Rasayana ayurvedic drug contains *Whitania somnifera* (Ashvagandha) which has its action on bone marrow and reproductive organs.Leaves ,bark and roots are used as a medicine.

Fig. 1.9.1 ***Withania somnifera***

1.10 Rationale and Research gap

- As the rate of survival of in vitro plants during hardening is very less through conventional hardening techniques. Therefore, this experiment focuses on increasing the survival rate of in vitro grown plants when transferred from lab to fields.
- As the conventional hardening technique is very time consuming and parameters for optimization of hardening protocols varies from plant to plant . Therefore , in order to minimize time for hardening , different hydroponic system would be optimized.
- Hydroponic systems are mostly used for growth of horticulture and vegetable plants whereas , few medicinal plants have been targeted for propagation.
- No Hardening in hydroponics has been carried out yet.

1.11 Objective

1. Development of hydroponic system for propagating and hardening.
2. Optimization of growth conditions for improving the survival percentage of in vitro grown plants.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Micro propagation of plants:

Picrorhiza kurroa have many medicinal properties and therefore is used in large amounts. Plant was taken from its natural habitat that is Rohtang and was cultured in laboratory conditions. In vitro grown micropropagated shoots were kept in liquid and agar medium which were supplemented with Kinetin and BAP. Prior to hardening rooted plantlets were kept at 5^0 and 15^0 C for 10 days. Plants were then transferred to greenhouse in small jars. Plants were then sprayed with Hoagland solution. Data was recorded after 8weeks of transfer [12]. Whereas *Genitana kurroo* was also multiplied by using MS media having different concentration of Kinetin, BAP, IBA either or in combination for elongation. Plants were transferred to pots containing autoclaved mixture of farm yard manure and clay (1:1).Pots were then covered with plastic sheet or glass beaker to maintain the humidity. Plants were transferred to the greenhouse for hardening. It was kept under observation for 45 days in earthen pots[11]. Use of combination of growth regulators for callus regeneration and for regeneration of different explants (leaves,petioles,roots) of *Gentiana kurroo* Royle responded differently accoding to the combination of growth regulators[20]. *Swertia chirayita* was also propagated through axillary bud culture and was hardened using different techniques. Shoots and roots developed were kept in MS medium with different concentration of IBA,IAA,NAA. For invitro hardening roots were transferred to 1\4th MS strength medium not having PGR's for 7 days. After they were kept in the containers containing mixture of soil: sand: manure (1:1:1) and were covered with perforated polythene bags and were kept under green house conditions. Plants were kept under observations[10].

2.2 HYDROPONICS :

Many factors are responsible for the deterioration of land used for cultivation of crops one of the major reason is global warming , other reasons include land pollution, over use of fertilizers which leads to depletion of fertile land. Effect of these factors can be seen on vegetation, which fails to fulfils the demands of huge population. Hydroponics ,in this scenario plays a vital role which can contribute in filling the voids. This is the soil less culture which helps in facing various challenges. Hardening of in vitro grown plants (medicinal plants) is another application of the soil less culture.

Techniques of hydroponics:

1) Circulating methods (cross flow solution cultured system)/continuous

2) Non circulating method

Therefore hydroponics not only meets the demands of the population but also affects the yield of production of the crop[1].Hydroponics focuses not only on the growth of medicinal plants but hardening is also major con-

cern. Various research claims that the concentration of bioactive substance can be varied when these plants are observed under controlled conditions.

The hydroponic system is a useful technique of cultivating various crops like lettuce and other leafy vegetables through optimizing various conditions like the temperature ,ph, humidity, light, irrigation and fertilization of the plants that are growing in a medium that provides not only the mechanical support but also provides sufficient nutrients . They can be rock wool, clay pebbles, vermiculite, perlite, coco peat, mixture of these and others. The purpose of cultivating propagated or other medicinal plants in hydroponic systems is to achieve sufficient growth associated with magnificent crop quality and large amount of bioactive substance. Having inspected all characteristics of the technique, hydroponic cultures can have varied results like change in yield, with different percentage of bioactive substances and this kind of system can be the way to cultivate the medicinal plants in commercial purpose .Not only the economical and chemical benefits , the hydroponic systems of cultivating medicinal plants, helps in protection of diverse flora and the variety of wild species[2].Hydroponics is also used to study abiotic stress tolerance.

Hydroponics allowed researchers to easily notice the effects of elemental deficiencies and toxicities and to study other aspects of plant development under specific conditions. With the help of this soilless water cultures people could conclude that plants absorbs nutrients in various forms and in varied concentration.

Transfer of plantlets to hydroponic system varies from one plant species to another.

A typical growth solution consists of the following essential macro-elements: nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg) and sulphur (S); and micro-elements: iron (Fe), boron (B), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn),

Molybdenum (Mo) and chlorine (Cl), and, for leguminous species requiring N fixation, cobalt (Co). Sometimes, silicon (Si) and selenium (Se). Along with these components medium is supplemented with carbon source either organic or inorganic. Sometimes growth hormones are accompanied with these components.

pH of the medium is also very essential factor to be taken care of as it affects the dissociation of ions and uptake of nutrients by the plants .pH can be maintained by automated systems attached with the system. Acid and Base can be dispended into medium whenever there is alteration in the ph of the medium. Hydroponics can control the situation in abiotic stress condition like droughts, salinity, toxicity(due to boron and aluminium) and element deficiency in the plant[3].Different nutrient solution influences the content of pharmaceutical compounds in medicinal plants[18].

2.3 HARDENING :

Hardening simply means introducing the plants to various conditions like direct sunlight , dry air , humidity etc. This is generally a tedious process which involves several techniques and methods. Medicinal plants like *Picrorhiza kurroa*, *Swertia chirata* are hardened using copious methods .

The hardening of these in vitro raised plantlets is vital for better stick out and victorious establishment. Direct transfer of tissue culture raised plants to field/wild is impractical because of high rate of death, Direct transfer to sunlight also causes charring and scorching of leaves and drooping of the plants . Therefore it is necessary to habituate plant to its surroundings through the hardening process [4].Secondary metabolites of medicinal plants are worthwhile as they give rise to several health benefits. Extracts of roots and rhizomes of *P. kurroa* have shown hepatoprotective activity in manifold models of liver toxicity. *P. kurroa* contains iridoid glycosides like picroside I, II, III, pikuroside, kutkoside and 6-feruloyl catalpo, cucurbitacin glycosides, androsin , apocynin, and organic acids such as vanillic and cinnamic acids. It is appropriate to note its ayurvedic properties of tikta rasa, laguruksha guna, and katuvipaka. Based on such properties, one may anticipate its pharmacodynamic activity on lipids specifically related to lipid disorders.Picroside I has earlier been shown to be active in several models of liver toxicity. In India where ayurveda is at it's peak plant like *Picrorhiza kurroa* are used in curing many diseases related to liver and thus shows their hepatoprotective properties. *Picrorhiza kurroa* is known to heal diseases like jaundice. Ayurvedic formulation Arogyavardhi containing 50% P. kurroa also found functional in a double-blind trial in viral hepatitis [5].

This shows the importance of the plant and therefore hardening is vital step in plant growth. conventional hardning of other in vitro grown medicnal plant like *Garcinia indica chois* was performed.seeds of the plant were innoculated in MS Medium along with sucrose and agar. Seeds were sterlised under aseptic conditions . After the growth of the plant, treatment of IBA was prefered for the rooting of invitro grown shoots. Rooted plants were shifted to different mixtures of mediums. mixtures were as cocopeat + sand + soil, cocopeat + sand, cocopeat.Plants grown in cocopeat showed better results than other two mediums.The in vitro elongated shoots can also be rooted with the help of synthetic hormones like Rootex-1 and Rootex-3. The hardened plants were victoriously hardened in greenhouse conditions and were shifted to field conditions[6]. Researchers have worked on different techniques of hardening to increase the survival rate of tissue grown plants such as hardening of tissue cultured raised tea plants through the use of rhizosphere bacteria(Anita Pandey et al, 2000) [9].

There are several factors affecting the growth of in vitro grown plants when hardened in hydroponics. Salinity is one of the important factor which contributes to the growth of the plants under different conditions and different

media . This is measured with the help of electrical conductivity meter therefore range at which plant shows optimum growth is between 1.5- 2.5 ds m [21]. Dissolved oxygen is another important factor contributing to the plant growth . various studies depicts the effect of dissolved oxygen in the proliferation of the roots [22]hence it is necessary to supplement the media with oxygen using air pump.

Quantification of 2° metabolites is of prime importance in order to explore content accumulated in in-vitro grown tissue cultured plants. Various quantification has been done [23].Advantage is that when in small quantity , content is analysed which gives lead to further analyses of the plant metabolites. HPLC is one such technique which helps in the analysis of the secondary metabolites present in the plant.

CHAPTER 3

MATERIAL

AND

METHODS

3.1 Plant source

Picrorhiza kurroa ,*Swertia chirayita* , *Gentiana kurroo* and *Withania somnifera* were obtained as source of explants from previously maintained cultures from the culture room of Department of Biotechnology and Bioinformatics JUIT Waknaghat.

3.2 Mass propagation of plants on defined media

- **Media Preparation-**MS media was prepared using the previously prepared stock solutions A-100ml/L, B-50ml, C,D,E,F,G,H-10ml\L poured in a flask and volume was made up using distilled water.
- Growth hormones were used as growth regulators (Kinetin:IBA)(3:1) that is 3ml of Kinetin and 1ml of IBA in 1L of MS media and 30g of sucrose was added to it.
- The pH was checked and maintained in the range of 5.5-5.7 .
- After the pH was checked agar was added in the proportion of 9g\L.
- The media was heated before autoclaving to solubilize the agar.
- After the media was boiled it was transferred to the glass jars for autoclaving.
- The media was autoclaved for 30 minutes at 121°C and 15 lb $inch^{-2}$pressure.
- After autoclaving the media was allowed to solidify.
- Explants of *Picrorhiza kurroa,Swertia chirayita*, *Gentiana kurroo* and *Withania somnifera* were sub cultured on MS media in LAF(laminar air flow).
- The cultures were maintained in culture room for a month.

Fig-3.2.1-Micropropagated *Gentiana*

Fig-3.2.2-Micropropagated *Swertia*

Fig.3.2.3-Micropropagated *Picrorhiza*

Fig.3.2.4-Micropropagated *Withania*

3.3 Fabrication of Hydroponic system

Different systems were setup for propagation and hardening of invitro grown plants.

- **Setup 1(Fig.3.3.2)** was setup using thermocol box and lid. Thermocol cups were used to hold the plants and pipe of oxygen pump was immersed in solution .
- **Setup 2(Fig.3.3.3)** was setup using plastic tray and hydroponics tray.It was partially covered with thermocol sheet to avoid fungal contamination. Compressed Cocopeat blocks were used to hold the plants and oxygen pump was used for oxygenation.
- **Setup 3(Fig.3.3.4)** was setup using waste plastic box. Netted cups were used to hold the plants.Oxygen pump was used.hydroponic system.
- **Setup 4(Fig.3.3.8)** was commercial hydroponic system manufactured by **FUTURE FARMS** and named **as LEAF PYRAMID ONE.**

Table 3.1.1 Table represents hydroponic setups with different medias.

SETUP	MEDIA	TEMPERATURE (˚C)
1	MS + 40g sucrose	20-25
2	MS+sucrose(40g,20g,10g)	18-20
3	No sucrose,5g sucrose,Hydroponics solution	15-18
4	MS + Sucrose (10g)	20-25

3.4 Effect of different sucrose concentration and Commercial Hydroponics Solution in plant hardening.

Method-

1. Different Hydroponic systems were set up along with different media composition.

A. **Setup 1**- (**Fig.3.3.2**).This sytem was fabricated using themocol box and cups. MS media with a sucrose concentration of 40g\L was used as growing media.

B. **Setup 2(Fig.3.3.3)**-This system was fabricated using plastic trays which was partially covered with thermocol to avoid fungal contamination, it contained MS media with variable sucrose concentration of 40g\L sucrose(autoclaved) , 20g\L sucrose (autoclaved) ,10g\L sucrose (autoclaved) .

C. **Setup 3**(**Fig-3.3.4)** This system was setup using plastic boxes , it contained MS media with 5g\L sucrose (not autoclaved)(**Fig-3.3.7)** no sucrose(not autocalved) **Fig(3.3.6**) and commercial hydroponic solution(**Fig -3.3.5)** the media was dissolved in tap water.

D. **Setup 4(Fig -3.3.8)** This system was commercial hydroponic system fabricated by the company named **FUTURE FARMS** under the name of **LEAF PYRAMID ONE.** It contains the water pump which circulates the media from the bucket to the various pipes containing hydroponic cups which are composed of the mixture of cocopeat and the pebbles, which holds the plant. Media used was MS media with 10g\L media(not autoclaved).

2. Setup 2 used compressed coco peat blocks that were soaked in water for 1 minute and were used to hold the plants in the hydroponic tray , *Picrorhiza* was use as the model plant.

3. Setup 3 used Coco peat mixture along with netted cups(4-5 cm height) to hold the plants ,it used *Picrorhiza, Gentiana* and *Swertia* as the model plant.

4. Setup 4 used netted hydroponic cups along with the mixture of coco peat which helps in holding of the in vitro grown plants *Picrorhiza kurroa, Gentiana, Swertia* and *Withania somnifera.*

5. In vitro cultured plants 3-4 cm in height were placed in hydroponic system along with the air pump(24h running)with a flow rate of **400ml\min** for oxygenation purpose.

6. Factors such as PH and EC (Electrical Conductance) was maintained that is PH ranges from 5.8-6.0 and EC was 0.5-1.5 mScm-1.

7. In vitro cultured plants 3-4 cm in height were placed in the hydroponic setups.

8. Systems were kept under different temperature and humidity conditions in green house.

9. After a week of hardening in hydroponic system the plants were shifted to potting mixture(perlite:vermiculite:cocpeat in the ratio of 1:1:1) and were not covered with glass jars or polythene bags.

10. The data was collected for *Picrorhiza, Gentiana, Swertia* and *Withania somnifera* on the basis of Increase in number of shoots , number of days , Increase in shoot length and biomass.

11. Plant extract was prepared and used for further analyses.

Fig 3.3.1-Compressed cocopeat blocks

Fig 3.3.2- Setup 1(40g sucrose)

Fig -3.3.3 –Setup2(40g sucrose)

Temp.(18-20°C)

Fig-3.3.4- Setup 3 made from plastic box

Temp.(15-18°C)

Fig -3.3.5-Setup 3 contains Hydroponic Solution(5ml\L)

Fig-3.3.6- Setup 3 contains MS media+No Sucrose

Fig-3.3.7-Setup 3 MS media+ 5g/L sucrose

Fig 3.3.8 Set up 4 (Leaf Pyramid One) MS media 10g\L sucrose.

3.5 Quantification of a marker compound by HPLC:

After 4 weeks of hardening of *Picrorhiza kurroa* fresh shoots of the was taken for the estimation of the secondary metabolites or the marker medicinal compound using chromatography technique known as HPLC (High Performance Liquid Chromatography).

Fresh plantlets of the plant was carefully uprooted from the hydroponic cups and were transformed into fine powdered form by crushing the plant in the presence of liquid nitrogen. 100 mg of this powder was mixed with 10ml of 80% methanol and kept overnight. The samples were vortexed and mixed thoroughly,this was followed by sonication for 10 minutes with 2 seconds pulse at 30% amplitude. After sonication samples were centrifuged at 10,000 rpm for 10 minutes. Supernatant was kept and pellet was further discarded. Filteration of supernatant was done using 0.22 µm milipore filter. This filtered extract was used for the estimation of P-I and P-II. Retention time for P-I is 14.154 (Figure) and for P-II is 8.626 (Figure). This quantification was done on Waters HPLC System equipped with Waters 515 HPLC pumps, Waters 717 autosampler,Waters 2996 photodiode array detector and Empower software. Waters Spherisorb reverse phase C18 column (4.6 mm ×250 mm,5µm) was used as stationary phase for the quantification of the secondary metabolites. The mobile phase used for the analysis of the metabolites of *Picrorhiza kurroa* was solvent A (0.05 % trifluoro-acetic acid in water) and solvent B (1:1 methanol : acetonitrile mixture). Isocretic method was used for solvent A: solvent B was 70:30(v\v) throughout the processhaving flow rate of 1ml\min at detection wavelength of 270 nm.the cycle the for the quantification was 30 min at 30ºC . The presence of the secondary metabolites was analysed on the basis of the retention time and comparison of UV spectra with specific standards of the plant procured from Chroma Dex. Inc. and calculated in µg/mg.

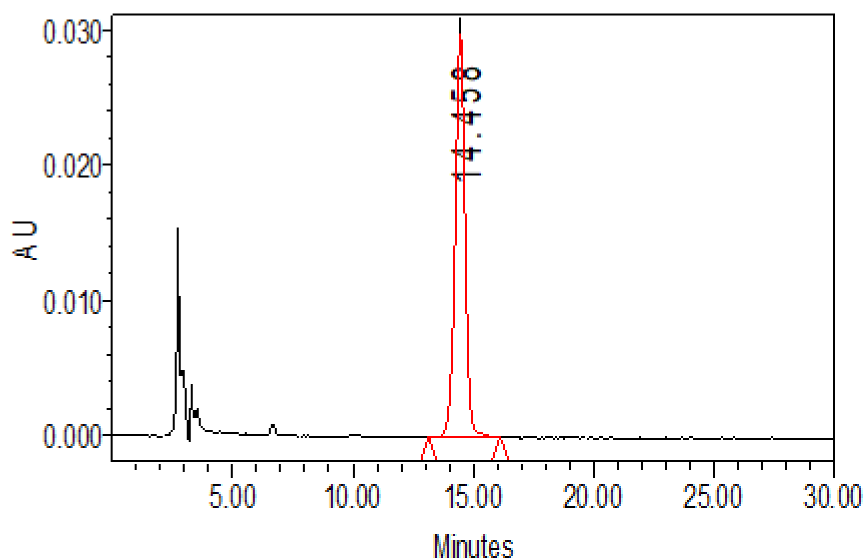

Fig3.5.1- HPLC Chromatogram of P-I standard of P. kurroa.

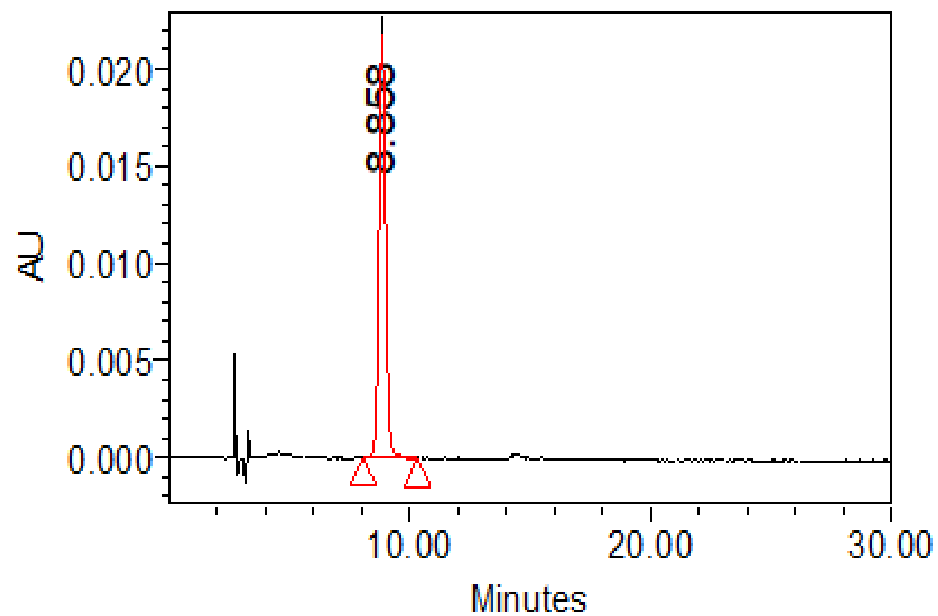

Fig 3.5.2 HPLC chromatogram of P- II standard of *P. kurroa*.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Invitro shoot multiplication

Invitro shoots of *Picrorhiza kurroa ,Swertia chirayita , Gentiana kurroo* and *Withania somnifera* were cultured and cultured in MS media with growth hormones IBA:Kinetin(3:1) at 15℃±1 and 25℃±1.

The shoots multiplied on the define media further gave 5-10 shoots per explants after sub culturing which was utilized for hardening experimentation with hydroponic setups.

4.2 Hardening in hydroponic system:

In **Setup 1(Fig 3.3.2)** thermocol box was used along with thermocol cup to hold the plants,this sys tem was futile as it could not retain media for longer period of time. Leakage of the media was observed.

In **Setup 2(Fig -3.3.3)** Invitro grown *Picrorhiza* was kept in hydroponic system having different concentration of sucrose (40 g/L, 20 g/L, 10 g/L). Fungal growth was observed within 3 days in the system containing MS media with 40g sucrose. Similarly set up 2 containing solution with 20g/L sucrose was also contaminated with fungus within a time period of 3 days. However with decrease in sucrose concentration a gradual increase in the days of fungal growth was observed. Therefore decreased concentration of sucrose to 10g/L in MS media ,fungus contamination was observed after 4 days. System was continuously supplied with oxygen pump having air flow rate of 400 ml/min. Five plants of *Picrorhiza* were kept in each media out of which 3 plants survived in media containing 40g/L and 10 g/L sucrose whereas 4 plants survived in 20g/L of sucrose after 1 week of hardening in the system itself.

In **Setup 3(Fig-3.3.4)** Invitro grown *Picrorhiza,Swertia* and *Gentiana* were kept in Hydroponics system containing MS media with 5g\L sucrose , no sucrose and hydroponic solution in which fungus was observed within 6 days and no fungus was observed respectively. All plants survived during hardening of one week in the system.

In **Setup 4 (Fig-3.3.5)** Invitro grown *Picrorhiza , Swertia ,Gentiana* and *Withania* were kept in commercial hydroponics containing MS media with 10g\L sucrose . *Withania Somnifera* showed the best results and formation of flower and fruit was observed after 3 weeks of hardening.

(Table 4.2.1- The table represents the results of "Survival of in vitro cultured plants in different hydroponic setups, for 1 week").

MS Media	Fungal growth	No. of cultured plants taken for hardening	No. of plants that survived (1 week)	Initial Plant Biomass (g)	Initial number of shoots
40g/L sucrose	Yes(within 3 days)	5	3	1.62g-1.85g	Pico(7-10)
20g/L sucrose	Yes(within 3 days)	5	4	1.58g-1.90g	*Pico*(8-10)
10g/L sucrose	Yes (within 4 days)	5	3	1.72g-1.85g	*Pico*(8-10) *Withania* (6) *Swertia* (5) *Gentiana* (5-6)
5g/L sucrose	Yes (within 6 days)	6	6	1.69-2g	*Pico*(10-12),*Swertia*(7-10),*Gentiana*(10-12),*Withania* (5-6)
No sucrose	No fungus	6	6	1.80g-2g	*Pico*(7-10),*Swertia*(8-10),*Gentiana*(10-12),*Withania*(4-5)
Hydroponic Solution	No fungus	6	6	1.75g-2g	*Pico*(7-10),*Swertia*(8-12),*Gentiana*(7-11),*Withania* (5-6)

Fig 4.2.1-Hardening of *Picrorhiza*(40g\L sucrose +MS)

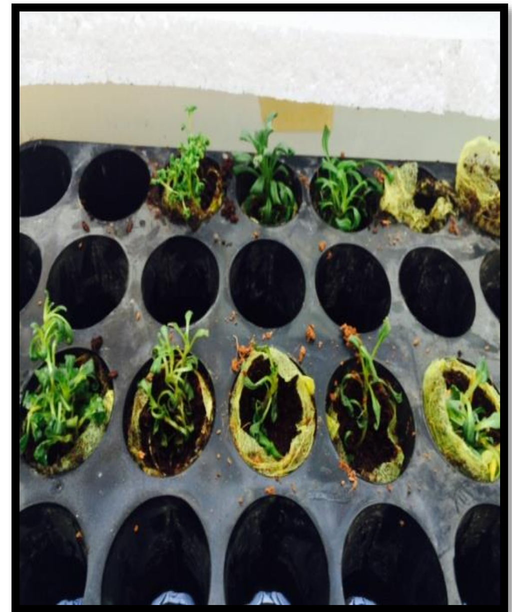

Fig. 4.2.2- *Picrorhiza* in 20g sucrose +MS

Fig. 4.2.3-Hardening of *Picrorhiza* in 10g\L sucrose + MS media

Fig.4.2.4-*Picrorhiza ,Swertia, Gentiana* (2 each) in 5g\L sucrose+MS .

Fig.4.2.5- In vitro plants in no sucrose +MS.

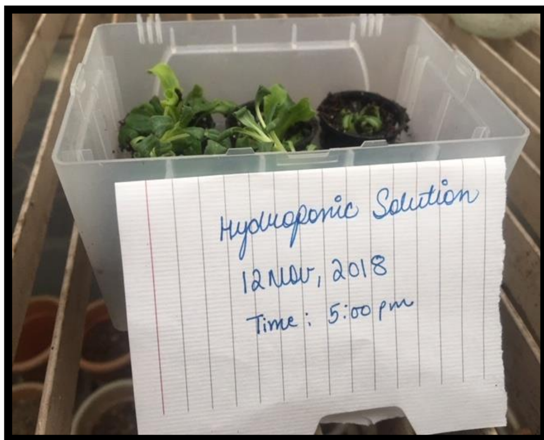

Fig.4.2.6- *Picrorhiza, Swertia, Gentiana*(2each) In hydroponic solution.

Fig 4.2.7- *Withania* in hydroponic System.

4.3 Transplantation to potting mixtures:

After 1 weeks of hardening of in vitro grown medicinal plants they were transferred to the potting mixture containing cocopeat , perlite and vermiculite in the ratio 1:1:1. They were not covered with any polythene bags or glass jars. The pots were watered thrice a week and were kept in greenhouse with a temperature range of 15-18°C.The data was collected for *Picrorhiza, Gentiana, Swertia* on the basis of increase in number of shoots , number of days , Increase in shoot length and biomass.In the first week of transfer there was no change in plant physiology with respect to shoot length, biomass and number of shoots. In the second week of transplantation there was minor increase in shoot length and biomass as well as number of shoots. However, in the fourth and sixth week there was a significant increase in shoot length and number of shoots.

Table 4.3.1-*The* table represents the effect of nutrient solutions after transplantation to potting mixture in medical plants.

MS Media	Transplantation To potting mixture after following days	No. of plants that survived in 1 week of transfer	NO. of plants survived(2 weeks)	NO. of plants survived (4 weeks)	NO. of plants survived (6 weeks)
40 g/L sucrose	3	3	3	1(*Pico*)	1(*Pico*)
20 g/L sucrose	3	4	4	2(*Pico*)	2(*Pico*)
10g/L sucrose	4	6(*Withania*) 5 (*Swertia*) 6 (*Pico*) 5(*Gentiana*)	6(*Withania*) 4(*Swertia*) 6(*Pico*) 4(*Gentiana*)	6(*Withania*) 4(*Swertia*) 4(*Pico*) 3(*Gentiana*)	6(*withania*) 3(*Swertia*) 2(*Pico*) 2(*Gentiana*)
5g/L sucrose	6	Died	Died	Died	Died
No sucrose	7	Died	Died	Died	Died
Hydroponic solution	7	6(*Withania*) 7(*Swertia*) 6(*Pico*) 8(*Gentiana*)	5(*Withania*) 6(*Swertia*) 4(*Pico*) 6(*Gentiana*)	3 (*Pico*) 4(*Swertia*) 5(*Withania*) 4(*Gentiana*)	2 (*Pico*) 3(*Swertia*) 4(*Withania*) 2(*Gentiana*)

Table 4.3.2-The table represents the effect of nutrient solution in growth and development of hardened *Picrorhiza,Swertia* , *Gentiana* , and *withania* after 2,4 and 6 weeks of transplantation.

MEDIAS (MS)	Increase in shoot length (cm)(2weeks)	Increase in number of shoots (2 weeks)	Increase in Biomass (g)(2weeks)	Increase in shoot length (cm)(4weeks)	Increase in number of shoots(4weeks)	Increase in Biomass (g)(4weeks)	Increase in shoot length (cm)(6weeks)	Increase in number of shoots(6weeks)	Increase in Biomass (g)(6weeks)
40g\L sucrose	**0.5-.75cm**	***Pico(2-3)***	**0.5-1g**	**1-2cm**	***Pico(5-6)***	**0.75g**	**2-3cm**	**5-6**	**1g-1.5g**
20g\l sucrose	**0.5-.75cm**	***Pico(1-2)***	**0.5-.75g**	**1-2cm**	***Pico(3-4)***	**0.5-0.65g**	**1-2cm**	**4-5**	**0.5g-1g**
10g\L sucrose	**0.25-0.5cm**	***Pico(2-3)***	**0.5-1g**	**0.5-1cm**	***Pico(2-3)***	**0.70-0.80g**	**0.25-0.5cm**	**4-5 *Withania (8-9)***	**0.75-*1.5g Withania (4-5g)***
No sucrose	**No increase observed**	**No increase observed**	**No increase observed**	**Died**	**Died**	**Died**	**Died**	**Died**	**Died**
Commercial Hydroponic solution	0.5-0.75cm	***Pico(2-3) Swertia(1-2) Gentian(1-2)***	**0.5-1g**	**1-2cm**	***Swertia(1-2) Pico (2-3)***	**0.75-0.80g**	**0.35-0.65 cm**	**3-4 *Withania (6-7)***	**1-1.5g *Withania(3-4g)***

Fig.4.3.1 *Picrorhiza*(40g,2weeks)

Fig.4.3.2- *Picrorhiza* (20g,2weeks)

Fig.4.3.3-*Picrorhiza*(10g,2weeks)

Fig.4.3.4- Fungus in 5g sucrose within 6 days

Fig-4.3.5 Plants transferred from no sucrose solution (1 week)

Fig4.3.6 Plants transferred from Hydroponics solution(1 week)

Fig-4.3.7 Plants status after 2 weeks of potting (Hydroponics solution and no sucrose)

Fig.-4.3.8 Death of *Picrorhiza* and *Swertia* from no sucrose solution (2weeks)

Fig-4.3.9 Better survival of *Picrorhiza* and *Swertia* taken from hydroponics solution

Fig.4.3.10-*Picrorhiza* (40g\L,6weeks)

Fig.4.3.11-*Picrorhiza*(20g\L,6weeks)

Fig.4.3.12-*Picrorhiza*(10g\L,6weeks)

Fig 4.3.13. ***Withania*** **(10g\L,6 weeks)**

Fig 4.3.14 ***Withania*** **roots (10g\L,6 weeks)**

4.4 Quantification of the marker compound by HPLC

4.4.1. Effect of hardening in hydroponic system on P-I and P-II production in P. kurroa

HPLC analysis of plantlets hardened in hydroponic system containing MS media was done to analyse the production of P-I and P-II.

Different peaks were observed.

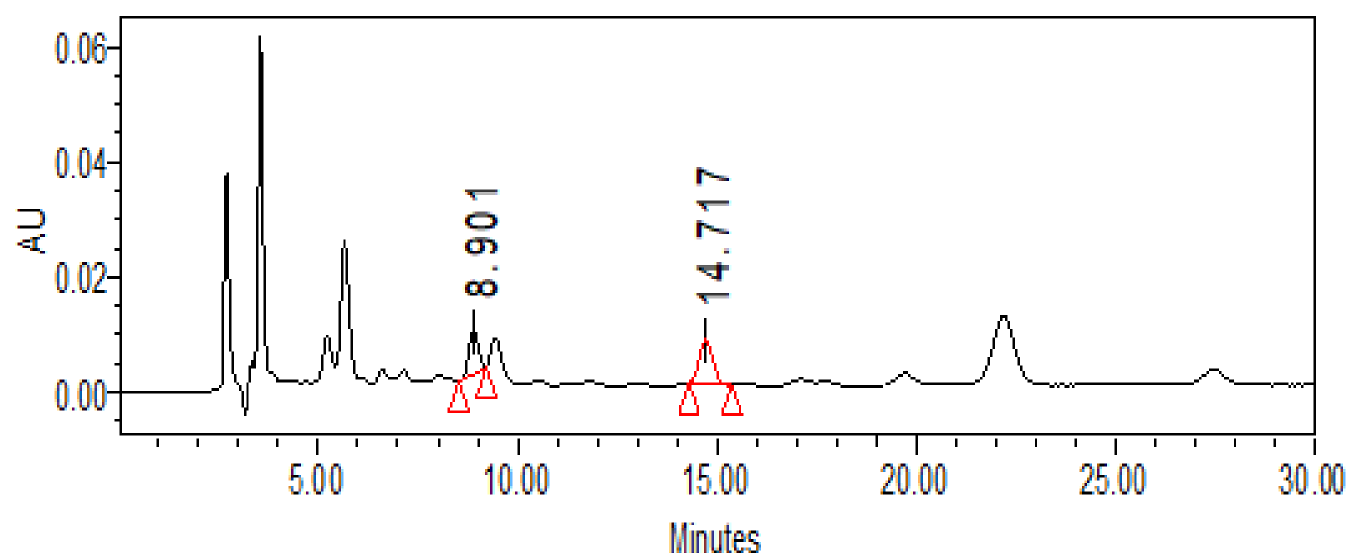

Fig 4.4.1. HPLC chromatogram showing peaks of P-II and P-I of *P. kurroa*

Name	Retention Time	Peak Area	% Area	Concentration
Picroside I	14.717	178117	58.52	2.01µg\mg

Table 4.4.2. HPLC analysis for P-I *P.kurroa*

4.5 Discussion :

Hardening is one of the essential requirement for better survival rate and enrichment of the plants. The process is tedious as plants require ample time for its modification in morphology, anatomy and physiology. If plants are directly transferred to fields from in vitro conditions to ex vitro conditions it may have consequences like photoinhibition . Therefore to prevent such ramifications hardening comes into play. Various other factors during acclimatization affects plant growth , like concentration of sucrose along with the growth hormones. According to Rajib Bandopadhay et al (2010) there are many factors which affects plant stabalization when shifted from in vitro conditions to ex vitro conditions . Plants in invitro condition are generally kept under low light intesity frequently between 1300-3000 lux . Temperature range varies from 20-25 degree celcius whereas if transferred to direct light whose intensity ranges from 4000-12000 lux, leaves of plantlets may undergo wilting and probably dies off. Humidity directly affects the transpiration rate due to modification of cuticle, epicuticular waxes after the transfer of plants . Carbohydrate concentration also plays the vital role [8]. Sucrose molecules serves as the origin of fixed carbon which is distributed all around the plant consistently.The sucrose molecule is hydrolysed into glucose and fructose and therefore act as an energy source. Therefore in our work we have used varied concentration of sucrose for the acclimatization of plants along with MS Media , Growth hormones and Hydroponic solution .Plants were shifted from in vitro conditions to the natural environment in different systems containing MS media, Growth hormones, different amount of sucrose like 40g, 20g, 10g,5g and no sucrose. Hence we observed that Media containing more amount of sucrose was contantaminated with Fungus and few plantlets died, after transferring the plants to potting mixture . Plants transferred to media containing no sucrose died as component(sucrose) that act as carbon source , required for the growth of plants was missing. Better survivability of *Withania somnifera* was observed in MS media having 10g\L sucrose.Increase in number of roots and shoots were observed. Flowering was also observed in *Withania somnifera.* Orange colour fruits were also observed after 4 weeks of hardening in Hydroponics.

CHAPTER 5

Conclusion

Hardening techniques have expanded and developed over the past few years and hydroponics has been an advanced ground in it. In this project we have aimed to propagate and harden the invitro grown medicinal plants through hydroponic system . From the experiments performed in this project we were able to draw an abstractive conclusion that with the use of different concentration of sucrose in the media of hydroponic system, it hardened the plants but as sucrose concentration was decreased in the media there was a significant decrease in survival of plants. Whereas with the increase in sucrose concentration, fungal contamination were observed, and led to the plant death.

It was observed that 66.6% plants survived in commercial hydroponics system, 40% plants survived in 20g\L and 10g\L sucrose solution and 20% survival rate of plants was seen in 40g\L sucrose solution. The survival of plants in 40g\L sucrose solution can be increased if the fungal growth is controlled. The plants have a better growth rate in 40g/L sucrose solution. No plants survived in “no sucrose” media as there was no available carbon source for plant growth.

Withania somnifera showed the best results and flowering was observed after 4 weeks of hardening in MS media having 10g\L sucrose .

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