Development of Micropropagation Technology for *Aconitum heterophyllum*

By

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CERTIFICATE

This is to certify that the work entitled, "Development of Micropropagation Technology for *Aconitum heterophyllum*" submitted by Astitva Kachiar in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology 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 this or any other degree or diploma.

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"I have not failed. I've found 10,000 ways that won't work."

Thomas A. Edison

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Place: JUIT, Waknaghat.
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Astitva Kachiar

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LIST OF ABBREVIATIONS

MS Media	MURASHIGE AND SKOOG MEDIUM
NAA	1-naphthaleneacetic acid
BAP	6 benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
IBA	indole-3-butyric acid
UV	ULTRA VOILET

OBJECTIVES

Aconitum heterophyllum Wall. Commonly known as 'Atis' or 'Patis' belongs to family of Ranunculaceae and is reputed for its medicinal and pharmaceutical values since long. It is perennial herb, distributed over temperate parts of western Himalaya, extending from Kashmir to Kumaon. Its chief habitat is the alpine and sub-alpine region at altitude of 3000-3500 m. The plant is used for treatment of diseases of the nervous system, digestive system, rheumatism and fever. *Aconitum* has biological and pharmacological activities such as anti-fungal, anti-bacterial insecticidal activities. *Aconitum* is an endangered species due to deforestation and because of its extensive use for medicinal purpose. *Aconitum* is not harvested commercially and there is need to save it from extinction.

Hence, in this study an attempt has been taken to develop a micropropagation technology for aconitum heterophyllum with the following objectives:

- The Initiation of Callus on MS media from *A.heterophyllum* leaf and petiole explant.
- Shoot-Proliferation from callus derived.
- Direct axillary shoot-initiation, proliferation and elongation.
- The root induction of well-developed micro shoots.
- Based on above experiments development of most suitable micropropagation technology for *Aconitum Heterophyllum*.

ABSTRACT

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction. Aconitum is an endangered species because of deforestation and its extensive use. Micropropagation technology is required for rapid multiplication and exploits its commercial potential. Hence, an attempt has been taken by performing various experiments to develop micropropagation technology for *Aconitum Heterophyllum.*

CHAPTER 1

INTRODUCTION

1.1 *Aconitum heterophyllum*

Aconitum heterophyllum Wall. Commonly known as 'Atis' or 'Patis' belongs to family of Ranunculaceae and is reputed for its medicinal and pharmaceutical values since long. It is perennial herb, distributed over temperate parts of western Himalaya, extending from Kashmir to Kumaon. Its chief habitat is the alpine and sub-alpine region at altitude of 3000-3500 m. The tuberous roots of genus *Aconitum* contain alkaloids: benzoylmesaconine, mesaconitine, aconitine, hypaconitine, heteratisine, heterophylline, atidine, isotisine, hetidine. The roots are reported to posses' significant antipyretic and analgesic properties and a high therapeutic index. The plant is used for treatment of diseases of the nervous system, digestive system, rheumatism and fever. *Aconitum* has biological and pharmacological activities such as anti-fungal, anti-bacterial insecticidal activities.

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. Therefore it is needed to develop a micropropagation technology for *Aconitum heterophyllum* to exploit its potential.

BOTNICAL CLASSIFICATION OF *Aconitum heterophyllum*	
Kingdom	Plantae
Division	Magnolophyta
Class	Magnoliopsida
Family	ranaunculaceae
Genus	Aconitum
Species	heterophyllum

TABLE 1.1: BOTANICAL CLASSIFICATION OF *A.heterophyllum*

1.2 MICROPROPAGATION

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

1.2.1 Establishment:

Micropropagation begins with the selection of plant material to be propagated. Clean stock materials that are free of viruses and fungi are important in the production of the healthiest plants. Once the plant material is chosen for culture, the collection of explant(s) begins and is dependent on the type of tissue to be used; including stem tips, anthers, petals, pollen and others plant tissues. The explant material is then surface sterilized, usually in bavistin and mercuric chloride and finally rinsed in sterilized water. This small portion of plant tissue, sometimes only a single cell, is placed on a growth medium, typically containing sucrose as an energy source and one or more plant growth regulators (plant hormones). Usually the medium is thickened with agar to create a gel which supports the explant during growth. Some plants are easily grown on simple media but others require more complicated media for successful grow; some media include vitamins, minerals and amino acids. The medium is sterilized during preparation to prevent fungal and bacterial contamination, which can outgrow and smother the growing explant. Autoclaves and filter sterilization are used to remove potential contaminates. The plant tissue grows and differentiates into new tissues depending on the medium.

1.2.2 Multiplication:

Multiplication is the taking of tissue samples produced during the first stage and increasing their number. Following the successful introduction and growth of plant tissue, the establishment stage is followed by multiplication. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds or thousands of plants. Depending on the type of tissue grown, multiplication can involve different methods and media. If the plant material grown is callus tissue, it can be cut into smaller pieces and recultured on the same type of culture medium to grow more callus tissue. If the tissue is grown as small plants called plantlets, hormones are often added that cause the plantlets to produce many small offshoots that can be removed and recultured.

1.2.3 Pretransplant:

This stage involves treating the plantlets/shoots produced to encourage root growth and "hardening." It is performed *in vitro*, or in a sterile "test tube" environment.

Root growth does not always occur in the earlier stages in plant cell culture, and is of course a requirement for successful plant growth after the micropropagation procedure. It is often performed *in vitro* by transferring the plantlets to a growth medium containing auxin(s) which stimulate root initiation. The pretransplant stage is not always performed; some plants are micropropagated and grown in culture and normal cuttings are made that are then rooted ex vitro.

"Hardening" refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in "ideal" conditions, designed to encourage rapid growth. Due to lack of necessity, the plants are likely to be highly susceptible to disease and often do not have fully functional dermal coverings and will be inefficient in their use of water and energy. In vitro conditions are high in humidity and plants grown under these conditions do not form a working cuticle and stomata that keep the plant from drying out, when taken out of culture the plantlets need time to adjust to more natural environmental conditions. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to what would be considered a normal growth environment for the species in question. This is done by moving the plants to a location high in humidity, such as a green house with regular mist watering.

1.2.4 Transfer from culture:

In the final stage of plant micropropagation, the plantlets are removed from the plant media and transferred to soil or (more commonly) potting compost for continued growth by conventional methods.

This stage is often combined with the "pretransplant" stage.

CHAPTER 2

MATERIALS AND METHODS

2.1 REQUIREMENT AND APPARATUS

- Glass Test Tube – Initially experiments is started with 30 test tubes
- Explant - Plantlets of Aconitum heterophyllum were procured from Himalayan Forest Research Institute (HFRI), Panthaghati, Shimla.
- Media components - Stock solution (VII) were prepared, agar-agar, sucrose, growth hormones.

Plantlets of Aconitum heterophyllum were procured from Himalayan Forest Research Institute (HFRI), Panthaghati, Shimla and grown in campus for explant purposes. Initially experiment is conducted in 30 glass test tubes. Seven stock solutions were prepared for media preparation, agar-agar is used as gelling agent and different growth hormones were used in the experiments. Other components required were pH meter, Hot Plate, Autoclave, Sucrose, Beaker, Chemicals Reagents, Double distilled water and Flasks etc.

2.2 MEDIA PREPARATION

MS (Murashige and Skoog medium) Media was prepared by using above components chemicals. pH set to 5.6 and required growth hormones is added in it. After pouring the media into glass test tube and covering it with cotton plugs, it was autoclaved (15-20 min). After solidification media was left for 1-2 days to see if there is any contamination, after that media is ready for inoculation.

2.3 INOCULATION

Inoculation was carried out in clean room in Laminar Air Flow Chamber only. Glass test tubes, petri plates, scalpel and forceps, burner, ethanol, double distilled water were placed in Laminar Air flow for 20 minutes for sterilization by UV light.

Different explant were taken (leaf, petiole) and surface sterilized with .5% Bavistin and .1% Mercuric Chloride followed by repeated washing with dd water. Now, these explants were injured and inoculated in test tubes.

A.heterophyllum leaf and petiole explants were cultured for Callus initiation on MS media fortified with NAA + BAP and with 2,4-D Kinetin. For direct shoot-proliferation explants were cultured on MS media fortified with Kinetin and Kinetin + IBA. Previously published research papers referred for concentration of different growth factors required.

For subculutring callus is used for inoculation.

2.4 INITIATION OF PROJECT

Project started by inoculating fresh explants into MS Media in sterilized condition in laminar air flow. Inoculate test tubes were kept in tissue culture room under standard condition (Temperature, photoperiod etc). Observations were taken after regular intervals.

2.5 USE of GROWTH HORMONES

Different growth hormones like NAA, BAP, 2,4-D, Kinetin, IBA were used in the preparation of MS media and inoculation. NAA and BAP were used for callus initiation while Kinetin and IBA were used for direct shoot proliferation.

Observations were taken after regular intervals.

CHAPTER 3

METHODOLOGY

3.1 METHODOLOGY

For development of micropropagation technology for *Aconitum heterophyllum* we use common method of varying concentration of different growth hormones for inoculation. Following methodologies were adopted in the study:

I. Shoot proliferation rate is quite good for *A.heterophyllum*; therefore major thrust is on direct shoot initiation and proliferation using regenerating part of shoot as explant. Kinetin and IBA were major growth hormones used for shoot proliferation.

II. Callus initiation using leaf and petiole with varying concentration of NAA, BAP, 2,4-D and use of plant tissue culture techniques for development of micropropagation technology for *A.heterophyllum*

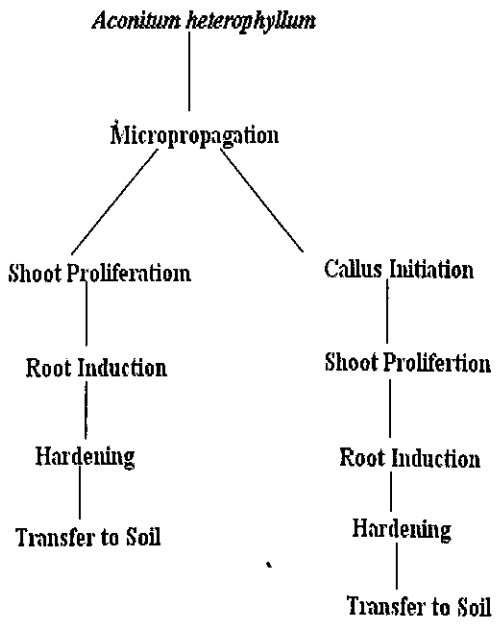

Figure 3.1 Methodology

CHAPTER 4

RESULTS AND DISCUSSION

Experiments conducted in the study have not given any callus or shoot proliferation. Explants cultured on MS media with varying concentration of different growth factors have not shown any response. More permutations and combinations of growth factors are required to be tested for development of micropropagation technology of *Aconitum heterophyllum.*

Cultured petiole explants have better survival rate in MS media than those of leaf explant of *A. heterophyllum.*

Aconitum heterophyllum plants have quite good shoots regeneration rate, but rooting is not so good at lower altitudes. Therefore we have gone for direct shoot proliferation. Also, *A.heterophyllum* plants require humus rich soil for better rooting of the plants and grow better in shade than under direct sunlight.

	Development of Micropropagation Technology for *Aconitum heterophyllum*				
No. of Test Tubes used in experiment.	**Growth Hormones for Callus Induction**				**Results**
	NAA	**BAP**	**2,4-D**	**Kinetin**	
15	0.5 mg L^{-1}	0.25 mg L^{-1}	NA	0.5 mg L^{-1}	Contaminated
15	NA	NA	1 mg L^{-1}		Contaminated
20	0.5 mg L^{-1}	0.25 mg L^{-1}	NA		No Response
15	NA	NA	1 mg L^{-1}	0.5 mg L^{-1}	No Response

Table 4.1: Growth Hormones used for Callus Induction of *A.heterophyllum*

	Development of Micropropagation Technology for *Aconitum heterophyllum*		
No. of Test Tubes used in experiment.	**Growth Hormones for Shoot Proliferation**		**Results**
	Kinetin	**IBA**	
15	0.5 mg L^{-1}	NA	No response
15	1 mg L^{-1}	NA	No response
20	1 mg L^{-1}	1 mg L^{-1}	Explants dead
12	.5 mg L^{-1}	NA	No response
12	.5 mg L^{-1}	5 mg L^{-1}	No response

Table 4.2: Growth Hormones used for Shoot Proliferation of *A.heterophyllum*

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