In Vitro Seed Germination and Callus Induction of *Aconitum spicatum* (Bruhl) Stapf

A Dissertation Submitted for the Partial Fulfillment of the Requirement of a Master's Degree in Botany

BY

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Declaration

This dissertation entitled "***In Vitro* Seed Germination and Callus Induction of *Aconitum spicatum* (Bruhl) Stapf"**, which is being submitted to the Central Department of Botany, Institute of Science and Technology, Tribhuvan University, Nepal for the partial fulfillment of the requirement of Master of Science in Botany has been carried out by me under the supervision of Associate Professor **Dr. Krishna Kumar Pant**. This research is authentic in all sense, and has not been submitted to any universities or institutions for any academic degree.

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Recommendation

This is to recommend that **Pooja Gautam** has carried out the dissertation entitled "***In Vitro* Seed Germination and Callus Induction of *Aconitum spicatum* (Bruhl) Stapf"** for the partial fulfilment of the requirements of M. Sc. in Botany under my supervision. To my knowledge, this work has not been submitted to any other institutions for any academic degree. She has fulfilled all the requirements of the Central Department of Botany, Institute of Science and Technology, Tribhuvan University, Kritipur for the submission of the dissertation for the M. Sc. in Botany.

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December, 2024

Letter of Approval

The dissertation work entitled ***In Vitro*** **Seed Germination and Callus Induction of** ***Aconitum spicatum*** **(Bruhl) Stapf"** submitted at the Central Department of Botany (Amrit Campus or Degree Campus), Institute of Science and Technology, Tribhuvan University by **Pooja Gautam** has been accepted for the partial fulfilment of M. Sc. in Botany.

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Abstract

Aconitum spicatum (Bruhl) Stapf commonly known as 'Bikh' is an herbaceous perennial plant belonging to the family Ranunculaceae. It is one of the highly poisonous plants but characterized by significant and valuable medicinal uses. Low seed germination in nature and uncontrolled harvesting practices, as well as a lack of concerned conservation efforts, lead to the rapid declination of this vital plant species; however, no comprehensive and reliable protocol has been developed till date to produce plant materials for conservation as well as for pharmaceutical purposes. Therefore, this study was designed to develop an efficient, simple, and reproducible *in vitro* seed germination, direct shoot-roots organogenesis as well callus induction protocol using seed as an explant. The highest seed germination percentage was recorded on Murashige and Skoog's (MS) basal medium, with a germination rate of 77.78% in a mean germination time of 14 days. Among the various plant growth regulators examined, 1.5 mgL^{-1} BAP proved to be effective for maximum shoots and root induction after 42 days, The maximum callus was induced in MS medium supplemented with 1.0 mgL^{-1} NAA within 49-60 days. To our knowledge, this is the first study on *in vitro* seed germination and callus induction of this plant. This study could provide a basis for germplasm conservation and sustainable utilization, with implications for the isolation of unique and pharmacologically active compounds from callus or regenerated plantlets.

शोध सार

Aconitum spicatum औषधीय र बिषाक्त गुण भएको उच्च हिमाली क्षेत्रमा पाइने बिरुवा हो। Tissue culture विधिबाट यसको बिउ प्रयोग गरेर पहिलो पटक callus र बिरुवा उत्पादन गरियो। करिब १४ दिनमा ७७.७ प्रतिशत बिउहरू MS- medium मा अंकुरण भए भने औसत ४२ दिनमा १.५ मिलिग्राम प्रति लिटर BAP राखिएको MS- medium मा बिरुवा उत्पादन भयो। १० मिलिग्राम प्रति लिटर NAA राखिएको MS- medium मा callus उत्पादन गरियो। यस अध्ययनले यो विरुवाको Gene संरक्षण र औषधीय प्रयोगको लागि औषधि उत्पादनमा tissue culture लाई दिगो उपयोग गर्न मार्ग प्रदर्शन गर्दछ ।

List of Abbreviations and Acronyms

µg	Microgram
µm	Micromillimeter
^{0}C	Degree Celsius
2,4-D	2,4-Dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6-benzylaminopurine
EDTA	Ethylenediamine Tetra Acetic Acid
et al	Et alia
FMS	Full Murashige and Skoog media
g/L	Grams/liter
GA_3	Gibberellin
HCl	Hydrochloric acid
$HgCl_2$	Mercury(ii) chloride
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
Mg/L	Milligrams/liter
MGT	Mean germination time
Mm	Millimeter
MS	Murashige and Skoog media
N	Normal
NAA	Naphthaleneacetic acid
NaOCl	Sodium hypochlorite

NaOH	Sodium hydroxide
PGR	Plant growth regulator
P^H	Potential of Hydrogen
Psi	Per square inch
RT	Response time
SPSS	Statistical Package for Social Science
UV	Ultraviolet

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1. Introduction

1.1 Background

Plant tissue culture has established a strong position in biotechnology and is a promising method for plant production and trait modification (Haque et al., 2022). It is based on the concept of the cellular totipotency hypothesis stated by Gottlieb Haberlandt (1902), which states that each and every plant cell has the capacity to develop into an entire plant provided with the right nutrition in an appropriate environmental condition. Since then, plant tissue culture has been used for various applications like genotype conservation, large-scale multiplication of disease-free plants, the production of new varieties, encapsulated seeds, rapid cloning, etc.

Plant tissue culture, also referred to as *in vitro* propagation, is a process that produces a large number of plantlets by isolating, sterilizing, and culturing certain plant cells, tissues, and organs in an aseptic environment that leads to cell multiplication and plant regeneration (Njukwe et al., 2007). The explants can be either a single cell, tissue, or organ derived from a healthy plant. *In vitro*-grown plants are independent of growing season and geographical and climatic factors, which can be beneficial for the production of significant compounds and molecules to meet the needs of the pharmaceutical industry. *In vitro* propagation procedures like *in vitro* seed germination and callus induction are crucial for conservation and commercial production of plants. *In vitro* seed germination provides a controlled environmental condition that can enhance the seed viability and germination rates, overcoming the challenges such as seed dormancy and poor germination under natural conditions (Mosoh et al., 2024). Furthermore, callus induction is a vital aspect of plant tissue culture technique, particularly for the production of secondary metabolites in medicinal plants. Due to the various advantages over traditional plant multiplication techniques, *in vitro* propagation of industrially and medicinally valuable plants is gaining more popularity in today's world (Hasnain et al., 2022). It can also be used to preserve sterile and endangered plant species and is a very effective and economical method for biosynthesis, bioproduction, and the production of phytochemicals (Hasnain et al., 2022). Biotechnological research through plant tissue culture significantly contributes to the sustainable utilization and preservation of medicinal plant species (Aremu et al., 2012).

Among the various significant medicinal plants, the *Aconitum* species holds a special place in both cultivation and conservation. The genus *Aconitum* belonging to the Ranunculaceae family of flowering plants comprises more than 400 species all over the world (Chan et al., 2021). They are primarily indigenous to the mountainous regions of North America, Europe, and Asia in the Northern Hemisphere. This species is found in open rangelands, scrublands, and damp humid forests from the eastern, central, and western regions of Nepal, including Rasuwa, Jumla, Kaski, Dolpa, Sindhupalchok, Taplejung, and Darchula (Ghimire et al., 2008; Lama et al., 2001; Shrestha et al., 2022).

A total of 28 *Aconitum* species are known from Nepal (Shrestha et al., 2022). Since ancient times, *Aconitum* species has been widely used in numerous traditional Chinese medicines, Unani Siddha, and Ayurvedic rituals. In traditional Chinese medicine, *Aconitum* species were classified as the "warm" medications, which have the ability to rejuvenate the body, remove dampness and humidity, and reduce discomfort (Chan et al., 2021). They were used as anti-inflammatory, analgesic, antipyretic, antiemetic, antirheumatic, and antidiarrheal drugs in Ayurvedic medicine (Hikino et al., 1980; Shyaula, 2012; Jaiswal et al., 2013; Tai et al., 2015). A natural neurotoxic alkaloid called "aconitine" found in the roots and tubers of *Aconitum* provides the plant with its therapeutic qualities (Rawat et al., 2013). The genus *Aconitum* contains a wide range of components, including gums, mucilage, proteins, free fatty acids, saponins, polysaccharides, glycosides, cardiac glycosides, and various types of alkaloids (Rahman, 1993; Pala & Mir, 2014). In addition to these therapeutic benefits, alkaloids have a high degree of toxicity in humans and animals; however, this toxicity can be readily reduced by heating or treating with alkaline solutions through oxidation, deacetylation, or debenzylation (Beyer et al., 2009; Lu et al., 2009; Rawat et al., 2013). Due to growing understanding of its toxicology and detoxification methods, the medicinal uses of *Aconitum* species are becoming more prominent nowadays (Chan et al., 2021).

Among the many *Aconitum* species recognized for their therapeutic qualities, *Aconitum spicatum* is one of the most valuable and lethal poisonous plants (Shyaula et al., 2012). After being processed, the tubers of *Aconitum* are mostly used in herbal traditional remedies to treat fever, headaches, lung infections, and to heal cuts and wounds (Tsarong, 1994; Lama, et al., 2001; Uprety et al., 2010). Locally, it is also used to cure intestinal problems, as well as an antidote for food poisoning and snake bites. Additionally, it has been used to make sedatives and balms for analgesia. Aconitine, hypoconitine, and mesaconitine, which are known to have antibacterial, anti-inflammatory, analgesic, and enzyme inhibition

properties, are the main alkaloid components of this plant, which is also poisonous and these alkaloids show a direct correlation to the acute toxicity of the plant extract (Srivastava et al., 2010).

As the plant carries high medicinal properties, illegal and unsustainable collection of plants from the wild for commercial interest and trade without considering domestication of this species has led to the rapid declination of the plant. So, there is an urgent need for germplasm conservation of plants. The application of these tissue culture technique not only support the conservation of plant by significantly reducing the burden on natural populations but also provide an alternative sustainable source of raw materials to the pharmaceutical industry.

1.2 Rationale of the study

Aconitum spicatum is a highly demanded medicinal plant. It is one of the ten medicinal plants from the Himalayas of Nepal that are traded the most (Olsen & Larsen, 2003). According to Rana et al. (2020), the trade of *Aconitum* rhizome is increasing; >300 kg of rhizome is traded annually (DOF, 2017), and the majority of them are exported to India in order to manufacture the homeopathic medicines (Olsen, 2005). Furthermore, the Nepalese government has designated it as one of the 30 national priority herbs for its growth, study, and production (DPR, 2012). Under a natural environment, a number of sprouting buds of *Aconitum spicatum* developed into 1-5 daughter tubers only while exhibiting limited seed germination capacity, and the seed can remain dormant for a protracted period of time (Srivastava et al., 2010). Tubers also exhibit frequent dormancy. *Aconitum spicatum* was previously utilized in small amounts by the local people as a traditional medicine, but in recent years, the commercialization of plant-based medications has boosted demand for the plant, and its great medical value results in unsustainable harvesting, which eventually accelerates the rapid declination of medicinal plants (Chapagain et al., 2019). According to the flora of Nepal, it is one of the medicinal plants threatened through overcollection for the export trade. To improve biomass and productivity, the only method that can produce significant numbers of "elite" planting material is *in vitro* propagation. (Sharma et al., 2020). The development of *in vitro* culture techniques for this medicinal plant will be significantly justified by the concerns about possible extinction of species due to overexploitation and its delayed regeneration in nature.

1.3Research Questions

- What are the possibilities to develop an efficient protocol for *in vitro* seed germination of *Aconitum spicatum*?
- What will be the response of the phytohormones during *in vitro* propagation of *Aconitum spicatum*?
- What will be the response of the phytohormones to the callus culture of *Aconitum spicatum?*

1.4 Research objectives

1.4.1 General Objective

- The objective of this research is to produce plants and induce callus from explants excised from *in vitro* germinated seedlings of *Aconitum spicatum.*

1.4.2 Specific Objectives

- To develop an effective protocol for *in vitro* seed germination.
- To develop a reproducible propagation protocol for *Aconitum spicatum* by utilizing the best possible combinations of phytohormones.
- To study the response of different phytohormones on the callus induction of *Aconitum spicatum.*

1.5 Limitations

This study primarily presents a seed germination protocol, *in vitro* propagation and callus induction. Because of number of limitations like mass contamination, slow growth and irregularity in power supply and various lab condition, acclimatization of *in vitro* grown plantlets to ensure the high survival rate and vigorous growth as well as callus proliferation indirect organogenesis and genetic homogeneity test cannot be carried out.

2. Literature Review

2.1 Traditional uses of *Aconitum spicatum*

Aconitum species are generally poisonous, but some species show high medicinal properties and have also been used in Ayurveda and traditional Chinese medicine. *Aconitum spicatum* is one of the most important floras of the Himalayas, often used in the traditional medicine system.

The root of *Aconitum* is extremely bitter and is used in combination with *Piper longum* for pain in the bowels, diarrhea, and vomiting. Externally, its paste is applied for rheumatism, bruises, and injuries as an anti-inflammatory and antiseptic agent (Khare, 2004). According to Dosmann (2002), in the Rasuwa district, *Aconitum spicatum* is used to treat fever, headaches, and muscular and skeletal issues. In addition to its medicinal properties, *Aconitum spicatum* is greatly valued in the horticultural trade for its dark blue to purple floral displays. In Jumla, root paste of *Aconitum spicatum* is used as an arrow poison to kill rats and wild animals (Manandhar, 1986; Rokaya et al., 2010).

2.2 Seed Germination

According to Forcella et al. (2002), two of the most significant factors influencing seedling emergence, a crucial stage in the life of annual plants, are seed germination and dormancy. These processes have a major influence on the distribution of rare species (Herranz et al., 2010). Several medicinal plants in the Ranunculaceae family have limited seed germination because of various environmental variables or because of undeveloped embryos that result in morphological and physiological dormancy (Shrivastava et al., 2011). *Aconitum* species show limited seed germination capacity in natural environments and the seed can remain dormant for a prolonged period (Shrivastava et al., 2010).

Vandelook et al. (2009) reported that chemical stimulants such as nitrate, thiourea, and gibberellic acid (GA_3) are considered to break the dormant state. Seeds of *Aconitum ferox* Wall. treated with IAA were found to be germinated within four weeks of inoculation on the basal medium, whereas seeds treated with BAP and GA_3 remain dormant (Singh et al., 2019). After being chilled at -4^0 c for 20 days, *Aconitum chasmanthum* stapf Ex Holmes seeds in an MS basal medium showed better germination rates of 47.59 0.53% with a mean germination period of 10.78 0.21 days (Shah et al., 2021). In *Aconitum vilmorinianum*, seed germination was increased up to 66.00% on Murashige & Skoog (MS) medium fortified

with 2.0 mgL^{-1} 6-benzylaminopurine (BAP), 0.1 mgL^{-1} 1-napthaleneacetic acid (NAA), and 30 gl^{-1} sucrose (Mou et al., 2022). The germination rate of the seeds of *Aconitum violaceum* was increased when they were soaked in tap water for ninety-six hours during the winter and early spring before being cultured. Of the various PGRs that were utilized, the MS basal medium enriched with 0.5 mg L−1 Kn and 1.0 mg L−1 Kn showed the highest rate of *in vitro* seed germination in the winter and spring, with MGT values of 27.22 ± 0.70 and 26.88 ± 0.16 days and percentage germination values of 77.32 ± 0.38 and 75.33 ± 0.14 (Hadi et al., 2022).

The highest rate of germination for *Aconitum nagarum* was obtained from seeds that were stratified using filter paper for 48 hours at 4°C. The seeds favor low temperatures for germination (Langhu & Deb, 2014). In *A. sinomontanum,* stratification enhances seed germination, and Gibberellins and cold pre-chilling break seed dormancy, resulting in the maximum percentage of germination (90.8%) after 84 days of stratification (Dosmann, 2002). Treatments with sodium hypochlorite at 4% concentration and 8 minutes of exposure time have a greater influence on the germination percentage of the seed of *Dracocephalum moldavica* L (Babaei et al., 2015).

2.3 *In vitro* propagation of *Aconitum* species

In vitro propagation protocols of several species of *Aconitum*, such as *A. heterophyllum, A. ferox, A. napellus, A. violaceum, A. chasmanthum,* etc., Have been reported. But no study on *in vitro* propagation of *Aconitum spicatum* (Burhl) Stapf has been recorded so far. Some of the previous studies of the genus *Aconitum* are discussed.

Watad et al. (1994) studied the micropropagation of *Aconitum napellus* using the shoot tip as an explant and discovered that the maximum shoot multiplication was achieved when 2 mg/l of BAP was given to the multiplication medium, which was 77% greater than the control without additional cytokinin. According to Rawat et al. (2013) from the nodal explant, the highest shoot proliferation in terms of induction and elongation of *Aconitum violaceum* Jacq. was recorded on MS medium fortified with 0.5µg BAP + 0.1µg NAA within 3 weeks.

In vitro shoot multiplication rate of *Aconitum heterophyllum* was maximum on 1.0 MS medium supplemented with 0.25 mg/l indole acetic acid (IAA) and 0.25 mg/l kinetin. Up to 100% roots were obtained for shoots grown on 1.0 MS medium accommodated with 1.0 mg/l IAA (Belwal et al., 2016). The addition of 1.0 mg/l BAP, 0.5 mg/l, and 1.0 mg/l GA3 to

MS medium resulted in shoot growth of 46.50% in *Aconitum heterophyllum* Wall, with an average number of shoots of 2.7 = 0.48 and an average shoot length of 2.63 = 0.35 (Mahajan et al., 2015). Singh et al. (2019) reported that the MS medium in combination with 3μm IAA and 6μm BAP produced the greatest result in *Aconitum ferox* Wall with plantlets having well- grown shoots and roots from callus.

The study by Rafiq et al. (2021) on *Aconitum chasmanthum* showed that micropropagation of 20-40-day-old seedlings using nodes, leaves, and stems was tested in MS medium with cytokinin, auxins, and adenine sulphate. Nodal explants showed direct multiple shoot regeneration with 7 ± 0.36 shoots with an elongation of 5.51 ± 0.26 cm in the MS medium containing 0.5 mg/L BAP and with a response time (RT) of 10.41 ± 0.51 days and a percentage culture response of 77.77 ± 2.77% and rooting, while rhizome formation was observed after 8 weeks, with a 60% germination rate and plantlet development. Using the stem segments as an explant, the highest number of shoots per explant (2.39) and 100% shoot induction were achieved by using BAP at 2.0 mgl^{-1} in combination with NAA at 0.3 mgl^{-1} in *Aconitum vilmorinianum* (Mou et al., 2022).

2.4 Callus inductions

An irregular, undifferentiated, and unorganized but actively dividing mass of parenchymatous cells is called a callus. In laboratory conditions, it is generated by culturing explants in an appropriate culture medium that has been supplemented with specific growth regulators. In nature, callus is developed on the plant as those masses of cells that cover the wound. Generally, callus is divided into two types: compact callus and friable callus. Compact callus is green and robust, whereas friable callus is white to creamy yellow in color, breaks readily, and can be utilized to create somatic embryos (Vladimir et al., 2006). Skoog & Miller (1957), using tobacco callus, found that depending on the adequate proportion of plant hormones in the culture medium, the callus can eventually divide to develop shoots and roots. Basically, callus is capable of forming an entire new plant through indirect organogenesis or embryogenesis. Callus and suspension cell cultures are utilized in various fields such as pharmacology, Chinese medicine, agriculture, and horticulture. Using gene technology methods, genetically engineered callus cultures can produce bioactive secondary metabolites and improve plant tolerance against salt, draft, diseases, and pests (Efferth. T., 2019).

In *Aconitum heterophyllum* Wall. Nodal segments on MS media supplemented with (0.5mgl^{-1}) NAA and (0.25mgl^{-1}) BAP initiate the formation of calluses (Jabeen et al., 2006). The

study by Singh et al. (2019) on *Aconitum ferox* Wall. demonstrated that the most effective medium for producing and sustaining the callus was the MS medium enhanced with 2.26μM 2,4-dichlorophenoxyacetic acid (2,4-D). Stem segments of *Aconitum vilmorinianum* Kom. cultured on MS medium containing 2.0 mg L^{-1} 2,4-D, showed a high callus induction percentage of 96.67 (Mou et al., 2022).

2.5 Research Gap

The majority of earlier studies of genus *Aconitum* mainly focus on the field of herbal remedies due to its ability to treat a variety of diseases, while studies regarding *in vitro* propagation and callus induction are limited to certain species like *A. heterophyllum* (Belwal et al., 2016), *A. chasmanthum* (Rafiq et al., 2021), *A. vilmorinianum* (Mou et al., 2022) etc.

Aconitum spicatum has been the subject of some research, including ones on the extraction of secondary metabolites and other bioactive compounds (Shyaula et al., 2012), plant density and life history (Chapagain et al., 2019), therapeutic uses (Tsarong, 1994; Lama et al., 2001; Uprety et al., 2010) and its toxicity (Srivastava et al., 2010). However, there is still a dearth of research on *in vitro* methods for large-scale propagation, regeneration of entire plantlets from the induced callus, and the isolation of bioactive compounds through callus.

Therefore, by addressing these research gaps, this study includes both the understanding of *in vitro* techniques for *Aconitum spicatum* and the development of better propagation and conservation strategies for this species, and this is the first approach on seed germination and plant propagation of *Aconitum spicatum* that can contribute to the plant's conservation, especially in the face of climate change or habitat loss.

3. Materials and Methods

3.1 Plant Material

Healthy seeds of *Aconitum spicatum* (Bruhl) Stapf were used as an explant. The seeds were collected from Deurali of Annapurna Rural Municipality in Gandaki province. It is located in Kaski district at an elevation range of 3220m. The coordinates of the collection site are 28.52714^0 N and 83.892083^0 E. The seeds were cleaned, sterilized, and cultured for shoot/root development and callus induction in the Plant Biotechnology Laboratory of the Central Department of Botany, TU, Kritipur, Kathmandu.

Botanical description of *Aconitum spicatum* (Bruhl) Stapf

Common Name: Monkshood, Devil's Helmet, Blue Rocket

Nepali Name: Bikh

Flowering and fruiting time: July – September and August – November respectively (Lama et al., 2001).

Taxonomic description: *Aconitum spicatum* is an herbaceous mountainous plant distributed at an elevation range of 1800-4800m (Ghimire et al., 2008; Shrestha et al., 2022). It is a perennial herb that grows up to 2m high on an upright stem. Leaves are mostly cauline, lobed, and infrequently divided and dentate and dark green in color. At the top of the stem, there are racemes containing large zygomorphic flowers with numerous stamens in hues of blue and purple.

Figure 1: Habitat photograph of *Aconitum spicatum* (Bruhl) Stapf. A: whole plant in natural habitat; B: Inflorescence

Their unique name, monkshood, comes from a cylindrical helmet-shaped galea on the posterior sepal, one of the five petaloid sepals that makes them immediately distinguishable. 1-110 fruits are produced by each plant with an average of 41 seeds. Five to six follicles come together to form each fruit (Chapagain et al., 2019).

3.2 Plant tissue culture

For this experiment, MS medium in combination of different phytohormones was utilized. This medium was prepared from the liquid stock solutions of salt and vitamins. The further process of plant tissue culture is described below.

3.2.1 Preparation of stock solutions

The nutrient medium used for the culture was Murashige & Skoog (1962) medium (MS) as the basal medium (BM). The chemical composition of the medium is as follows:

Table 1: Preparation of different stock solutions

Components	Composition of final concentration	Final volume taken
Macronutrients (Stock A)	**g/L**	
Potassium nitrate (KNO_3)	19.0	
Ammonium nitrate (NH_4NO_3)	16.5	
Calcium Chloride ($CaCl_2.2H_2O$)	4.4	100 ml
Magnesium sulphate ($MgSO_4.7H_2O$)	3.7	
Potassium biphosphate (KH_2PO_4)	1.7	
Micronutrients (Stock B)	**Mg/L**	
Manganese sulphate ($MnSO_4.4H_2O$)	22.30	
Zinc sulphate ($ZnSO_4.4H_2O$)	8.60	
Boric acid(H_3BO_3)	6.20	
Potassium iodide (Kl)	0.83	
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25	1ml

Copper sulphate ($CuSO_4.5H_2O$)	0.025		
Cobalt chloride ($CoCl_2.6H_2O$)	0.025		
IRON SOURCE (Stock C)	**Mg/L**		
Sodium EDTA (Na_2-EDTA)	37.30		10ml
Ferrous sulphate ($FeSO_4.7H_2O$)	27.80		
VITAMINS (Stock D)	**Mg/l**		
Glycine	2.0		
Nicotinic Acid	0.5		
Pyridoxine Hydrochloride	0.5		1ml
Thiamine Hydrochloride	0.1		
Myo-Inositol	0.1g/L	(Freshly added)	
OTHERS	**g/L**		
Sucrose	30		
Agar	0.8		

The stock solutions A, B, C, and D were prepared, which consisted of macronutrients or major salts, micronutrients or minor salts, an iron source (Fe-EDTA), and vitamins, respectively, as Murashige & Skoog (1962) media. Sucrose was used as a carbon source and agar was used for solidifying the medium. During the preparation of each stock solution A, B, C, and D, the above chemicals were weighed accurately and dissolved in distilled water. To dissolve the chemicals completely, the solution was stirred by a magnetic stirrer. All these stock solutions were stored in a dry, sterile, and labeled bottle and stored in the refrigerator at 4^0c.

3.2.2 Preparations of Hormones

For this experiment, both auxins (IAA, IBA, and NAA, 2,4-D) and cytokinins (BAP, KN) were utilized as hormones or plant growth regulators to enhance the growth and development of cultured explants. For the preparation of all hormones, 50 mg of

hormones were weighed and dissolved with a few drops of either 70% ethanol or 1N NaOH. The final volume was 50 ml using double- distilled water. The prepared hormones were then stored in the refrigerator for further use.

3.2.3 Preparation of 1L media

For full MS-Media, all the stock solutions and other chemical compounds, such as freshly prepared myo-inositol, sucrose, and KI, were mixed according to the MS-Media protocol, and it was made up to 1 liter solution by adding sterile water in a 1-liter conical flask. For ½ strength media, and ¼ strength media ½ and ¼ amounts of all the stocks A, B, C, D, and myo- inositol were mixed, respectively, and a final volume of 1litre was made by adding sterile water.

The pH of the medium was adjusted to 5.8 by using 0.1N NaOH and 0.1N HCl before autoclaving the medium. Then the medium was equally distributed in 10 sterile glass jars, and 0.8 g of agar was poured in each jar. The medium-containing jars were heated in a micro-oven for 5-6 minutes to dissolve the agar properly. The medium in the jar was then divided evenly between two jars, each holding 50 ml of media, or six test tubes, each holding approximately 16.67- 17 ml of media. The jars or test tubes were labeled, covered properly with aluminum foil, and made airtight with the help of rubbers. After being sterilized in an autoclave, they were sifted to the culture room, where culture tubes were kept in a slanting position.

3.2.4 Sterilization

Sterilization is the process of entirely eliminating or destroying all microbial life (including vegetative and spore forms). It is accomplished through a variety of physical and chemical techniques. In plant tissue culture technology, it is the vital step that assures a high incidence of explant survival and a reduction in contaminants.

3.2.6 Surface sterilization of glass wares and metal instruments

All the necessary glassware, such as test tubes, glass jars, beakers, conical flasks, measuring cylinders, funnels, petri dishes, and watch glasses, were first soaked in detergent water for 24 hours and washed properly by scrubbing with a bottlebrush and rinsing with tap water. Other essential materials, such as petri dish, forceps, and blade holders, were sanitized properly, then wrapped in aluminum foil and placed together in an autoclave and ran at a temperature of 121 °c for at least 45 minutes by using saturated steam under 15 psi. For 45-60 minutes, the autoclaved material was placed in a hot air oven set at 150 °C to achieve

dry sterilization. The heat- sterilized materials were kept inside the laminar air flow and UV- sterilized for 45 minutes before culturing.

3.2.6 Sterilization of culture medium and surface sterilization

The prepared medium was autoclaved for 15 minutes at 121 ^{0}C and 15 psi pressure, where it was wet sterilized. Before the explant was inoculated, the medium was once more UV sterilized for 45 minutes. The sterilization of the culture room, incubation room, and laboratory was fumigated by using formalin, which is prepared by adding formaldehyde (40%) to water. The desk, table (on which media preparation was done) and UV Chamber was sterilized using 70% alcohol.

3.2.7 Sterilization and Pre- sowing treatment of the seed

Various sterilization processes and pre-showing treatments were performed using different chemicals to discover the optimal method for the initiation of tissue culture of *A. spicatum* using seeds as an explant.

Firstly, the seeds were washed under running water for 10-15 minutes tied in muslin cloth, then the seeds were subjected to Tween-20 for 5 minutes and rinsed off using running water for 30 minutes. After that, they were sterilized with 0.1% Bavistin for 15 minutes, followed by three times rinsing with distilled water.

The semi- sterilized seed emerged in the distilled water and underwent chilling treatment at 4^0c for 4 days. Then the further sterilization process was carried out under the laminar air flow. The surface sterilization of soaked seed was achieved by using 0.1 % mercuric chloride ($Hgcl_2$) for 3-5 minutes or by using 1% sodium hypochlorite (NaOCl) for 7-10 minutes. After that, seeds were subjected to 70% ethanol for 1 minute. Both treatments were followed by four times rinsing with sterile distilled water. This process was modified from the previous study.

Seeds were also cultured following all the sterilization processes except for the chilling treatment. The optimized sterilization protocol favorable for maximum seed germination was followed.

3.2.7 Inoculation and culture

After completing all the sterilization processes, three seeds were inoculated in various strengths of MS medium, with or without phytohormones, under a clean, aseptic laminar air flow cabinet. Three seeds were inoculated in each test tube and six test tubes were used

per treatment. The cultured test tubes were then moved to the culture room. The cultures were kept at 25±2°C and 3000-4000 lux of artificial light under 8-16h photoperiod with 60-70% relative humidity. The cultures were regularly monitored, and data was taken.

3.2.8 Seed germination

Seeds with a radicle emergence of ≥1.5 mm were considered germinated *in vitro* (figure 4, B). The seeds were routinely observed, and the seed germination percentage was calculated. The well-developed seedlings were then employed for direct organogenesis and callus induction.

3.2.9 Direct shoots/roots organogenesis

Various explants, such as leaves, nodes, and roots, were excised aseptically from 5–6-week-old seedlings and cultured separately on different phytohormone regimes to develop the propagation protocol of *A. spicatum*. The explants were cultured in the MS medium supplemented with varying combinations and concentrations of auxin and cytokinin, including BAP, NAA, 2,4-D, and IAA. The data were taken after 6 weeks of inoculation.

3.2.10 Callus induction

The explants (leaves, nodes, and roots) obtained from *in vitro*- grown seedlings were employed for the callus induction. The leaves were placed with the abaxial side down, and all three explants were inoculated individually in the test tube with MS medium that had been enriched with varying concentrations of different phytohormones. The phytohormones like NAA, BAP and 2,4-D were employed separately or in combination. 1-2 explants were cultured in each test tube, and six replicates of each experiment were conducted. The culture test tubes were wrapped by aluminum foil to maintain the darkness suitable for callus induction (figure 9, D). The nature of the callus and callus induction percentage were determined after the 8th week of inoculation.

3.3 Statistical analysis

All the experiment were conducted based on completely randomized design to ensure that results are not biased by any extraneous factors. Six replications including three explant (seeds) per test tube for seed germination stage and 1-2 explants for *in vitro* propagation and callus induction were maintained. Each experiment was repeated thrice and reported data are the means of three experiments. All data are presented as ± standard error to measure the accuracy of mean value. MS- Excel 2019 and SPSS software were used to

analyze the data. MS- Excel 2019 was used to analyze the seed germination data. Mean differences between different treatments were determined by analysis of variance (One - Way ANOVA), and significant differences among mean values were estimated using Duncan's new multiple range test. All the stages of experiments is represented in (figure 2).

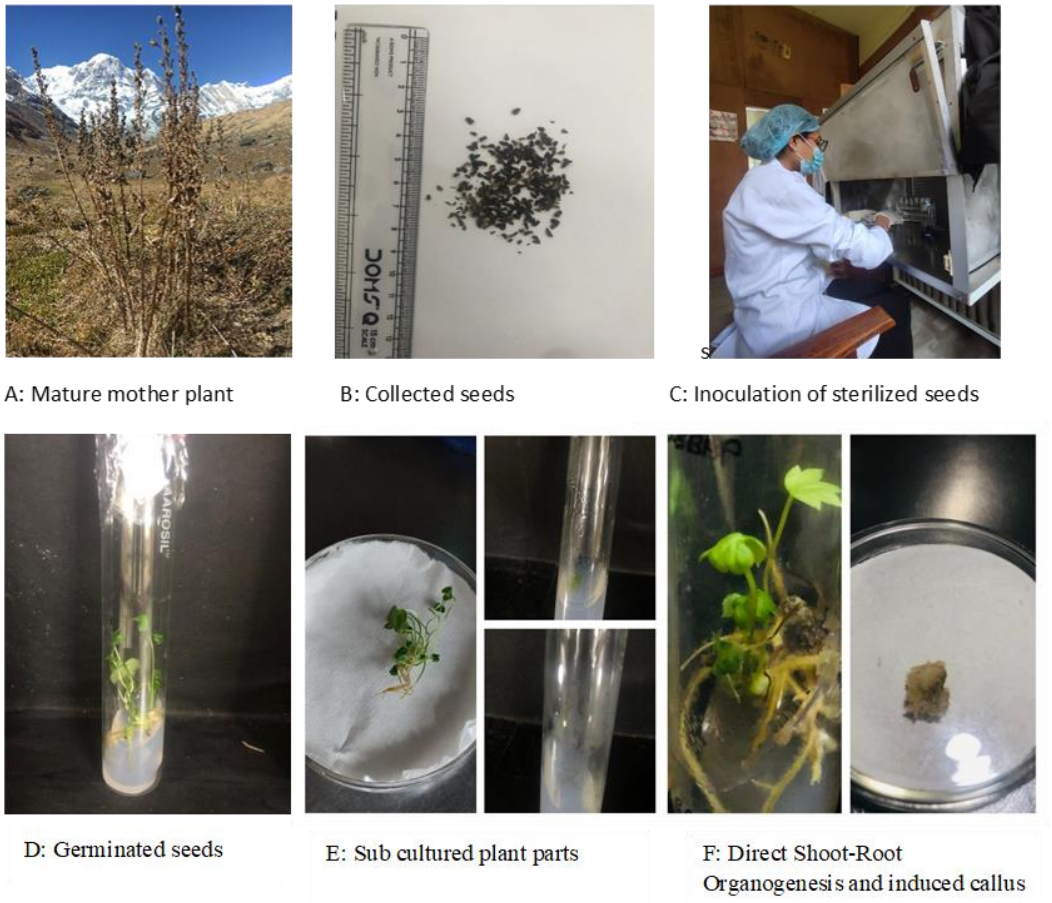

Figure 2: Represent the overall methods of *in vitro* propagation.

4. Results

4.1 Sterilization and Seed germination

Sterilization of seeds before inoculation in the medium is mandatory to ensure the success of plant tissue culture and to prevent contamination. Two different sterilizing chemicals of different concentrations on varying time exposure, with or without giving chilling treatment, were tested. Out of which seeds chilled at 4^0c for 4 days and treated with 1% NaOCl for 10 minutes showed the positive response on seed germination.

Table 2: Effect of pre-showing treatment and sterilant on seed germination

Pre-showing treatment	Sterilant	Germination response
Chilling treatment at 4^0c for 4 days	1% NaOCl for 7-10min	+
Chilling treatment at 4^0c for 4 days	0.1% $Hgcl_2$ for 3-5min	_
Without chilling treatment	1% NaOCl for 7-10min	_
Without chilling treatment	0.1% $Hgcl_2$ for 3-5 min	_

After the seeds were sterile, they were inoculated in different strengths of MS-Medium, which included MS-medium (MS), 1/2 MS-medium, and 1/4 MS- medium, free of phytohormones. Additionally, MS medium enriched with phytohormones GA_3 and KN was utilized. Altogether, 7 distinct media combinations were examined. Within an average of 14 days, the seeds sterilized with 1% NaOcl for 10 minutes inoculated in MS exhibited the highest germination rate of 77.78%, followed by 1/2MS at 66.67% and 1/4MS at 50%. On the other hand, the MS-Medium enriched with GA_3 and KN showed a relatively low germination rate of 22.22% and 5.55%, respectively. All the germinated seeds were developed into healthy plantlets. Figure 3 represents the overall data.

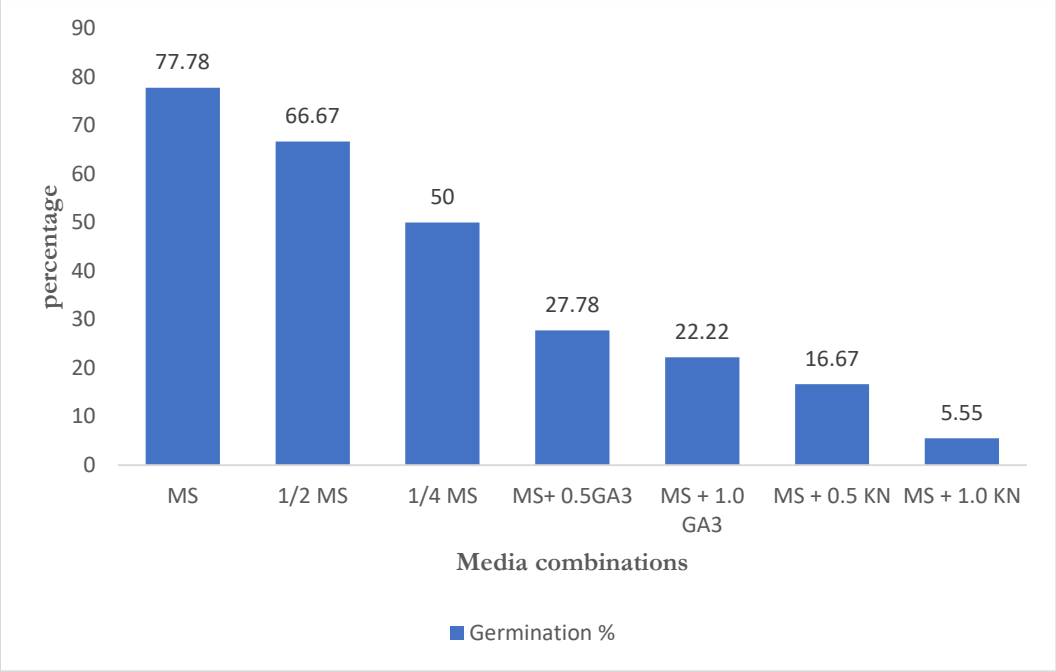

Figure 3: The percentage of seed germination on different media combinations.

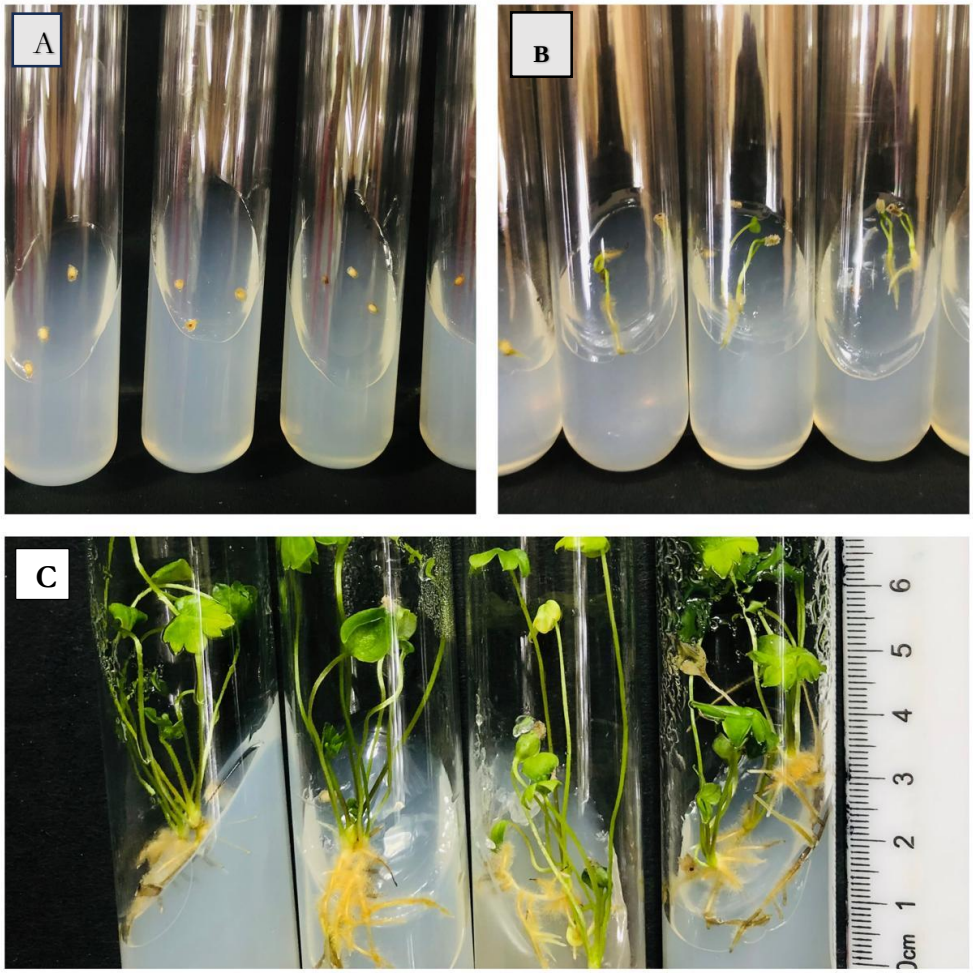

Figure 4: Different stage of seed germination. A: Seed just after inoculation; B: Seed after the two weeks; C: Seed after 5-6 weeks of inoculation in different strength of MS medium

4.2 Direct shoots/ roots organogenesis

For the shoots and roots development, several explants, such as leaves, nodes, and roots from *in vitro* -grown seedlings, were inoculated on MS medium containing different phytohormones. But the leaves and nodal explant did not show any response, so they were avoided. Only the root explants were utilized for the experiment. For these thirteen different media combinations were tested using hormones BAP and 2,4-D individually of different concentrations (0.5, 1.0, 1.5, 2.0, 2.5) or in combinations (BAP+2,4-D). The use of BAP showed the best result among all the combinations (figure 6). No explant induces shoots and roots on MS medium supplemented with other hormones. All other hormones except BAP showed zero shoot/ root initiation. From figure 5 it is observed that concentration of BAP differs significantly from each other in term of average shoot number and within the BAP combination 1.5 BAP mg/L shows the best result than others. On the other hand, there is no significant difference in term of average shoot length.

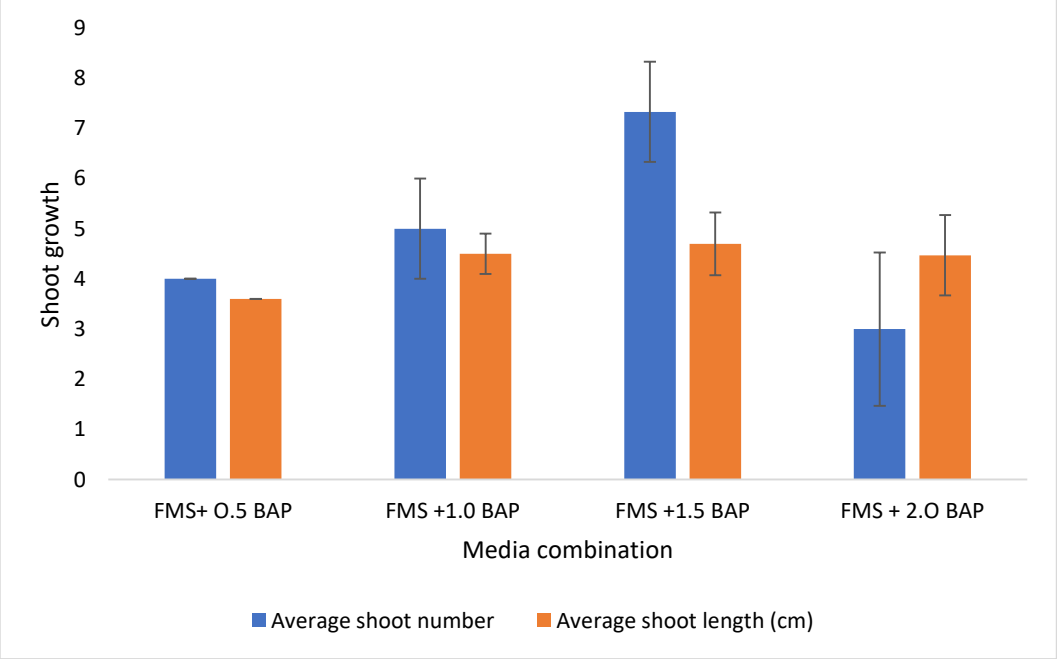

Figure 5: Effect of BAP on shoot induction of *Aconitum spicatum* after 6th weeks of culture.

Results are expressed as mean new shoot number and shoot length along with standard deviation. All are significantly different at p value less than 0.05 using Duncan Multiple range tests. Culture condition: Artificial light with a 16/8 light/dark cycle at 24±1^{0}C

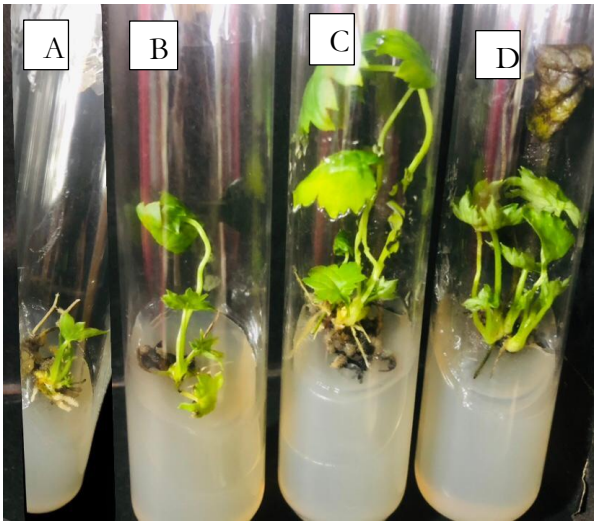

Figure 6: Shoot/ Root induction of *Aconitum spicatum* on different media combination. A: 0.5 mg/L BAP; B: 1.0mg/L BAP; C: 1.5mg/L BAP; D: 2.0mg/L BAP

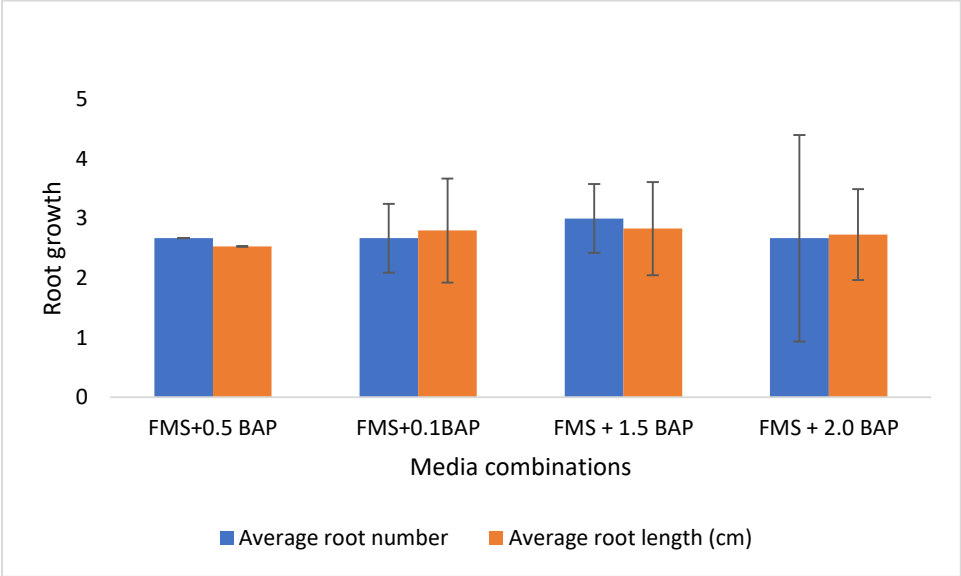

Figure 7: Effect of BAP on root length and root number of Aconitum spicatum.

Among the various concentrations of BAP in MS medium, the maximum number of new shoots 7.33 and shoot length 4.7cm, along with the root number 3 and root length 2.83cm, were observed in the 1.5 mg/L BAP followed by 1mg/LBAP with shoots number 5 and shoot length 4.5cm, having root number 2.67 and root length 2.8 cm but there are no such significant differences among the various concentration of BAP on root growth.

4.3 Callus induction

To induce callus, *in vitro* aseptic explants (leaves, shoots, and roots) were used. These were cultured on MS media that had been treated with 2,4-D and NAA either independently or

in combination, at various concentrations between 0 and 3.0 mg/L. The amount of callus developed varied depending on the hormone combination and concentration. No callus initiation was observed in the explant inoculated in only MS media devoid of cytokinin and auxin. Based on visual inspection i.e. progresses to cover the inoculated explant, the callus induction was determined and denoted by the symbols -, +, ++, and +++ for no callus, ~ 0.1×0.5 cm, 0.5 ×1.0 cm, and more than 1.0 ×1.5 cm, respectively. Additionally, the nature of callus was reported. For the callus induction, NAA was determined to be a better combination than 2,4-D out of all the combinations evaluated. Maximum white and friable callus induction was observed in the basal MS medium containing 1.0 mg/L NAA (figure 8, B) and 1.5 mg/L NAA (figure 8, C) while the least callus induction was observed in MS media containing 2,4-D (figure 8, F). Additionally, the leaf explant induces the maximum callus out of three explants. The callus induction was observed after 7-8 weeks of inoculation (figure 8). In the presence of light, no callus was reported; instead, browning of the explant was observed (figure 9, C). The overall data for callus induction is illustrated in Table 3.

Table 3: Effects of different phytohormones on callus induction

S. N	Media combination	Callus presence	Nature of callus
1	MS	-	No callus formation
2	MS+ 0.5 2,4-D	+	Whitish small callus formation
3	MS +1.0 2,4-D	+	Whitish small callus formation
4	MS +1.5 2,4-D	+	Whitish small callus formation
5	MS +2.0 2,4-D	+	Whitish small callus formation
6	MS +0.5 NAA	+	Whitish friable callus formation
7	MS +1.0 NAA	+++	Whitish friable callus formation
8	MS + 1.5NAA	+++	Whitish friable callus formation
9	MS + 2.0 NAA	++	Whitish friable callus formation
10	MS + 2.5 NAA	++	Whitish friable callus formation

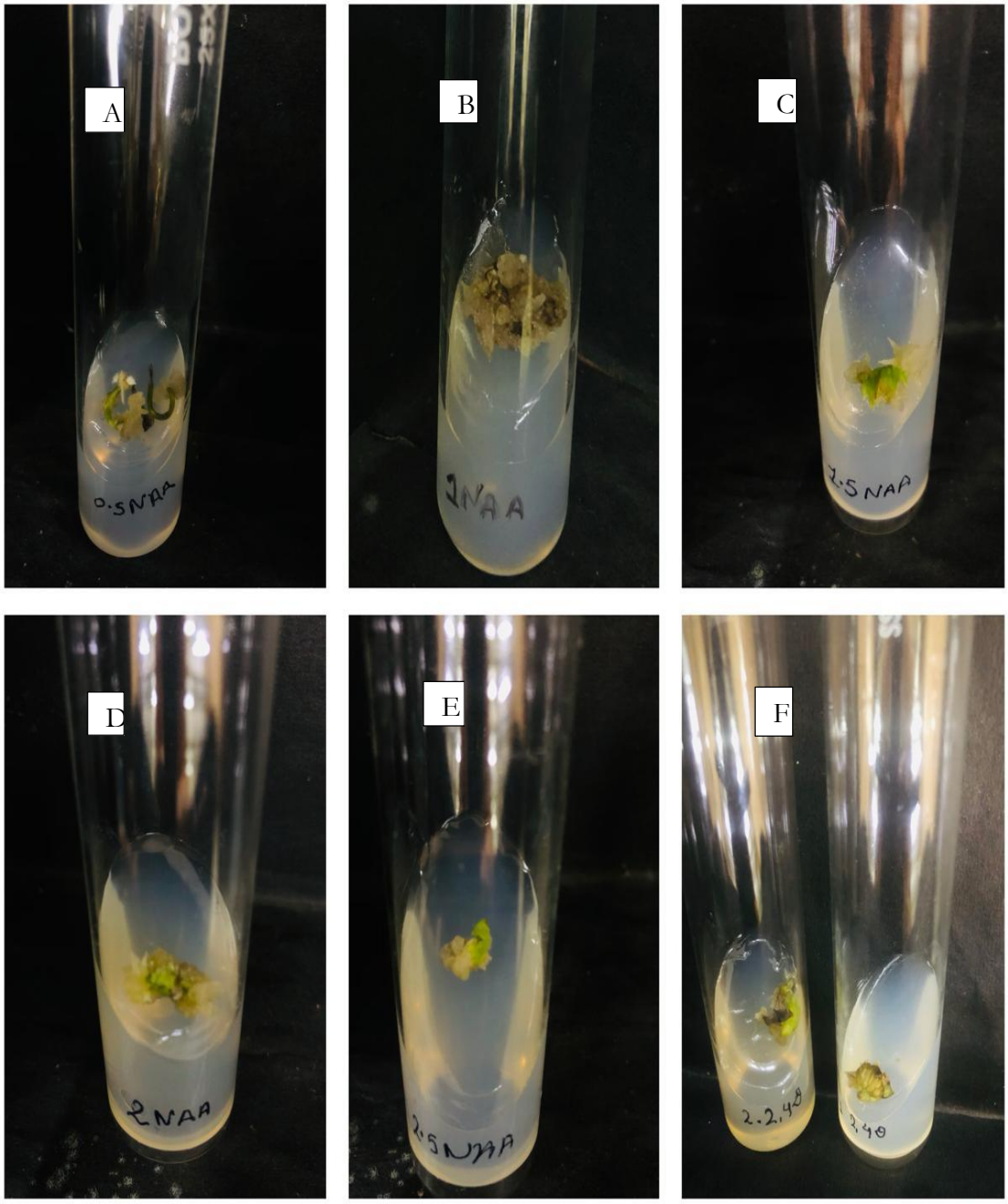

Figure 8: (A-F): Callus induction of *Aconitum spicatum* in MS media supplemented with different concentration of phytohormones

5. Discussion

5.1 Sterilization and Seed germination

Seed dormancy is defined as the inability of a viable seed to germinate under favorable conditions (Bewley, 1997; Finch-Savage and Leubner–Metzger, 2006). To optimize germination over time, the seed enters a dormant state (Bewley, 1997). This property is controlled by environmental factors such as light, temperature and time of seed dry storage as well as by genetic factors. Such dormancy can be overcome by germination promoting factors such as after-ripening, light, cold treatment (also called stratification) and by the application of chemicals like gibberellin (Bentsink & Koornneef, 2008).

The challenges posed by seed dormancy in *Aconitum* species is well known which is frequently brought on by the underdeveloped embryo, prevents seed germination and seedling establishment in some *Aconitum* species**,** e.g., *A. napellus, A. lycoctonum,* and *A. heterophyllum,* (Engell et al., 1995; Pandey et al., 2004; Vandelook et al., 2009; Hernaz et al., 2010). The species' specific habitat requirement amplifies these difficulties (Shrivastava et al., 2010; Rawat et al., 2013). Moreover, germination of seeds is significantly influenced by the morphological features of the seed coat as well as the concentration and duration of contact with sterilizing chemicals.

Similar problems were noted in the current investigation, where a major barrier to germination was seed dormancy. However, this dormancy was successfully broken by chilling them at 4^0c for 4 days before sterilizing them with 1% NaOCl for 10 minutes within 14 days of inoculation on MS medium. Evidence shows that chilling treatment influences a wide range of processes such as activation of particular gene regions, enhancement of embryo maturation, modification of seed hormone levels, stimulation of several enzyme activities, elimination of inhibitory substances as well as mimics the environment needed for germination to begin (Deng et al., 2021). And in a number of *Aconitum* species, including *A. heterophyllum and A. sinomontanum,* chilling treatment has been demonstrated to successfully break dormancy by promoting embryo development and subsequent germination (Dosmann, 2002; Beigh, 2005). These findings support our result, as seeds exposed to chilling treatment exhibited the highest germination rates. After being chilled, seed sterilized with 1% NaOCl had a considerably better germination rate than those sterilized with 0.1% $Hgcl_2$, which did not promote any germination at all. This could be due to the phytotoxic nature of $Hgcl_2$. The seed treated with NaOCl showed the most

positive response, being less toxic and by softening the seed coat, making it more permeable to water and nutrients required for seed germination, consistent with earlier research by Agogbua & Okoli (2022).

However, the use of phytohormones like GA_3 and KN in the MS medium did not yield positive outcomes in this study. Similar findings were reported by Vandelook et al. (2009) in *Aconitum lycoctonum,* where GA_3 has no effect even after chilling and embryo development and germination occur mainly at low temperatures less than 10^0c. Similar results were shown by Pandey et al. (2000) in *Aconitum heterophyllum,* where GA_3 inhibits the germination of seeds. This indicates that the seed may have morphological dormancy rather than physiological dormancy, so giving chilling treatment was enough, and the GA_3 hormone, best known for seed germination, did not provide the desired effect, as they are often utilized to end physiological dormancy, which is absent in this species. Furthermore, this might be due to the negative impact of 13- hydroxylation pathway on the plants' biological activities (Magome et al., 2013). Lower germination rate in 1/2 and 1/4 media indicate that nutrient availability is essential for successful germination and the growth of robust seedlings.

5.2 Direct shoot/root organogenesis

According to Giri et al. (2016), auxins and cytokinins are effective in inducing shoots and can be effectively utilized for plant regeneration. Generally, previous research indicates that NAA, at lower concentrations, along with higher concentrations of BAP, are vital for plant regeneration in several *Aconitum* species, such as *A. balfourii* and *A. violaceum* (Pandey et al., 2004; Rawat et al., 2013). However, in this case, BAP alone shows the substantial result on plant regeneration. This indicates that an exogenous phytohormone is required for shoot multiplication in *Aconitum spicatum*. Ashraf et al. (2014) provided evidence that BAP was more remarkable among various cytokinins that significantly affect explant development, including shoot induction, shoot multiplication and cell division stimulation to produce the essential organs. Moreover, BAP is a synthetic plant hormone which is difficult for plant enzymes to breakdown, it implies that BAP stays in the plant for an extended time without being easily broken down by the plant's natural enzymes so it can promote the initiation and proliferation of shoots effectively (Kurnianingsih et al., 2009). This result is in agreement with the earlier study of genus the *Aconitum napellus,* where the maximum shoot multiplication was achieved on MS medium containing 2 mg/l of BAP (Watad et al., 1994). Similar results were also observed in the genus *A. carmichaeli,* and *A.*

chasmanthium, where MS medium containing BAP only showed the multiple shoot induction and proliferation (Hatano et al., 1988; Rafiq et al., 2021). But like the previous work by Singh et al. (2019) in *A. ferox* found further increases in BAP concentration decreased the shoot proliferation.

For *in vitro* regeneration of plants, root explant cultured on MS medium supplemented with cytokinin showed the positive response on shoot induction, and the rooting of the plant was witnessed in the same media as previously demonstrated by the work of Singh et al. (2019) in the same genus, A. *ferox*. This phenomenon may be attributed to the presence of endogenous auxins, which can facilitate rooting (Deswiniyanti & Dwipayani, 2020).

5.3 Callus induction

This study illustrates that auxin has a major impact on the formation of callus. The absence of callus formation in the control group (MS medium devoid of regulators) highlights the importance of phytohormones to generate the callus. This is consistent with the earlier research in the same genus like *Aconitum heterophyllum, Aconitum vilmorinianum,* and *Aconitum ferox,* which emphasizes the function of phytohormones in the development of callus (Jabeen et al., 2006; Mou et al., 2021; Singh et al., 2019). Among the hormone combinations that were tested, NAA was notably more successful than 2,4-D in inducing callus. A significant amount of white and friable callus development was observed at 1.0 mg/L and 1.5 mg /L NAA. The reason could be that the NAA stimulate the activity of plant cells, particularly in meristematic (actively dividing) regions promoting the rapid division of undifferentiated cells, leading to the formation of a callus (Adhikari et al., 2013). Despite being applied at similar concentrations, 2,4-D notably shows less callus induction. This is in contrast to many studies where 2,4-D was responsible for production of higher amount of callus on same genus *Aconitum ferox* Wall (Singh et al., 2019). This might be due to imbalance in endogenous phytohormone. In addition, this greatly depend on the species being cultured (plant's genetic response) where 2,4-D may trigger a response that is more aligned with organogenesis (formation of organs like roots and shoots) than callus proliferation (Dar et al., 2021).

The outcomes underscore NAA's superiority as a growth regulator for this particular application, corroborated by earlier research that found NAA to be effective in inducing callus in a variety of species. The result is supported by the observation in callus induction of *Aconitum heterophyllum* (Jabeen et al., 2006). Similarly, Nurwahyuni et al. (2020) reported that the heaviest callus was produced on MS media containing NAA.

In addition, compared to shoots and roots, leaf explant proved to be the most responsive tissue type for callus induction based on our observation. This might be due to the physiological characteristics of leaf tissues, which might have a higher capacity for regeneration, meristematic activities and dedifferentiation in response to certain hormonal conditions. The observation of callus formation after 7-8 weeks of inoculation suggests that a longer incubation period would be necessary for ideal callus growth. A similar result was recorded in *Aconitum chasmanthum* by Rafiq et al. (2021). The white and friable callus was observed in the dark; this might be due to the fact that natural auxin accelerated under lower light intensity or darkness (Chory et al., 1994). Lack of callus induction and browning of explant they might be caused by oxidation of polyphenols in the explant, which is accelerated by light (Soni et al., 2014; Taranto et al., 2017).

6. Conclusions and Recommendations

6.1 Conclusions

This study indicates that pre-sowing treatment and seed sterilization are significant factors in enhancing the germination rate of *Aconitum spicatum*. Seeds sterilized with NaOCl showed a promising result in comparison to $HgCl_2$. On the other hand, negligible germination response was obtained in the absence of chilling treatment. Furthermore, for shoot and root organogenesis, BAP was found to be the most effective hormone among the various media combinations, yielding optimal growth rates for both shoots and roots. MS media fortified with 1.5 mg/ l BAP was most effective for shoot and root development. Regarding callus induction, NAA was superior to 2,4-D at producing a comparatively larger amount of friable callus from leaf explant, particularly at concentrations of 1.0 mg/l and 1.5 mg/ l. Remarkably, in the absence of phytohormones, callus development was not observed, which highlights the necessity of plant growth regulators for callus induction.

6.2 Recommendation

Besides being highly therapeutic, *Aconitum spicatum* has been hardly explored. Very limited research has been conducted thus far. Therefore, there is immense potential for further research. Some of the recommendations are listed below:

- Additional research can be done to standardize the propagation procedures that might be employed for large- scale multiplication.
- The study recommends the isolation and identification of biologically active secondary metabolites from *in vitro*- derived callus.

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Appendix

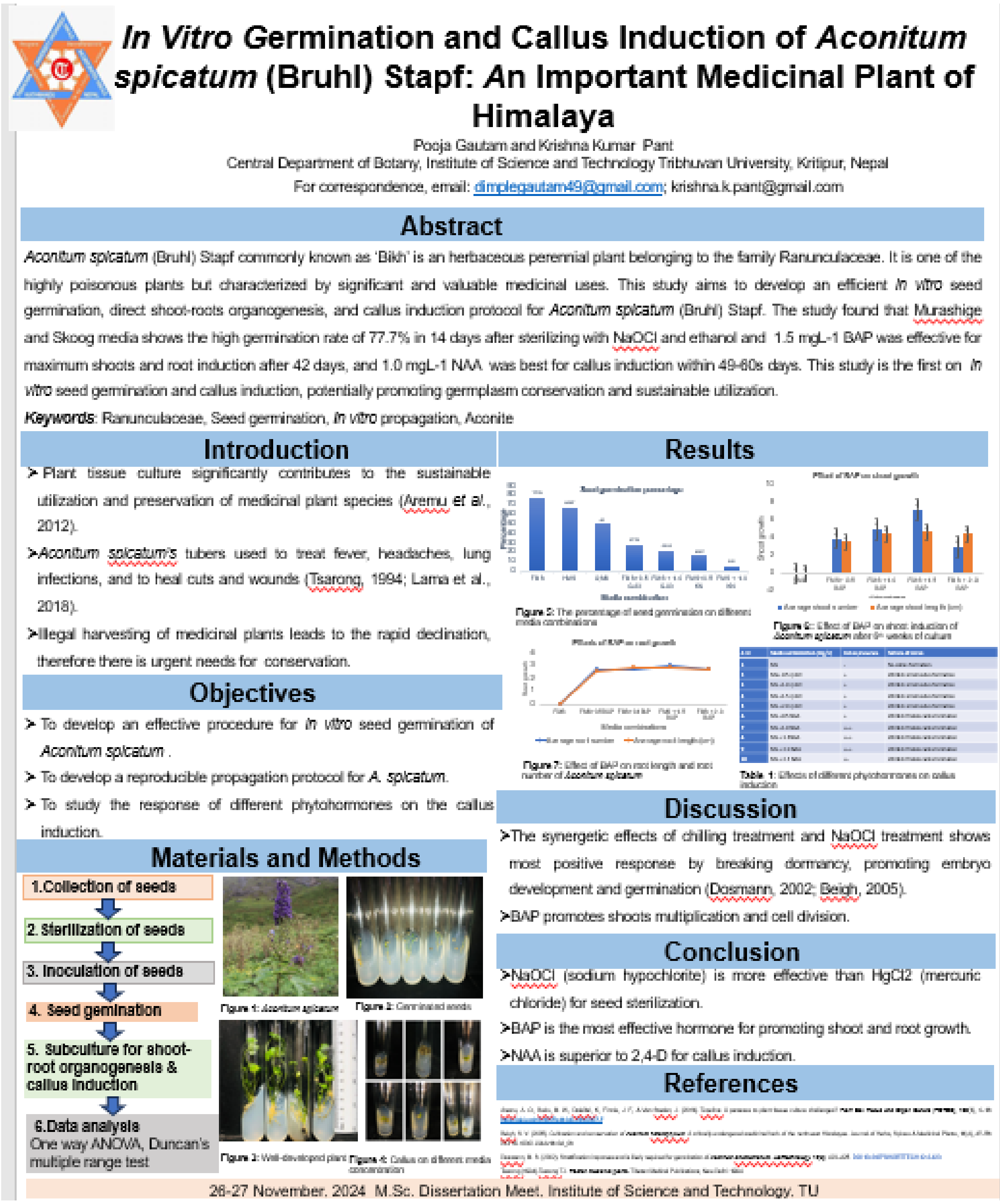
Appendix 1**:** Effect of BAP on shoot induction of *Aconitum spicatum* after 6th weeks of culture. Results are expressed as mean new shoot number and shoot length.

Media combinations	**Shoot number; Mean ± S. E**	**Shoot length; Mean± S. E**
FMS	0.0±0.0	0.0±0.0
FMS+0.5 BAP	4.00±0.57	3.60±0.23
FMS+ 1.0 BAP	5.00±0.57	4.50±0.36
FMS +1.5 BAP	7.33±0.88	4.70±0.46
FMS +2.0 BAP	3.00±0.57	4.47±0.37

Appendix 2: Effect of BAP on root length and root number of *Aconitum spicatum*. Results are expressed as mean new root number and root length

Media combinations	**Root number; Mean ± S. E**	**Root length; Mean± S. E**
FMS	0.0±0.0	0.0±0.0
FMS+0.5 BAP	2.66±0.33	2.56±0.33
FMS+ 1.0 BAP	2.66±0.33	2.80±0.33
FMS +1.5 BAP	4.0± 1.0	2.83±1.0
FMS +2.0 BAP	2.66±0.33	2.73±0.33

Appendix 3: Poster presented at M.SC. Dissertation Meet, 2024



Appendix 4: Photo plates

Figure 9: Photograph taken during the Laboratory works; A: Media preparation; B: Non-germinated seeds; C: Browning of the sub-cultured plant parts; D: Test tubes wrapped with aluminum foil to avoid browning; E: Contamination; F: Regular inspection & data

नेपाल सरकार
वन तथा वातावरण मन्त्रालय
राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण विभाग
(..... शाखा)

फोन नं. : ४२२०८५०
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फ्याक्स नं. ४२२७६७५

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पत्र संख्या :- ३२
चलानी नं :- ०७८/७९ इको २८०

मितिः २०७८/५/२०

विषयः अध्ययन अनुमति सम्बन्धमा ।

श्री अन्नपूर्ण संरक्षण क्षेत्र आयोजना, हरियोखर्क, पोखरा ।

प्रस्तुत विषयमा तहाँ संरक्षण क्षेत्रमा निम्नानुसारको अध्ययन अनुसन्धान अनुमति प्रदान गरिएको व्यहोरा निर्देशनानुसार अनुरोध छ ।

अनुसन्धानकर्ताको नाम	**Bharat Babu Shrestha**		
ठेगाना	शहिद लखन थापा गाउँपालिका-०९, गोर्खा	इमेलः Shresthabb@gmail.com	फोन नं. ९८४१२४१४८४
समद्ध संस्था	वनस्पतिशास्त्र केन्द्रीय विभाग, त्रिभुवन विश्वविद्यालय, किर्तिपुर, काठमाण्डौं ।		
अनुसन्धानको प्रकृती	व्यक्तिगत		
पद	सह-प्रध्यापक		
अनुसन्धानको तह	राष्ट्रिय स्तर		
अनुसन्धानको शिर्षक	**Impact of climate change on germination, growth, pollination and distribution of Himalayan medicinal herbs *Aconitum spicatum* and *A. naviculare***		
अनुसन्धान विधि	Transplant Experiment in field	नमुना संकलन	नमुना परिक्षण कहाँ गर्ने
		गर्ने	नेपालमा
अनुसन्धानको अविध	२० भाद्र २०७८ देखि १९ भाद्र २०८० (दुई बर्ष)		

शर्त :

१.अनुसन्धानकर्ताले राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण ऐन, २०२९ र नियमावली, २०३० तथा मातहतका सबै नियमावलीहरुको पूर्ण पालना गर्नु पर्नेछ ।

२. अध्ययन गर्दा सम्बन्धित संरक्षित क्षेत्र कार्यालयसंग समन्वय गरी कार्यालयमा कार्यरत कर्मचारीको रोहबरमा गर्नु पर्ने पर्नेछ ।

३. अनुसन्धानकर्ताले आफ्नो अनुसन्धानको प्रस्ताव सम्बन्धित संरक्षित क्षेत्र कार्यालयमा समेत पेश गर्नु पर्नेछ ।

४.अनुसन्धानकर्ताले अनुसन्धान समाप्त भएपछि प्राप्त तथ्याङ्क, एक प्रति कागजी प्रतिवेदन र एक प्रति इलोक्ट्रोनिक प्रतिवेदन यस विभाग र सम्बन्धित संरक्षित क्षेत्र कार्यालयमा बुझाउनु पर्नेछ ।

५. अनुसन्धानकर्ताले नतिजाहरु प्रकाशित गर्दा अनुसन्धानमा संलग्न यस विभाग र अन्तरगतका कर्मचारीको योगदानको आधारमा सहलेखकको रुपमा समावेश गराउनु पर्नेछ ।

६. सङ्कलित नमुना विदेश लैजान पाईने छैन ।

७. तोकिएका शर्तहरुको पालना नगरेमा विभागले कुनैपनि समयमा अनुमतिपत्र रद्द गर्न सक्नेछ ।

हेम राज आचार्य
सहायक इकोलोजिष्ट

बोधार्थः

श्री **Bharat Babu Shrestha:** सम्बन्धित संरक्षित क्षेत्र कार्यालयसंग समन्वय गरी अध्ययन अनुसन्धान गर्नु हुन र अनुसन्धान समाप्त भएपछि एक प्रति कागजी तथा विद्युतीय प्रतिवेदन सम्बन्धित कार्यालय र विभागमा बुझाउनु हुन अनुरोध छ ।

श्री अन्नपूर्ण संरक्षण क्षेत्र सम्पर्क अधिकारीको कार्यालयः जानकारीको लागि अनुरोध छ ।

वनस्पतिशास्त्र केन्द्रीय विभाग, त्रि.विः जानकारीको लागि अनुरोध छ ।

NATIONAL TRUST FOR NATURE CONSERVATION
ANNAPURNA CONSERVATION AREA PROJECT
Headquarters, Pokhara

Headquarters, Pokhara

Ref: 80 /078/079 Date: 2078-05-30

Dr. Bharat Babu Shrestha
Associate Professor
Central Department of Botany
TU, Kathmandu

Re: Permission to conduct research in Annapurna Conservation Area

We received your request letter regarding permission to conduct research on **"Impact of climate change on germination, growth, pollination, and distribution of Himalayan medicinal herbs *Aconitum spicatum and A. naviculare*"**. You have been given permission to carry out your field research in ACA with the following terms and conditions.

1. The research must be for scientific and academic purpose with the aim of making contribution in conservation and development of conservation area.
2. This permission will be **valid up to August 31, 2023** (2080 Bhadra 14).
3. You have to follow the ACAP Minimum Impact Code and the Conservation Area Management Regulation 2053.
4. You have to follow the terms and conditions mentioned in the research permit provided by Department of National Park and wildlife Conservation.
5. You are **allowed to collect sample only** from the study area.
6. You will have access to the NTNC-ACAP Resource Library in Pokhara.
7. Upon the completion of the research, **you must submit a hard copy and digital copy of your report** to the NTNC-ACAP Headquarters, Pokhara.
8. You have to **coordinate with ACAP Unit Conservation Offices** while performing your field research work.
9. You and your research **team have to strictly follow all rules, guidelines and social norms to keep in safety from COVID-19 while doing your fields work.**
10. Any dispute arose during the execution periods will be solved by mutual understanding.
11. Any unsolved disputes will be handled as per the existing law of Nepal government.

Thank you and wish you all the best.

Raj Kumar Gurung
Project Chief

CC:
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