OPTIMIZATION OF TISSUE CULTURE CONDITIONS FOR THE MICROPROPAGATION OF *ACONITUM HETEROPHYLLUM*

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CERTIFICATE

This is to certify that the thesis entitled **"Optimization of tissue culture conditions for the micropropagation of Aconitum heterophyllum"** submitted by **Ms. Neha Katyal and**

Ms. Saumya Sachdev to the Jaypee University of Information Technology, Waknaghat in partial fulfillment of the requirement for the award of the degree of **Bachelor of Technology in Biotechnology** is a record of bona fide research work carried out by them under my guidance and supervision and no part of this work has been submitted to any other university or institute for this or any other degree or diploma.

[Dr.(Mrs.) Hemant Sood]

DECLARATION

We hereby declare that the work presented in this thesis has been carried out by us under the supervision of Dr. (Mrs.) Hemant Sood, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for this or any degree or diploma to any other university or institute. All assistance and help received during the course of the investigation has been duly acknowledged.

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Needless to say, errors and omissions are ours.

(Neha Katyal)

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Summary

Aconitum heterophyllum Wall. (family Ranunculaceae) is an important medicinal plant of sub-alpine and alpine regions of Himalaya, distributed between 2800 and 4500 m.The root tubers of *A. heterophyllum* are used for various therapeutic actions such as anti-arthritic and anodyne. The marker ingredients for industrial use are atisine and aconitine. Its immense medicinal importance and high price in the market have led to an indiscriminate harvesting of tubers of this species from the wild and the species is identified as critically endangered in status. However, the cultivation of this species is difficult due to poor seed availability and the lack of superior germplasm .**Current market rates approximately** can fetch amounts up to Rs.1000-2800/- per kg in the market. But due to the lack of an organized supply channel, the rates often fluctuate drastically between Rs.1000-3500/- depending on the quality of the produce and the market demand.According to our studies callusing was achieved on MS media supplemented with 2-4-D(3mg) and NAA(2mg). Seed based multiplication is the most effective, realistic and convenient means for most of the species. But cultivation through seed in most of the species of *Aconitum* e.g., *Aconitum heterophyllum* is difficult due to poor seed availability and lack of superior germplasm. However ,our studies indicate that chilling treatment gave a good percentage (72%) rate of germination. We also tried shoot base for direct shoot multiplication. As the recent advances propell the use of hairy root induction for enhancing the metabolite production so the experiments covered this technique were also entertained in our research work.

Signature of Students
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LIST OF ABBREVIATIONS

Abbreviation	Meaning
°C	Degree Celsius
µM	Micro molar
BAP	Benzyl Amino Purine
cv.	Cultivars
gm	Gram
HCl	Hydrochloric Acid
IBA	Indole -3Butyric Acid
Kg	Kilogram
KN	Kinetin
l / lt.	Litre
mg	Milligram
MS media	Murashige and Skoog Media
NaOH	Sodium Hydroxide
ppm	Parts per Million
GA_3	Gibberilic Acid
2-4 D	2, 4 dichlorphenoxyacetic acid

CHAPTER 1

Introduction

Description of the plant :

Aconitum heterophyllum Wall. is a critically endangered wild medicinal herb of alpine Himalaya and cultivation is recommended owing to its large demand in the herbal market and to ensure the conservation of wild habitats. It is a perennial plant with an average height of 150cm.

Morphology :

The plant grows to a height of up to 1 m. The long stem is erect, cylindrical, yellowish-green and unbranched. The alternate, simple leaves have an ovate blade with a serrate margin, ending in an acute apex and an auricular base clasping the stem. Flowers are bright blue to greenish blue with purple veins in long many flowered peduncles. The five petaled flowers have two small lower petals, two middle round petals and a tubular hood-like upper petal which covers the lower lying petals like a cap. The hood is rounded and broad. The fruit consists of a bunch of five follicles, which are downy when young, but hairless when matured. Each of the follicles splits along the inside, releasing numerous blackish-brown, 3-4 mm long seeds with winged angles.

Habitat of the herb :

Usually found on humus-rich soils in the alpine and subalpine zones, and in forests, 2300 - 2900 metres.

Life cycle of the plant :

The plant roots remain dormant in the winter from October-November. New shoots sprout with the melting of snow in April-May. The vegetative phase continues till July and the plants start flowering in August. Seed setting takes place in September and the seeds reach physiological maturity by end of September-October.

Medicinal use of *Aconitum heterophyllum* :

It is used in India in the treatment of dyspepsia , diarrhea and coughs. It is also used in tibetian medicines, where it is said to have a bitter taste and cooling potency. It is used to treat the poisoning from scorpions or snake bites , the fevers of contagious diseases and inflammation of the intestines. Even though *Aconitum heterophyllum* belongs to aconitum family , it is non toxic if properly used. In Ayurvedic medicine it is used for children experiencing fever and diarrhea. The recommended doses of *Aconitum heterophyllum* depend on the condition being treated . Different formulations may be toxic ,therefore, strict supervision by a qualified herbalist or physician is advised before using this herb. Being rich in substances having potential biological significance, such as benzoylmesaconine, mesaconitine, aconitine, hypaconitine, heteratisine, heterophyllisine, heterophylline, heterophyllidine, atidine, isotisine, hetidine, hetisinone and benzoylheteratisine and other nutrients (Zhaohong et al., 2006) the plant has been reported to possess antifungal

(Anwar et al., 2003), cytotoxic (Anwar et al., 2003), antiviral (Pandey et al.,2004) and immune-stimulant properties (Atal et al.,1986).

Aconitum heterophyllum is reputed for its various medicinal and pharmaceutical properties. Due to its high demand in the local, national and international drug manufacturers, illegal, unscientific and indiscriminate extraction of *Aconitum heterophyllum* Wall from its wild habitat has increased. Micropropagation provides a best tool for large scale production of propagules and its conservation especially in case of endangered medicinal herb, where explant material is available in a very small quantity. Viability of seeds, age of explant and the tissue source from which the explant is excised are very important for high frequency of regeneration.

Known hazards :

The whole plant is highly toxic - simple skin contact has caused numbness in some people. One report says that this plant does not contain the toxic alkaloid aconitine, and so is not poisonous.

Propagation of the herb :

Although, many research works had been carried to conserve the *Aconitum sp*. through cultivation, but then also no complete effective method has been primed to bring it in conservation programmes. Tissue culture opens up new area for conserving threatened *Aconitum sp*. as small amount of plant material can generate large no. of disease free propagules which can be re-introduced in their native habitat. In addition, it also overcomes the problems of *ex situ* conservation where seed availability is nearly mandatory. Furthermore, *in vitro* propagation also contributes in broadening of species genetic database; augmenting the yield and production of active constituents and secondary metabolites. *In vitro* propagation of plants holds tremendous potential for the production of high quality plant based medicine (Murch *et al*., 2000). Micro-propagation has many advantages over conventional methods of vegetative propagation which suffer from several limitations (Nehra and Kartha, 1994). Numerous factors are reported to influence the success of *in vitro*propagation of different medicinal plants (Hu and Wang, 1983; Hussey, 1990; Bhagyalakshmi and Singh, 1988; Short and Roberts, 1991). Also the production of secondary metabolite *in vitro* is possible through plant cell culture (Tripathi and Tripathi, 2003). Zenk (1878) has reported cell lines capable of producing high yields of secondary compounds in cell suspension cultures. Approach for improving secondary products in suspension cultures, using different media for different species; have been reported by Robins (1994).

Previously Giri *et al.* (1993) had standardized the procedure for *in vitro* propagation of *A. heterophyllum* Wall. In this study, they observed that callus induction occurred on MS medium supplemented either with 1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kinetin with 10% coconut water or with 5 mg L^{-1} NAA and 1 mg L^{-1} BAP by maintaining them on MS medium with 1 mg L^{-1} NAA. Whereas, in Jabeen *et al.* (2006), for a second time carried out the *in vitro* propagation of *A. heterophyllum* Wall and reported that callus induction occurred at extremely low concentration of growth regulators NAA (0.5 mg L^{-1}) and BAP (0.25 mg L^{-1}) supplemented in MS medium. Conditions for maintenance of the callus were same. In this report, *in vitro* shoot proliferation experiments revealed that MS medium containing 0.25 mg L^{-1} NAA with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} IAA was the best blend for shoot multiplication and *in vitro* rooting respectively, while Giri *et al.* (1993) optimized 1.0 mg L^{-1} IBA for *in vitro* rooting. Somatic embryogenesis was attained when callus (induced on 2, 4-D and NAA) was transferred to MS medium with 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA after 2 subculture passage (Giri *et al.*, 1993). Complete plantlet formation from these embryos was obtained after 4 weeks on medium with ¼ MS nutrient containing 1 mg L^{-1} IBA (either supplementing it in medium or by dipping embryos in it for 5 min).

Important aspects:

Cultivation of this endangered species : unsustainable exploitation of the medicinal plants for the drug industry and local medicinal use.

Medicinal Importance : antipyretic, anti-inflammatoty, analgesic, astringent and anti-diarrhoeal.

Commercial outlook : The anti-inflammatory activity of ethanolic root extract of *Aconitum heterophyllum* (225, 450 and 900mg/kg p.o) has been evaluated in cotton pellet-induced granuloma in rats. The extract has reduced inflammation as evidenced by decreased weight of cotton pellet in cotton pellet-induced granuloma in rats. The results demonstrate the anti-inflammatory properties of extract and the effects were comparable to diclofenac sodium, a standard non-steroidal anti-inflammatory drug.(verma et al.)

Objectives :

The objectives of the present investigation are

1) To carry out micropropagation of *Aconitum heterophyllum* .

2) To carry out hairy root induction in different explants .

CHAPTER 2

Review of Literature

Literature Review

Review is discussed in the light of available literature relevant to the research problem on *Aconitum heterophyllum.*

1. Somatic embryogenesis and plant regeneration from callus cultures of *Aconitum heterophyllum* Wall

1.1 General micropropagation

Giri et al. (2001) carried out in-vitro propagation comprising of various stages: selection of explants; aseptic culture establishment; multiplication of propagules; rooting and acclimatization of plantlets. Conservation through vegetative propagation is slow and time consuming but tissue culture offers an alternative tool for rapid multiplication and conservation of disease free propagules in a short period, which will further enable uninterrupted supply of raw material, *Aconitum heterophyllum* for drug preparation.

1.2 Culture conditions

1.2.1 Medium

Murashige and Skoog basal medium supplemented with appropriate cytokinins and auxins were used for inoculation. Medium was checked for the contamination before inoculation. Sterilized explants were trimmed suitably to remove sterilizing agent affected parts/brown parts. Explants and seeds were then inoculated on the appropriate medium and labeled properly. Regular and proper record for contamination, browning and growth/bud break/germination (seeds) were taken for 30 days.

1.2.2 Modified media

For plantlet formation, somatic embryos were placed on full, half and one fourth strengths of the MS nutrients by **A.Giri et al.** with varying concentrations of 1BA (0.5, 1 and 2 mg 1-1). The somatic embryos were also dipped in a concentrated solution of IBA (1 mg ml-1) for varying times and placed on a hormone free MS medium for complete plantlet formation.

1.2.3 Light

A.Giri et al. maintained plants of *Aconitum heterophyllum* Wall. in earthen pots under controlled environmental conditions (22 +2°C; 14:10 h light: dark regime and 70% relative humidity, rh).

1.3 Sterilization of explants

As *Aconitum heterophyllum* is an endangered medicinal herb optimum conditions like type of sterilizing agent, its concentration and time of exposure to that sterilizing agent are mandatory for asepsis of *Aconitum heterophyllum.* These sterilants are toxic to the plant tissue, hence the type, concentration, time of exposure and removal of traces of sterilizing agent becomes important in standardizing sterilization protocol (Falkiner, 1990).

Nodal segments of *Aconitum heterophyllum* were excised from the pot grown plants. These nodal segments were trimmed to approx 2 cm. in size and large fleshy leaves were removed. All the brown skins were cleaned thoroughly. Procedure of sterilization for *Aconitum heterophyllum* had been divided into two phases: Phase I (outside Laminar Air Flow) and Phase II (inside Laminar Air Flow). Three different kinds of sterilizing agents viz., Mercuric Chloride (HgCl2), Sodium Hypochlorite (NaOCl) and Hydrogen Peroxide (H2O2) are tested for explant sterilization by varying their concentration and time of exposure. Among all
the three sterilizing agents viz., HgCl2, NaOCl and H2O2, treatment with 0.1% (w/v) HgCl2 for 5 min. gave the 100% healthy shoots ($p<1.0\%$).

Seeds of *Aconitum heterophyllum* were subjected to float test for determining the viability. The Seeds are small in size; this makes its washing and sterilization little bit uneasy. As these seeds run out from the flask while washing, using glass pipette (20-25ml) or hollow glass rod with one end sealed can avoid this situation. Various sterilizing agent with different concentration and time of exposure were tested, out of which 7.5% (v/v) H2O2 for 5 min. gave the maximum (90%, $p<1\%$) germinated and healthy seedlings while less germination and more percentage of contamination was observed with NaOCl, while HgCl2 showed adverse effect on germination.

1.4Establishment of shoot cultures :

Initially, experiments (A.Giri et al.) for the induction of callus used sections of leaves and petioles obtained directly from plants that were maintained in growth chambers. However, early senescence of the explants due to the harshness of the sterilizing procedure inhibited callusing. Subsequently complete shoot tips were excised and aseptically grown *in vitro* on a hormone-free MS medium and on the same medium with 0.25, 0.5, 1 and 2 mg 1-1 BAP. The explants failed to respond on a hormone-free medium but shoot cultures survived and grew best on a 0.25mg 1-1 BAP containing medium to give rise to fresh explants. Thus, shoot tips were routinely subcultured on MS medium with 0.25mg 1-1 BAP to obtain material for experiments on callus induction and regeneration.

1.5 Hardening

A.Giri et al. transferes *In vitro* raised plantlets to earthen pots containing a mixture of soil, vermiculite and farm yard manure (1 : 1 : 1) and were maintained under controlled temperature (24-2°C) and humidity (80-90%) in a glass house for hardening.

2. PRODUCTION OF HAIRY ROOTS IN *ACONITUM HETEROPHYLLUM* WALL. USING *AGROBACTERIUM RHIZOGENES*

A.Giri et al. proposed a method for the production of hairy roots of *Aconitum heterophyllum* wall. for the first time. Embryogenic callus cultures were successfully transformed using *Agrobacterium rhizogenes* strains viz. LBA 9402, LBA 9360, and A4 for the induction of hairy roots. The transgenic nature of hairy roots was confirmed by mannopine assay using paper electrophoresis.

2.1CULTURE MEDIUM :

Best growth of transformed roots was obtained on 1/4 MS (Murashige and Skoog, 1962) medium with 3% sucrose. Total alkaloid (aconites) content of transformed roots was 2.96%, which was 3.75 times higher compared to 0.79% in the nontransformed (control) roots. Thin layer chromatography (TLC) analysis of the components of aconites in the transformed roots revealed the presence of heteratisine, atisine, and hetidine.

Establishment of aseptic and callus cultures of *Aconitum heterophyllum* wall. was performed as described earlier (Girl et al., 1993).

2.2EXPLANTS :

From among the different explants that were tested for the induction of hairy roots, the embryogenic callus cultures were most responsive for transformation. Other explants such as leaf, petiole sections, decapitated shoot tips, and nonembryogenie callus did not show any response in terms of hairy root induction and also bacterial growth.

2.3ESTABLISHMENT OF SHOOT CULTURE :

Axenic shoot cultures were maintained on MS (Murashige and Skoog, 1962) medium supplemented with 1.1 microM BA (6-benzyladenine). Young leaves and petioles from such shoots were used as explants for callus induction. Embrvogenic callus was induced on MS medium containing 4.4 microM BA and .5 microI NAA.

2.4LIGHT :

All the cultures were maintained at 25-4- 2 ° C at 60 p.E/m2/s of white fluorescent light.

Although a large number of plant species have been tested for **A.rhizogenes** infection and hairy root production, there are no reports of successful transformation and induction of hairy roots in vitro in Aconitum sp.

3.New diterpenoid alkaloids from *Aconitum heterophyllum Wall*: Selective butyryl cholinestrase inhibitors .

Two new diterpenoid alkaloids, heterophyllinine-A and heterophyllinine-B, along with two known alkaloids dihydroatisine and lycoctonine were isolated from the roots of *Aconitum heterophyllum* Wall.

4.Chemical stimulation of seed germination in Aconitum heterophyllum Wall, and A. balfourii Stapf. : Important Himalayan species of medicinal value .

The effect of plant growth substances (PGSs, namely, abscisic acid, 6-benzylaminopurine, gibberellic acid and zeatin riboside) and two nitrogenous compounds (thiourea and potassium nitrate) for enhancing and synchronising uniform germination was examined. GA had an inhibitory effect on *A.heterophyllum* . on *A.heterophyllum* lower concentrations of BAP was inhibitory as well as higher concentrations of ZR.

5. Callus induction and organogenesis from explants of *Aconitum heterophyllum*

5.1Medium :

Callus initiation occurred from nodal segments on MS media fortified with NAA (0.5 mg/L) and BAP(0.25mg/L).

5.2Establishment of shoot :

Response for shoot proliferation was obtained on MS media supplemented with NAA(0.25mg/L) and BAP(0.5mg/L). the well developed microshoots were transferred to root induction media containing MS basal media and IAA(1.0mg/L).

5.3Hardening :

Healthy plantlets were deflasked and transferred into pots containing sand , soil and vermiculite mixture(1:1:1).

6.Pre-germination & Cryopreservation

The endangered *A. heterophyllum* was successfully conserved and domesticated under *ex situ* conditions (greenhouse and open). Water treatment at 45°C and 50° C for 90 seconds not only yielded high seed germination but also improved the growth of the plants over the control. Germination of seeds was initiated during March-April under natural conditions. However, under *ex situ,* the germination and early seedling growth was in October-November and hence the duration and events of life cycle got modified and the seeds matured during late May-June.

The average biomass was also evaluated in plants germinated and established under greenhouse. 7.77 g and 9.56 g/ plant yield (fresh) were in 1 year and 2 years old respectively. Alkaloid content of the dried roots/rhizomes was 1.67 and 2.4 % in 1 and 2 years old plants respectively and was almost at par (2.9%) present in the rhizomes from Chamba.

In *A. heterophyllum,* the initial moisture percentage was low (6%) and were therefore directly stored under liquid nitrogen. The seeds were retrieved after regular intervals for evaluation of viability, germination and cryo injury. Seeds showed about 90-100 % germination after 30 days storage.

CHAPTER 3

Materials and Methods

Selection of plant material and establishment of axenic cultures

The *Aconitum heterophyllum* plants were procured from the HFRI ,Shimla H.P. India and planted in a pot in a polyhouse at the experimental area of the Deptt. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat. The seeds of *Aconitum heterophyllum* were taken from Y.S Parmar university of horticulture and forestery , Solan , H.P , India. Axillary shoot tips of pot grown plants were surface sterilized in 0.5% Bavistin and 0.1% Mercuric Chloride followed by 4-5 washings in sterile water. The sterile shoot apices were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins.

Preparation of media and incubation of cultures

Modifications of MS media supplemented with different concentrations and combinations of IBA, KN, GA_3, NAA and BAP were prepared (Murashige and Skoog 1962). The pH of the media was adjusted to be between 5.5 - 5.7 using 0.1N HCl and 0.1N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. The media were autoclaved at 121°C and 15 lb/in^2 pressure for 15-20 minutes. The autoclaved media were kept in the Laminar Air Flow hood for 1-2 days before inoculations to screen for inherent contamination. One set of cultures was incubated at 16hr light /8hr dark cycle at 25 ± 2^0 C in plant tissue culture chamber. The other set of cultures was incubated at low temperature 15 ± 2^0 C with the same light and dark cycle. Data were collected on days to multiple shoot formation, per cent shoot apices with multiple shoots, and number of shoots per explant. The data were analyzed for test of significance. The cultures were subcultured after every 4 weeks on shoot proliferation media for 2 months so as to obtain good growth, followed by subculturing on the rooting media prior to transfer to the potting mixtures for the acclimatization.

Activated charcoal Activated charcoal is used in water filters, medicines that selectively remove toxins, and chemical purification processes. Activated charcoal is carbon that has been treated with oxygen. The treatment results in a highly porous charcoal. These tiny holes give the charcoal a surface area of 300-2,000 m^2/g, allowing liquids or gases to pass through the charcoal and interact with the exposed carbon. The carbon adsorbs a wide range of impurities and contaminants, including chlorine, odors, and pigments. Other substances, like sodium, fluoride, and nitrates, are not as attracted to the carbon and are not filtered out. Because adsorption works by chemically binding the impurities to the carbon, the active sites in the charcoal eventually become filled. Activated charcoal filters become less effective with use and have to be recharged or replaced.

Liquid Cultures

The gelling agent or the agar used to solidifying the culture media comprises of about 70% of its total cost and hence by using liquid media we cut down on a major part of the incurred costs by omitting the agar component of the media.

Also in liquid cultures the nutrients are more readily available for the plantlets as they are in the ionized form and can be more easily absorbed by the plantlets. As a result the growth rate on liquid media is higher than that in the solid media. Aeration was provided by placing the culture vessels on the shaker set at the rate of 120rpm, this prevented the plantlets from submerging in the liquid media and made fresh air available to them.

Hairy root cultures

Prepare media containing IBA(1mg/l) and KN(3mg/l) and autoclave. Put the Kanamycin (3mg/l) in the autoclaved media which has been filter sterilized (using 0.22 micron filter).

Measure LB and raise with water to prepare 4 flasks of LB measuring

200(blank) ;100(LB1) , 100(LB2) and 100(LB3). Autoclave the LB media.

Make innoculations with *agrobacterium rhizogenes* into the flasks LB1 , LB2 and LB 3 respectively. Check O.D at 600nm at frequent intervals . Induce hairy roots when O.D reaches 0.9. Take leaf disc ; make cuts on the same. Dip the needle in *A.rhizogenes* and then on leaves . Place these leaves on the media.

CHAPTER 4

Results

Results

Sterilization of explants:

Sterilization is a term referring to any process that eliminates (removes) or kills all forms of life, including transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) present on a surface, contained in a fluid, in medication, or in a compound such as biological culture media. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol or bleach) is required. Mercuric chloride is seldom used as a plant sterilant today, as it is dangerous to use, and is difficult to dispose of. Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired.

The explants were subjected to bacterioside and fungiside agents for different time durations. Bavistin and $HgCl_2$ are the most commonly used agents(refer Table 1).

Table 1 : Different concentrations and time of exposure of explants of *Aconitum heterophyllum* to sterilizing agents.

Sterilizing agents	Concentration	Time of exposure (in mins)	Percentage survival (%age)
$HgCl_2$	0.05%	1 , 1.5 , 2	40
	0.1%	**1 , 1.5 , 2**	95
	0.15%	1 , 1.5 , 2	25
Bavistin	0.45%	2 , 3 , 5	30
	0.5%	**2 , 3 , 5**	97
	0.55%	2 , 3 , 5	25

Pretreatment to seeds :

Different *in-vitro* conditions were tested for germination of seeds of *Aconitum heterophyllum* on culture media and petri plates under appropriate moisture and temperature conditions . Pretreatments prove to be beneficial for aseptic germination as all the solid and fluid impurities are eliminated with the help of sterilizing agents. Here , we have used two conditions that have shown positive results in terms of showing percentage germination.(refer table 2 and 3).

Table 2 : Pretreatments given to seeds of *Aconitum heterophyllum* before put for germination.

Treatment	Surface sterilization	Time duration
75 % H_2SO_4 (1 min) ; wash with double distilled water(4-5 min) and keep at 25°c ; kept in dark.	Tween20 (rinse 3-4 times) Wash with DH_2O Bavistin(0.5%) 3 min	15 days
Chilling treatment : keep seeds in refrigerator after dipping in distilled water at 4°c and treatment with 0.1%$HgCl_2$; kept in dark .	Tween 20(rinse 3-4 times) Wash with DH_2O $HgCl_2$ (0.1%) 2-3 min Bavistin(0.5%) 3 min	15 days

The seeds are put on petriplates that have controlled moisture conditions with either cotton or filter paper as base . The appropriate water content needs to be maintained by regular monitoring of the plates .

Table 3 : Germination of seeds of *Aconitum heterophyllum* seen in *in-vito* conditions(petri plates)

Treatment	Number of seeds on petri plate	Number of seeds showing germination	%age germination
H_2SO_4	45	10	22.2%
Chilling treatment	**Plate 1 :25** Plate 2: 27	**18** 15	**72%** 55.5%

The seeds were subjected to different combinations of growth hormones in MS media and kept in culture room maintained at 25°C.

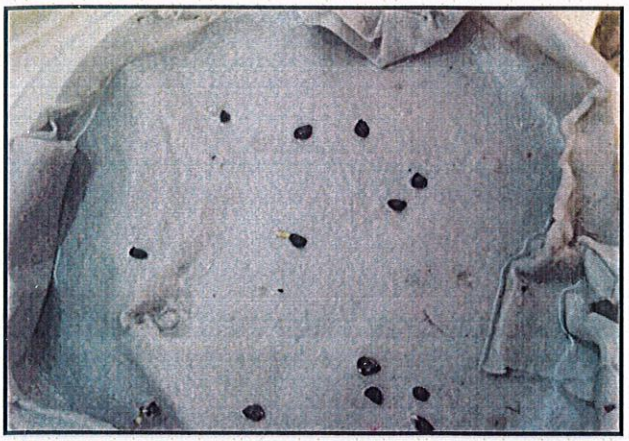


Figure 1: Treatment with H_2SO_4

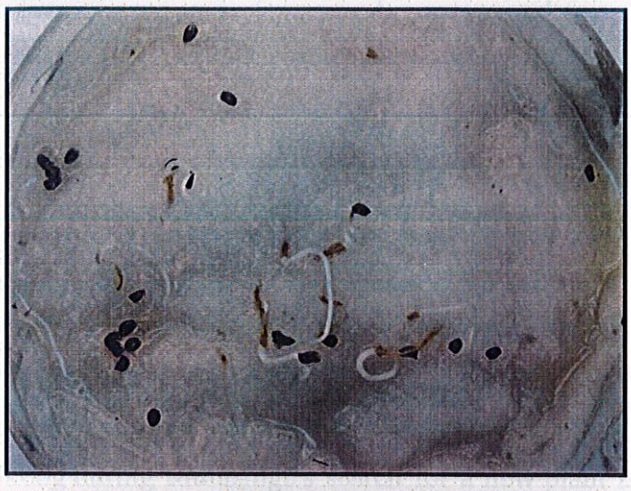


Figure 2: Treatment with $HgCl_2$(Plate1)

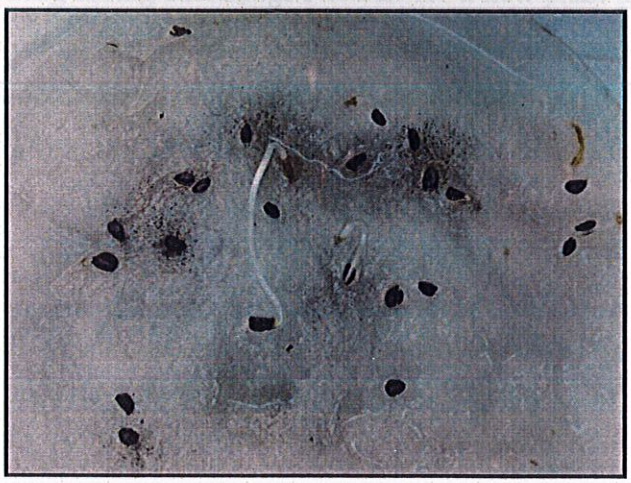


Figure 3: Treatment with $HgCl_2$(Plate2)

Table 4 : Germination of seeds of *Aconitum heterophyllum* seen in *in-vito* conditions(culture medium)

MS+growth hormones		Percentage of seed germination
NAA	BA	
0	0	
0	1	36
1	0	43
1	1	52
1	**2**	**60**
2	3	54

Media preparation:

Murashige and Skoog medium or is a plant growth medium used in the laboratories for cultivation of plant cell culture. MSO was invented by plant scientists Toshio Murashige and Folke K. Skoog during Murashige's search for a new plant growth regulator. It is the most commonly used medium in plant tissue culture experiments in laboratorium. The surface sterilized explants (leaf and petiole) were cultured on MS media containing 2-4-D (0-4 mg/l), NAA (0 -3 mg/l) and IBA (0-2 mg/l) in different combinations. Out of11 different media combinations tested for callus initiation, MS medium containing 2-4-D(3mg/l) + NAA(2mg/l)) was found to be the best with 60% and 65% of initiation is seen (Table 5; Fig.4a,b,c,d). With increase in 2-4-D and decrease in IBA concentrations , higher percentage of callus initiation was observed .

The surface sterilized axillary shoot tips were cultured on MS media containing KN (0-3 mg/l), GA_3 (0-2 mg/l) and BAP (0-2mg/l) in different combinations. Out of 9 different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN(3mg/l) +GA_3 (1mg/l)+ BAP(1mg/l) was found to be the best with 2-3 shoots per explants formation (Table 6; Fig.6a,b,c,d,e). increased concentrations of KN provided better results in terms of multiple shoot formation.

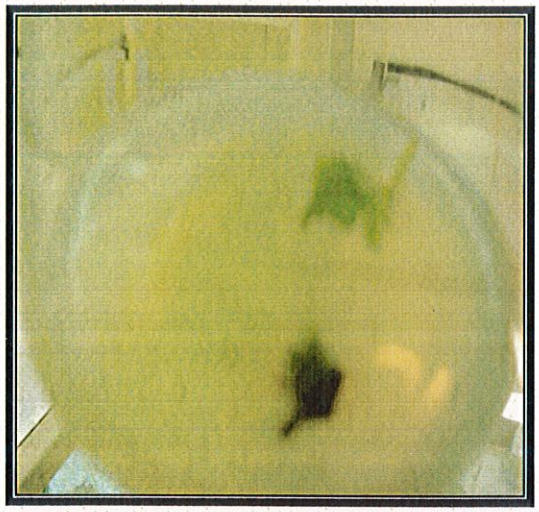
Table 5 : Different concentration and combinations of auxins tried for callus induction in *Aconitum heterophyllum.*

MS+growth hormones			Days to callus initiation	Percentage of callus initiation	
2-4-D	NAA	IBA		Leaf	Petiole
0	0	0			
1	0	1	26-27	22	18
1	1	0	23-25	28	23
1	0	2	19-22	28	22
1	2	0	18-19	35	32
2	0	1	16-19	32	27
2	1	0	15-16	48	42
2	2	0	12-13	54	51
3	0	2	15-16	41	35
3	**2**	**0**	**8-9**	**65**	**60**
4	3	0	8-9	59	58

Table 6 : Different concentration and combinations of growth hormones tried for shoot formation in *Aconitum heterophyllum*

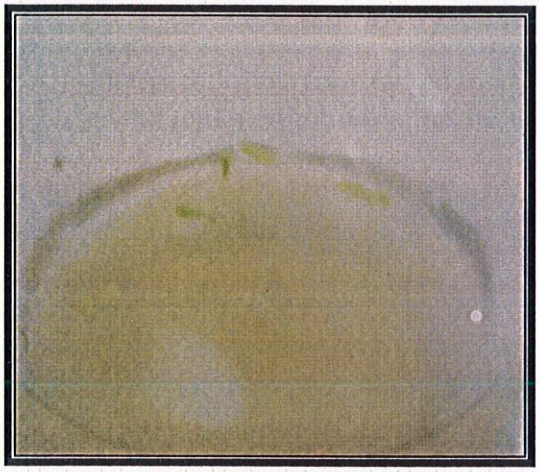
MS+growth hormones			Days to multiple shoot formation from shoot base	Number of shoots per explant
KN	GA_3	BAP		
0	0	0		
1	1	0	19-20	0
1	2	0	18-20	0
2	1	0	17-19	1
2	2	0	16-18	0
3	1	0	16-17	1
3	2	0	14-16	0
3	**1**	**1**	**12-15**	**2-3**
3	2	2	15-16	1

(a)



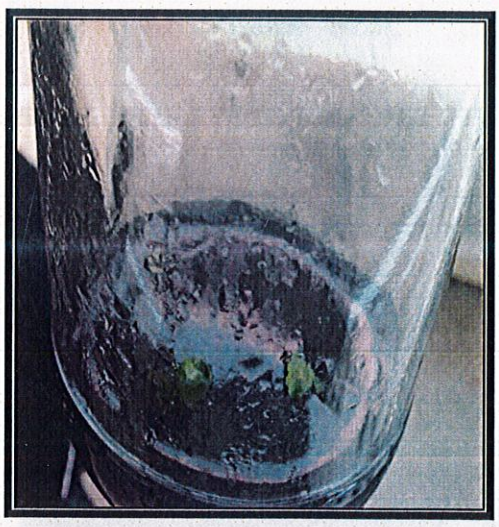
(b)

(c)

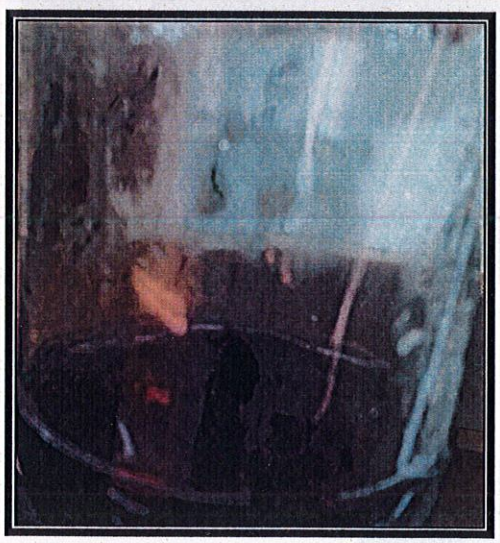


(d)

Figure 4 : Callus induction with leaf (a,b,c)and petiole(d)

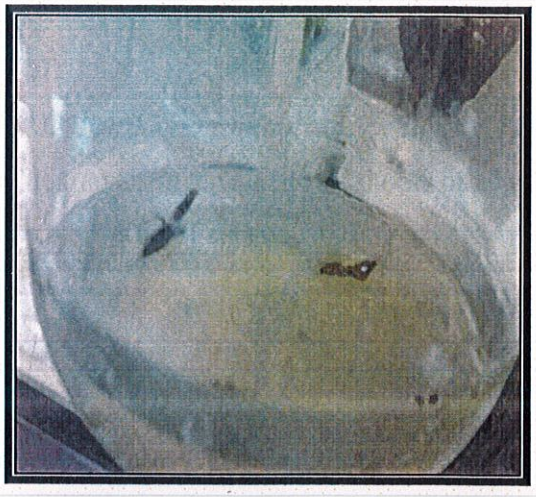


(a)

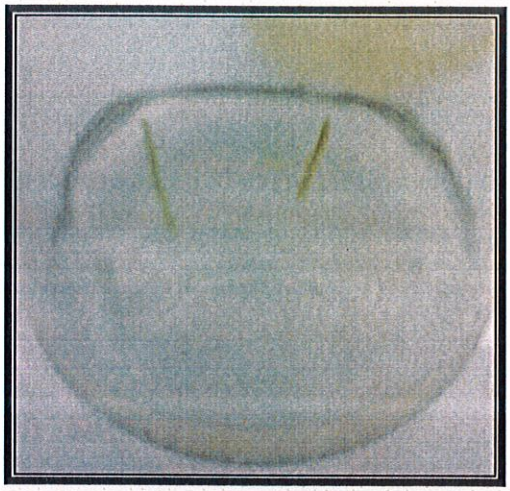


(b)

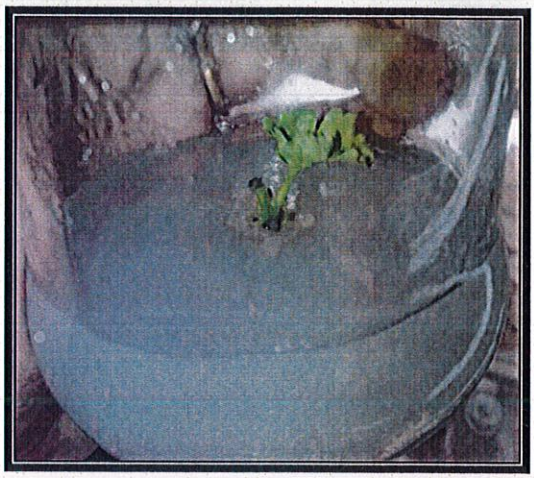
Figure 5: leaves transferred to activated charcoal medium forcallus induction



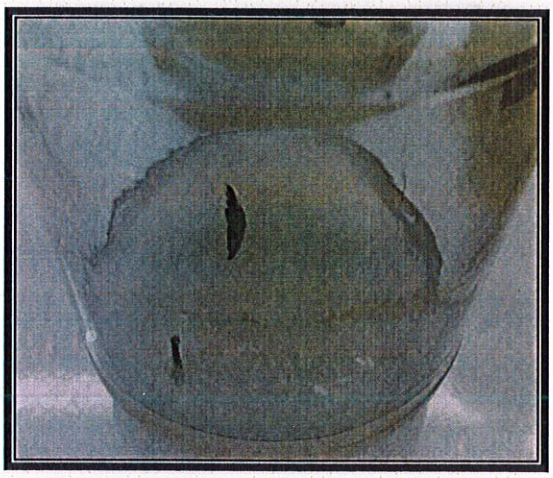
(a)



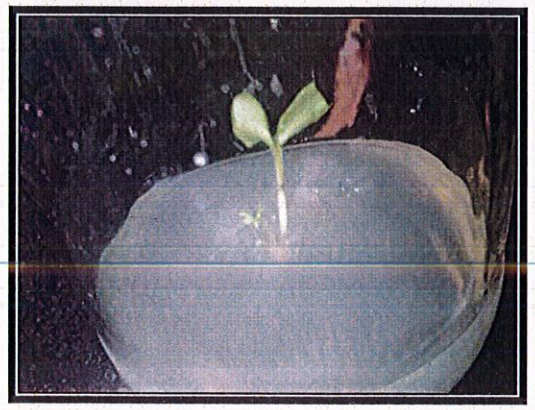
(b)



(c)



(d)

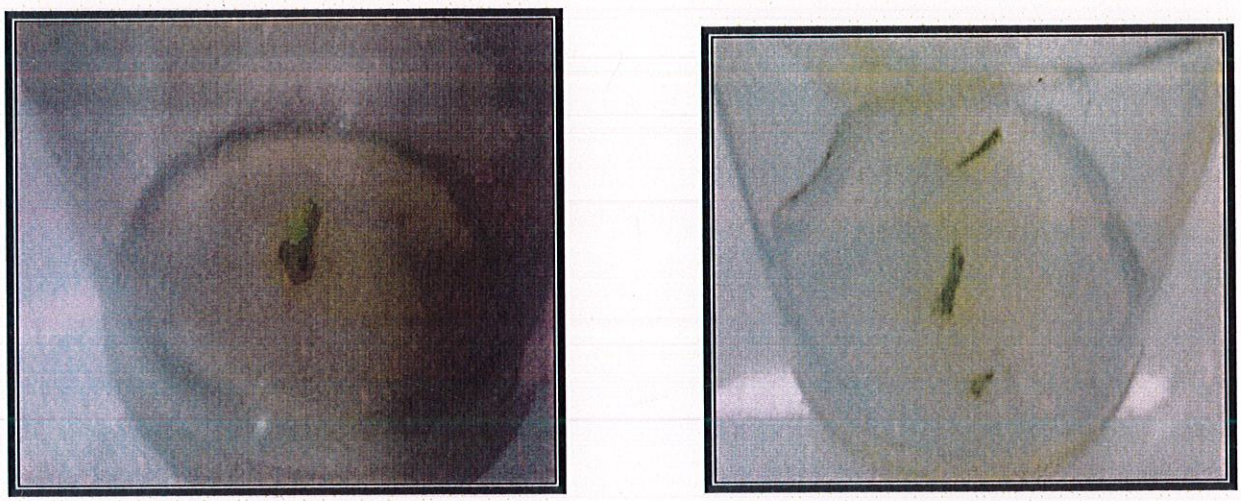


(e)

Figure 6 : Shoot formation from shoot base

Establishment of hairy root cultures

The transformed leaves and shoots of aconitum heterophyllum were cultured in antibiotic medium with 35-40% of survival and the shoots were cultured on different regeneration medium .



(a) *(b)*

Figure 7: Leaf (a) and Shoot (b) induced with *A.rhizogenes*

CHAPTER 5

Conclusion

CONCLUSION

Aconitum Heterophyllum has become an endangered species due to

- Habitat destruction and extensive exploitation for the drug industry and local medicinal system.
- These factors, coupled with overgrazing, prolonged seed dormancy, high seedling mortality, and ecological restriction of endemic population to localized niches, mean the herb is in danger of extinction.

In situ and *ex situ* conservation of the concerned species is thought to be the most easy and cost-effective mode of conservation. These modes need to be encouraged and practiced widely to reduce the pressure on the wild habitats of these species, eventually leading to their conservation.

According to our studies callusing was achieved on MS media supplemented with 2-4-D (3mg) and NAA(2mg). Seed based multiplication is the most effective, realistic and convenient means for most of the species. But cultivation through seed in most of the species of *Aconitum* e.g., *Aconitum heterophyllum* is difficult due to poor seed availability and lack of superior germplasm. However ,our studies indicate that chilling treatment gave a good percentage rate of germination . More over the techniques which we have deveopled could be used for its large scale propagation and reclaiming its natural population. As these tissue culture procedures not only exploited for their propagation but also act as an authentic source for the production of medicinally important metabolites.

APPENDIX – I

Media components (Inorganic)

Components		Amount (gm/lt.)
STOCK A [10X]		
	KNO_3	19.00
	$MgSO_4.7H_2O$	3.70
	KH_2PO_4	1.70
STOCK B [20X]		
	NH_4NO_3	33.00
STOCK C [100X]		
	$CaCl_2.2H_2O$	44.00
STOCK D [100X]		
	Na_2 EDTA	3.726
	$FeSO_4.7H_2O$	3.785
STOCK E [100X]		
	KI	0.083
STOCK F [100X]		
	H_3BO_3	0.62
	$CoCl_2.6H_2O$	0.0025
	$ZnSO_4.7H_2O$	0.86
	$CuSO_4.5H_2O$	0.0025
	$MnSO_4.4H_2O$	2.23
	$Na_2MoO_4.2H_2O$	0.025

APPENDIX – II

Media components (Organic)

Components		Amount (gm/lt.)
STOCK G [100X]		
	m – Inositol	10.00
	Glycine	0.20
STOCK H [100X]		
	Pyridoxin HCl	0.05
	Nicotinic Acid	0.05
	Thiamine HCl	0.01

APPENDIX – III

Media components for LB media

Components	Amount (gm/lt.)
Casein hydrolysate	10
Yeast extract	5
Nacl	10

APPENDIX – IV

Media components for yeast mannitol media

Components	Amount (gm/lt.)
Mannitol	5
Yeast extract	0.5
$MgSO_4.7H_2O$	0.2
$NaCl_2$	0.1
K_2HPO_4	0.5
Na gluconate	5

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Career objective:

To develop myself as a professional while working in a challenging and growing environment.

Education:

Pursuing Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat, Distt. Solan (H.P.) with the following marks till date:

Standard	College/School	Year	CGPA/Percentage
B.Tech (B.T)	Jaypee University of Information Technology, Solan	2011 (Expected)	8.3 (Up till 7th semester)
High School (C.B.S.E)	St Xavier's Senior Secondary School, 4 Raj Niwas Marg, Delhi-110054	2007	8O%
Intermediate (C.B.S.E)	St Xavier Senior Secondary School, 4 Raj Niwas Marg, Delhi-110054	2005	87.2%

Technical skills

- **Computer proficiency**: operating system-windows/vista/xp.

  Programming languages and software's-C, MS office, Data structures (DS).
- **Bioinformatics tools**: BLAST, FASTA, primer designing, MSA, GENSCAN.
- Performed basic wet lab experiments like electrophoresis, worked in LAF, etc.
- Done work in tissue culture techniques.
- Worked with PCR, Sonicator, lyophillizer, centrigufes, spectrophotometers.

AREA OF INTEREST

Genetics ,Stem cell research, plant and animal tissue culture, comparitive and functional genomics and immunology.

Also have a keen interest in professional development courses including financial management, project management and also principles of management .

PROJECT UNDERTAKEN

Currently working on a project titled optimizing tissue culture condition for Aconitum heterophyllum.

Also done a project based on the **preparation of orange flavoured vinegar from rhodendron wine .**

SUMMER INTERNSHIP

Organization-Max Healthcare Institute Ltd, Max House, No1, Dr Jha Marg, Okhla phase 3, New Delhi.

Synopsis- Worked in serology, histopathology and microbiology lab of Max Healthcare. Performed various experiments some of which included Immunohistochemistry staining, tissue processing, fixation and staining. Ag-Abs interaction experiments including sandwich and competitive ELISA. Abs detection for Vasculitis, Torch and Dengue etc.

- ## CO-CURRICULAR ACTIVITIES / ACHIEVEMENTS

  - Was a member of the **invitation committee** of the **JYC** (Jaypee Youth Club) in the college fest of 2009 and 2008.
  - Was a member of the **event management** club of the **JYC** (Jaypee Youth Club) in the college fest of 2009 and have been actively involved in the organization of various events.
  - Was a member of **Synapse (bio club)** in college, an active member of the club and have been a part of various activities of the club like the blood donation camp.
  - Member of college **anti ragging squad** in college.
  - Have been an active participant in various literary events in the college and school. Have been receiving academic distinction certificate from school.
  - Also have participated in activities regarding leprosy and cancer awareness in school.

STRENGTHS

Good communication skills, willing to learn new things, sense of responsibility, sincere and hardworking, down to earth.

Personal details

Father's name : Mr. Yashpal Sachdev
Mother's name : Mrs. Sammita Sachdev
Date of birth : June 25th, 1989.
Hobbies : listening to contemporary music, reading fiction novels.
Languages known: English, Hindi ,
German (currently pursuing)
Residential address: House no 1, pocket 3, sector 12, dwarka, New Delhi, 110075
Telephone : 9816734354, 9899277829

Declaration

I hereby declare that all the information given above is true and correct to the best of my knowledge.
Place : Waknaghat
Date : May,2011

Saumya Sachdev

NEHA KATYAL

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Waknaghat, Solan (H.P)
Cell: +91-9899711871
E-mail: katyneha@gmail.com

OBJECTIVE

To be part of an **organization**, where my **self motivated attitude** can trigger **symbiotic growth** and contribute to the all round development of the upcoming **biotech industry**.

PERSONAL SKILLS

Leadership quality, Self Motivated, Fast-Learner, Relationship Building at all levels.

ACADEMIC QUALIFICATIONS

Exam	Name Of School/University	BOARD	CGPA/percentage
Class X A.I.S.S.E 2005	Blue Bells Public School Gurgaon, Haryana	C.B.S.E	82.6 %
Class XII A.I.S.S.C.E 2007	Delhi Public School Gurgaon, Haryana	C.B.S.E	80.8 %
B.Tech Biotech Currently in 7th sem	Jaypee University Of Information Technology, Waknaghat, Solan (H.P.)		8.0

HOBBIES

- Reading fiction novels,Dancing,Socializing.Dramatics.

TECHNICAL SKILLS

- Lab practice at school and college level.

ACHIEVEMENTS

- President of Bio-club "Synapse" in college.
- Successfully organized BIO related events for le fiestus'09 and '10.
- Graduate of an NGO "art of living".
- Awarded first position at an intra-school level debate competition.
- Awarded first position in dance competition at district level.
- Been a part of invitation committee for le fiestus'09.
- Awarded first and second positions in debates and declamations organized at school level.
- Participated in Group dance competition in le fiestus'09.
- Participated in dramatics in college.

AREAS OF INTEREST

- Knowing latest news in the world of biotech.
- Learning about new equipments available in this field.
- Exploring new technologies.

PROJECTS UNDERTAKEN

- Currently working on a project titled optimizing tissue culture condition for Aconitum heterophyllum.
- Also done a project based on the **preparation of orange flavoured vinegar from rhodendron wine .**

SUMMER INTERNSHIP

- Worked with LAB INDIA pvt. Ltd. on projects regarding **human identification system , real time PCR and DNA sequencing.**

PERSONAL DETAILS

Date of Birth	: 11th May 1989
Father's Name	: Mr. Rajesh Katyal
Mother's Name	: Mrs. Ashu Katyal
Residential Address	: N-14/10 A , Ground Floor, DLF phase-2 , Gurgaon,Haryana
Phone No.	: 0124-4042997,9899711871

Declaration

I hereby declare that all the information given above is true and correct to the best of my knowledge.

Place : Waknaghat

Date : May,2011

Neha Katyal