Seed germination responses of a mountain medicinal herb *Aconitum spicatum* to different environmental conditions

A Dissertation Submitted for the Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science in Botany

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DECLARATION

I, Bishnu Sharma Gaire, hereby declare that this dissertation entitled "**Seed germination responses of a mountain medicinal herb *Aconitum spicatum* to different environmental conditions**" is entirely my original work and all the sources and information are duly acknowledged. This is submitted to the office of the Dean, Institute of Science and Technology, Tribhuvan University. I have not submitted it or any of its part to any other universities or institutions for any other academic award.

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LETTER OF RECOMMENDATION

This is to certify Ms. Bishnu Sharma Gaire has completed this dissertation work entitled "**Seed germination responses of a mountain medicinal herb *Aconitum spicatum* to different environmental conditions**" under our supervision. The result of this dissertation has not been presented or submitted anywhere else for the academic degree. It is hereby recommended for the acceptance of this dissertation as a partial fulfillment of the Master's Degree in Botany at the Institute of Science and Technology, Tribhuvan University, Kathmandu, Nepal.

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LETTER OF APPROVAL

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ABBREVIATION AND ACRONYMS

°C	Degree Centigrade
ACA	Annapurna Conservation Area
ANOVA	Analysis of Variance
CAMP	Conservation Assessment Management Plan
DoF	Department of Forest
FGP	Final Germination Percentage
GP	Germination Percentage
IUCN	International Union for Conservation of Nature and Natural Resources
LED	Light Emitting Diode
MAPs	Medicinal and Aromatic Plants
m asl	meter above sea level
MGT	Mean Germnation Time
MPa	Mega Pascal
MPD	Morpho Physiological Dormancy
NTNC	National Trust for Nature Conservation
PEG 6000	Poly Ethylene Glycol 6000
ROS	Reactive Oxygen Species
SD	Standard Deviation
SE	Standard Error
SPSS	Statistical Package for Social Sciences
TCM	Traditional Chinese Medicine
TI	Timson's Index

ABSTRACT

Germination studies of medicinally important mountain plants under different environmental conditions is potentially important to understand the impacts of climate and other environmental changes. Seeds from different populations of a species along the elevation gradient may respond in different directions and intensity to the changing environmental conditions. In this study, seed germination patterns of a mountain medicinal herb *Aconitum spicatum* were analyzed to understand variation under environmental gradients. Seeds of *Aconitum spicatum* collected from three different elevations (sub alpine to alpine) were germinated in growth chamber under different temperatures (low: 25/15°C; high: 30/20°C), light environment (12h photoperiod and complete dark, white and far-red light) and water potentials (−0.1, −0.25, −0.5, −0.75, −1MPa). Freshly collected as well as the seeds stored for one year were used in the germination experiments. Seed mass, Germination percentage (GP), Mean germination time (MGT) and Timson's index (TI) were calculated. The result showed that seeds from low elevation comparatively had higher seed mass and germination than high and mid elevation. At low temperature, seeds had higher GP and TI than in high temperature while MGT was longer in high temperature. With increasing level of water stress, germination decreased. Germination was highest in white fluorescent light than far-red light. Seed germination percentage and the Timson's index increased after long duration of cold storage (73 weeks). The results of this study advance our knowledge and comprehension of the potential effects of climate change on alpine plants. This study found that there wasn't always a consistent pattern associated to elevation and that germination responses varied greatly among elevational provenances. Another important conclusion of the current study is that it may not be enough to characterize a species' germination response by examining only one population since, as it showed, local environmental conditions may have an effect on seeds.

Key words: Elevation gradient, Timson's Index, Climate change, Environmental stress, Alpine plants.

CHAPTER 1. INTRODUCTION

1.1 Background

Germination is considered one of the important characteristic features of vegetation regeneration (Baskin and Baskin, 2014). In order to determine the sexual reproductive efficiency of any species and for the study of field distribution it is very crucial to analyze germination capacity (Ge *et al.*, 2020). Seed germination is one of the useful parameters comprising complex phenomenon of biophysiological and chemical changes that leads to the activation of the embryo (Parihar *et al*., 2014). The germination and seedling development phase are the initiation of plant life cycle (Wolny *et al*., 2018). It is essential to comprehend the molecular characteristics of plant seed germination and dormancy in order to investigate species distribution and their persistence in the environment (Tuan *et al*., 2019). Germination in natural habitats is largely unpredictable as it changes over time and different environmental conditions like temperature, light, humidity, water and seed maturation time, seed traits determines germination potential (Luo *et al*., 2022). Laboratory experiments also showed that the seed germination is influenced by water stress (Marcińska *et al*., 2012), light and temperature (Sharma and Sen, 1975). Germination is not only influenced by the immediate environmental conditions but also closely related to the characteristics of mother plant (Zhao *et al*., 2021) and habitat of the species (Okada, 2004). Plant species of different habitat have different stress tolerant capacity on germination behavior.

The elevation of the habitat is closely associated with seed germination variation among the species and population (Holm, 1994; Vera, 1997). Elevation gradients in mountain show continuous change in environmental variables which result in changes in species composition. Therefore, elevation gradients are important to find out the cause behind patterns in species diversity and to understand the threat of change in environment to seed germination of the species (Paudel *et al*., 2019; Ge *et al*., 2020). Germination studies of medicinally important mountain plants under different environmental conditions are potentially important to understand the impacts of climate and other environmental changes (Gairola *et al*., 2010). The distribution of plants in mountainous areas is primarily influenced by the elevational gradient (Körner, 2021). Although most plant species have relatively limited elevational distributions or ranges (Gurvich *et al*., 2014; Chiapella and Demaio, 2015), elevation clearly defines the boundaries of vegetation belts, other plant species can have extremely large elevational ranges and even exist in various ecological zones.

Climate change will have an especially negative effect on populations along environmental gradients, endangering a variety of habitats. To effectively estimate the effects of climate change on species, it is essential to comprehend the pattern of variation in sensitivity within populations and between developmental stages (Dawson *et al*., 2011). To highlight the potential role that seed features may play in protecting species against climate change, it is crucial to combine evidence addressing among-population variation in seed attributes.

Small seeds had greater germinability than large seeds (Ge *et al*., 2020). Similarly, seed dormancy status is greater at high elevations when temperature and rainfall are lower (Fernández-Pascual *et al*., 2013), seed mass increases as temperature rises (Liu *et al*., 2013). Germination rates (germinability), germination timing, reactions to light and dormancy levels are the seed properties that differ between plant populations of the same species (Pérez-García**,** 2009). Finding population variation in seed properties may help with conservation and management measures that function as a buffer against the loss of diversity if species with highly varied responses across their geographic range are more adaptable to change and diversify more readily (Cochrane *et al*., 2014).

Seed germination is considered a crucial test for plant growing and tolerating the global change in alpine habitat which determines whether they can persist or go into extinction (Fernández-Pascual *et al*., 2020). Seeds of alpine species are considered to have physiological dormancy caused by the seed's internal balance of phytohormones and it requires a long time exposure to dormancy-alleviating treatments (Schwienbacher *et al*., 2011; Sommerville *et al*., 2013; Baskin and Baskin, 2014). Seed mass is considered an important characteristic of a plant species with regard to seedling recruitment (Coomes and Grubb, 2003; Turnbull *et al*., 2004). Seed mass vary within a species or even within an individual plant (Harper *et al*., 1970; Schaal, 1980). Variation in seed mass within a species may affect seed germination (Schaal, 1980; Weis, 1982) and germination rate (Weis, 1982; Zhang and Maun, 1990). Germination and establishment success increases with increasing seed mass (Leishman *et al*., 1995). Seeds with large size show better germination and establishment success than small seeds (Salisbury, 1942; Weis, 1982; Leishman *et al*., 1995; Hodkinson *et al*., 1998) while small seeds germinate quickly (Howell, 1981).

The seeds of Ranunculaceae, one of the basal groups in eudicots (Martin, 1946; Engell, 1995) are of the primitive family (Cronquist, 1988). The seeds have undeveloped but fully differentiated embryos which need to grow for germination (Baskin and Baskin, 1998). Physiological mechanism delays embryo growth and germination in Ranunculaceae family causing Morpho-Physiological Dormancy (MPD). To break this dormancy chemical stimulants such as nitrate, thiourea and gibberellic acid are considered effective (Vandelook *et al*., 2009). According to Herranz *et al*., (2010), considered that among *Aconitum* species, *A. heterophyllum* and *A. sinomontanum* had intermediate complex Morpho-Physiological Dormancy (MPD). Species of *Aconitum* occurs mainly in grasslands (Okada, 2004). They bloom from late summer to early autumn and disperse ripe seeds in late autumn. This different timing of seed dispersal results in different germination behavior (Okada, 2004). Seed germination response changes over the season and the range of its response is determined by the changing level of seed dormancy (Gresta *et al*., 2010).

1.2 Justification of study

Aconitum spicatum (Brühl) Stapf, is one of the highly traded alpine plants (Olsen and Larsen, 2003) with high medicinal (Srivastava *et al*., 2010) and a trade volume of >300 kg/year of rhizome (DoF, 2017). It is listed in the vulnerable category of the Conservation Assessment Management Plan (CAMP) and threatened category of International Union for Conservation of Nature and Natural Resources -IUCN Red List (Gurung and Pyakurel, 2017). Being important from the medicinal perspective, rhizomes of plant is used for alkaloids extraction and has anti-pyretic, analgesic and anti-bacterial properties (Srivastava *et al*., 2010). *A. spicatum*, being poisonous at one side but also a valuable medicine due to its cytotoxic, anti-microbial, anti-arrhythmic, anti-epileptiform activities (Nyirimigabo *et al*., 2014).

Predicting the current and future suitable habitat for important Medicinal and Aromatic Plants (MAPs) is a prerequisite in implementing cultivation planning that could integrate mixed plantation systems. This is because one of the key strategies for the conservation of highly traded species is to identify suitable habitat for commercial cultivation for dealing with overharvesting issues due to high trade demand (Rana *et* *al*., 2020). Mountain species frequently inhabit distinct elevational zones. There might not be enough habitat available to support additional expansion, though. They are unable to migrate up into suitable habitats (Rana *et al*., 2020), thus they must either adapt to their surroundings or colonize new areas to survive, or they will go extinct.

During the growing season, environmental factors can impact the chemical composition of seed, mineral and phytohormone resources, which in turn affect germination (Baskin and Baskin, 1998). Therefore, studying germination strategies is very important for understanding plant species survival, their distribution and establishment. Understanding the species' germination potential and dormancy duration is important.

In addition to this, the major implication of this study is to investigate whether the seed collected from three different elevations show variation in germination rate or not. The study of their germination pattern is very useful in determining how well they respond to the changing environment. The result from their germination response will be baseline for further implication of other different environmental variables to the seed germination behavior. Furthermore, information on germination enlightens about the medicinal plant and their stress tolerance capacity in a spatial range. This further helps to know their survival potential and adaptability towards changing environmental conditions.

1.3 Hypothesis

There is variation in germination patterns among populations along the elevation gradient.

1.4 Objectives

A general objective of the study is to examine germination response of a mountain medicinal herb *Aconitum spicatum* (Brühl) Stapf.

The specific objectives are:

- To understand germination patterns of *A. spicatum* under different environmental conditions.
- To analyze changes in germination pattern along elevation gradient.
- To compare germination patterns in vitro.

1.5 Limitation

Seed germination could not be carried out simultaneously together under high and low temperature regime due to the availability of single growth chamber.

CHAPTER 2. LITERATURE REVIEW

2.1 *Aconitum* species of Nepal Himalaya

Distribution and habitat range

The country possesses 2,500 medicinal and aromatic plants because of its unique topography and bioclimates (Pyakurel *et al*., 2019; Kunwar *et al*., 2021). There are 2,331 MAPs species known to exist in Nepal, of which 300 species are being traded (Pyakurel *et al*., 2019). The genus, *Aconitum* comprises about 400 species worldwide (Ali *et al*., 2021) where, in Nepal, recently 28 species are listed in Plants of Nepal (Shrestha *et al*., 2022). *Aconitum spicatum* is one of the highly valuable and traded medicinal plants listed in the vulnerable category of the Conservation Assessment Management Plan (CAMP) and threatened category of International Union for Conservation of Nature and Natural Resources -IUCN Red List (Gurung and Pyakurel, 2017). According to Shrestha *et al*., (2022), *Aconitum spicatum* are distributed along alpine and subalpine, east to west in Nepal at the elevation range between 1800-4800m. *Aconitum* species (Fam.: Ranunculaceae) are the herbaceous perennial plants growing in area of moisture retention well-draining soils of mountain meadows (Shyaula, 2012). Okada, (2004) reported that, *Aconitum* species mainly occurs in the grassland, margin and understory of forest in the temperate zone and the flowers bloom from late summer to early autumn and disperse ripe seeds in late autumn.

Ethnobotanical uses

Since ancient times, *Aconitum* species have been employed as cardiotonic, analgesics, anti-inflammatory agents, and more in Ayurveda, Chinese, Tibetan, and homeopathic systems of medicine due to their medicinal potency and pharmacological activity. In alpine region of Indian Himalaya, *Aconitum balfourii* is also considered a threatened species due to its over and illegal exploitation (Sharma *et al*., 2012). Due to the high medicinal value, tubers of *Aconitum* are used as an antipyretic and analgesic in the far western Nepal among them *Aconitum ferox*, *Aconitum palmatum* and *Aconitum heterophyllum* are commonly used species in ayurvedic process (Shyaula, 2012). In Manang district, *Aconitum naviculare* is used for high blood pressure, cold, fever and jaundice (Shyaula, 2012). The root powder of *Aconitum bisma* is taken as an antidote in food poisoning and snake bite in Makalu-Barun and Kanchenjunga (Shyaula, 2012).

Aconitum spicatum is used for fever and head ache, cuts and wounds and musculo-skeletal problems in Rasuwa district (Dosmann, 2002). Not only the medicinal properties, *Aconitum spicatum* are greatly valued in the horticultural trade for their dark blue to purple floral displays (Dosmann, 2002).

2.2 Germination pattern of *Aconitum* species

Evolution and seed germination patterns have profound effects on demographics and are strongly tied to species dispersion (Rejmánek and Richardson, 1996). Comparative studies of the germination patterns of the same species in various habitats and elevation ranges are likely to help us comprehend their wide distribution. The variation in life history pattern and differences in seed dispersal timing may result in variation in seed germination properties (Okada, 2004). The seeds of *Aconitum* species show irregular germination in natural condition and have low germination potential and it is able to stay dormant for prolonged time (Srivastava *et al.*, 2010). Favorable environmental conditions promote high seed production and stressful condition causes low seed production (Sehgal *et al.*, 2018). Seasonal effects on seed development may have an impact on seed germination. Precipitation, moisture and temperature variations have a significant impact on species' seed biology (Walck *et al.*, 2011).

In *Aconitum lycoctonum*, germination occurred at low temperature below 10°C. Germination at 5°C after 7 weeks of incubation was 98% and at 10°C after 22 weeks was 80% (Vandelook *et al.*, 2009). In *A. sinomontanum*, Dosman (2002), reported stratification improves germination. Cold pre-chilling and gibberellins breaks seed dormancy and germination was highest (90.8%) achieved after 84 days of stratification in *A. sinomontanum*. The maximum germination was at 5°C (80%) followed by 0°C while germination was constant at 10°C in *A. grossedentatum* (Okada, 2004). According to Beigh *et al.*, (2002), the seeds of *A. heterophyllum* from natural populations in the northwest Himalaya germinate after dormancy during the winter. The most effective treatment for inducing seed germination in *A. heterophyllum* is pre-chilling for 30 or 90 days (Beigh *et al.*, 2002). Treatments of the seeds with gibberellic acid and sun drying of seeds are considered for better germination of seeds in *A. balfourii* (Sharma *et al.*, 2012). Some chemical treatments such as thiourea and ammonium nitrate also helped to improve the germination of seeds of *A. balfourii* and *A. heterophyllum* (Pandey *et al.*, 2000).

According to Herrranz *et al*., (2010), in *A. napellus,* seeds with deep complex Morpho-Physiological Dormancy (MPD) required cold stratification for dormancy loss and seeds with non-deep complex MPD required warm and cold stratification before they germinate. In *A. heterophyllum,* ethanol (germination stimulator) treated seeds showed faster rate of germination with low mean time of germination (Rana and Shreenivasulu, 2013). *A. napellus* seeds did not germinate even in 30 days by being placed on a moist substrate at different light, temperature conditions (Baskin and Baskin, 2005). Such response towards germination process concluded that they have MPD (Baskin and Baskin, 2005). For the seeds of *Aconitum nagarum*, maximum germination was achieved from the seeds stratified for 48 hours at 4°C using filter paper as they prefer low temperature for germination (Langhu and Deb, 2014).

2.3 Environmental factors affecting seed germination

2.3.1 Light

Germination is a complex physiological process greatly varied within the same species which is determined by the different environmental signals i.e. light, temperature, moisture as well as seed morphology and physiology (Bewley and Black, 1994; Cristaudo *et al*., 2007). Dependency of seeds on light for germination is due to phytochrome-induced signal (Toyomasu *et al*., 1993; Yang *et al*., 1995), which is regulated by temperature. According to the Milberg *et al*., (2000), the influence of light on germination is much stronger in small-seeded species than in large-seeded ones. The light requirement is common in species that possess very small seeds and the requirement becomes less frequent with the increase in seed size (Milberg *et al*., 2000).

Size is an important characteristic of seed quality because larger seeds with larger volume contain more resources and are likely to exhibit greater vigor than smaller seeds (Ellis, 1992). In a range of plant species, seed size shows its affect to germination rate, emergence rate, success of establishment and growth (Sanderson *et al*., 2002). The importance of light in seed germination depends upon seed size, life form (annual or perennial) and species habitat (Uysal *et al*., 2006).

Seeds evolved to use light as an indicator of whether they are under the soil surface or under or beneath the canopy or close to the other plant species that compete for light and for other resources such as water and temperature (Bu *et al*., 2016). Germination of alpine medicinal plants such as *Rheum emodi* improved through exposure to light to seeds significantly ($p < 0.05$) at 15°C and 20°C incubation (Kandari *et al*., 2012). Low temperature (≥15°C) and light improved seed germination of *Rheum emodi* which indicated photoblastic nature of those seeds (Kandari *et al*., 2012).

In some experiments, hypocotyl became longer in all the lights in comparison with white light (Sharma and Sen, 1975). The far-red light is most beneficial for hypocotyl but not sufficient for radicle growth. In *Merremia dissecta*, white light is found to be the best for radicle growth. Its growth is suppressed in all the light conditions (Sharma and Sen, 1975). In the germination experiment carried out by Song *et al*., (2011), *Ulmus pumila* seeds germinated within six days without light conditions. Higher germination energy was found under Light Emitting Diode (LED) (Red: Blue, 2:1) and dark conditions, while significantly lower energy was found under white fluorescence and a higher Red: Blue ratio (Lee *et al*., 2021). Light quality can influence the germination and a certain wavelength of light might inhibited germination (Lee *et al*., 2021). The light quality is another ecophysiological characteristic of the ability of this species to adapt to varying conditions in habitats. Germination is often influenced by light quality, light intensity (Kettenring *et al*., 2006), as a report showing that blue LED improved *Stevia* seed germination (Simlat *et al*., 2016).

2.3.2 Temperature

Temperature is one of the major factors in regulating dormancy, rate and speed of germination (Baskin and Baskin, 1998). The effect of warming on germination of any species can be predicted by carrying out experiment under highest temperature regime. Seed germination is governed by temperature by determining their germination capacity and percentage, by breaking primary or secondary dormancy (Bewley and Black, 1994). The mechanism that surrounds the dormancy break of seeds after seed set play a great role in establishing germination pattern (Cristaudo *et al*., 2014). Most of the alpine species shows positive response to cope up with high temperatures.

The germination response of seeds after seed set is not only influenced by environmental conditions but also related to the environmental condition experienced by the mother plant before seed set (Cristaudo *et al*., 2014). With the increasing temperature chemical reactions inside seeds also stimulate that leads to faster germination. Most of the study revealed that seeds with larger size required higher temperature to trigger germination than smaller seeds (Notarnicola *et al*., 2022). Most of the Himalayan species prefer 25°C for seed germination (Pradhan and Badola, 2012). Seeds of *Swertia chirayita* incubated at 25°C resulted in the highest germination percent compared to those incubated at 20°C and 30°C (Pradhan and Badola, 2012). Fresh seeds of *Sinopodophyllum hexandrum* populations germinated to 70% at the warmer temperatures (25/15°C) in light, but germination was only about 50.0% at 15°C (Peng *et al*., 2023).

Study on germination revealed that species those required fire to germinate indicates that larger seeds need lower temperature to reach maximum germination (Liyanage and Ooi, 2017). In *Wahlenbergia ceracea*, a perennial herb of alpine environment, it was found that warmer temperature tends to increase germination success of larger seeds (Notarnicola *et al*., 2022). Study by Sood and Thakur, (2011) on *Aconitum deinorrhizum* revealed that seeds exposed to continuous light conditions with 20°C temperature gave better response in terms of seed germination. Germination percentage was found higher (76.67%) as compared to dark condition (63.60%) at the same temperature. The study recommended that 20°C temperature is most suitable for seed germination in this species on the basis of peak value, germination value and germination energy (Sood and Thakur, 2011) while in *Aconitum heterophyllum*, it was found that low temperature (15°C) was suitable for seed germination (Srivastava *et al*., 2011).

2.3.3 Water stress

With the increasing climate change the intensity of drought and temperature extremes is becoming a serious threat to many natural ecosystems (Smith, 2011). Water is one of the main abiotic factors limiting seed germination (Ansari *et al*., 2013). Moisture availability is essential for seeds for absorption of water (Kestring *et al*., 2009) and osmotic solutions can be applied to determine germination patterns simulating drought (Bradford *et al*., 2013). Water stress negatively impact plant regeneration, growth due to the reduced osmotic potential of dehydrated seeds restricting their metabolism (Bradford *et al*., 2013). Effect of drought stress varies with species as different species have different adaptation strategies to survive (Baskin and Baskin, 2014).

Understanding population persistence and community assembly patterns, as well as how they are affected by climate change, requires an evaluation of drought resilience during seed germination (Mouquet *et al*., 2010). It may be crucial to understand the links between seed size and the germination response to water stress in order to understand functional regeneration techniques. Different species have different life cycles (annual vs. perennial) and responses to drought (tolerance vs. avoidance). According to Poorter *et al*., (2011), perennial species prevent water stress by decreasing transpiration and absorbing water through a robust root structure. This suggests that perennial species are able to transfer more nutrients to their roots and are drought tolerant. *Nepeta persica*, a medicinal plant native to Iran, germination rate fell from 60% to 5% when exposed to polyethylene glycol (PEG) solution (Mohammadizad *et al*., 2013).

The germination characteristics of the species above the tree line may have significant drawbacks in a changing environment, which results in slowed growth, whereas the species below the tree line may be thought of as being pre-adapted for prospective migration to higher elevation (Walder and Erschbamer, 2015). Under the driest conditions (−0.9 MPa), only one species, *Achillea millefolium* below the treeline was able to germinate (3.8%) according to the study by Walder and Erschbamer, (2015). Higher water stress (−0.6 MPa) considerably reduced the Final Germination Percentage (FGP) or even stopped the germination of all species below the treeline (Walder and Erschbamer, 2015).

Water stress inhibited germination of *Caragana korshinskill* (Zheng *et al*., 2004). *Lavandula mairei* seeds were prevented from germinating by increasing the PEG concentration. When the concentration was −0.53 MPa, germination drastically decreased (11.11%) (Hamdaoui *et al*., 2021). To determine survival rate of any species it is very important to determine germination behavior in relation to temperature, water stress. *Sophora davidii*, a possible medicinal plant from southwest China, germinated more quickly when the climate was favorable, with temperatures between 20 and 30°C and water stress of −0.4 MPa. (Puchang *et al*., 2016).

2.3.4 Duration of storage

Seed storage duration is one of the main factors that the germination process relies. Ageing is a series of detrimental events that happen inside the seed, reducing their viability and ultimately causing the seed to die (Kurek *et al*., 2019). The lifespan of seeds varies from species to species. This is because different times of storage have different effects on seed viability and seed vigor. According to their storage capabilities, seeds are categorized as orthodox (desiccation-tolerant) and recalcitrant (desiccation-intolerant). Reactive oxygen species (ROS) induce oxidative damage to seeds during storage that reduces their ability to germinate (Ratajczak *et al*., 2015).

Seeds of alpine medicinal plants require cold treatment, gibberellins to break dormancy (Prasad, 1999). *In vitro* studies reveal that germination percentage is low i.e. 31.66% (control) in *Arnebia benthamii*. Many pretreatments were used to increase germination percentage, with germination enhanced to 96.33% (Ganaie *et al*., 2011). Review work on seed storage potential of medicinal plants showed that among different medicinal plants, germination of seeds of *Ricinus communis*, had no significant changes during storage period (95-84% and 86% from 9 and 12 months of storage respectively) (Aghilian *et al*., 2014). While germination of seeds of *Malva sylvestris,* increased after storage period (8%-17% and 33% from 9 and 12 months of storages respectively) (Aghilian *et al*., 2014).

Seeds of *Abrus precatorius* showed minimum effect of storage duration on germination i.e. decrease in germination percentage from 63%-60%-58% within 30-60-90 days of storage respectively (Tiwari and Das, 2014). Similarly, germination percentage of *Costus speciosus* and *Coleus forskohlii* decrease from 20.5%-12.9% from 30-90 days of storage and 83.7%-19.2% from 30-90 days of storage respectively (Tiwari and Das, 2014). The storage behavior of species also can be determined through species responses to different storage temperature.

After drying seeds of endemic medicinal plants of Ethiopia, *Solanecio gigas* showed reduction in germination percentage after one year of storage time while *Euryops pinifolius*, *Kniphofia foliosa* and *Lobelia rhynchopetalum* showed higher germination percentage after one year of storage at sub-zero temperature (Dagnachew *et al*., 2023). Viability testing through germination are seen to be one of the most important steps for understanding how plants adapt, as well as a technique to spot issues with seed storage conditions (Walsh *et al*., 2003). The study of seed lifetime and desiccation sensitivity provides fundamental knowledge for conservation of long and short-term storage among species throughout storage settings (Berjak and Pammenter, 2013).

2.4 Seed traits and germination behavior along elevation

Different seed traits *viz.* seed mass and size, seed coat permeability, light and temperature requirement determine their dispersal potential, time for germination and their establishment (Saatkamp *et al*., 2019). In order to investigate the potential consequences of climate change, elevation gradient is considered one of the useful tools. Alpine and subalpine environments have extreme climate. Mostly species at higher elevation habitat are more vulnerable to climate change than at low elevation (Gottfried *et al*., 2012). High elevation habitat are characterized by unpredictable conditions i.e. low temperatures and short growing seasons so the seed quality seems to be poor as compared to low elevation (Gottfried *et al*., 2012).

According to Sales *et al*., (2013), *Miconia albicans* seeds were collected in grasslands, shrublands and woodlands for three years in the southeast Brazil. Germination of seeds from plants in the grasslands was slower and asynchronous, while germination of seeds from plants in the woodlands was faster and more synchronous which serve as cues signaling unfavorable conditions for seed germination. Findings of Amimi *et al*., (2023) revealed that all populations of holm oak successfully germinate at 5°C. Tunisian holm oak had a relatively low temperature requirement for germination.

Species of *Allium* at high elevation showed faster and earlier germination than those at lower elevation (Ge et *al*., 2020). Among the population of *Allium chrysanthum*, seeds that are larger, germinated slow and less (Ge *et al*., 2020). Seed germination of *Gymnocalycium monvillei*, is affected by elevation, temperature, light and water availability, with maximal germination occurring at the highest elevation (2230, 1940 and 1555 m asl). Germination at 32°C was less than at 25°C for the majority of elevational provenances, with the exception of 1250 m (Bauk *et al*., 2017). A high elevation tree in the Rosaceae family called *Polylepis autralis* was also shown to exhibit the pattern of greater germination at higher elevation (Marcora *et al*., 2008). There is a great variation in germination traits within the population analyzed, by Pérez-García, (2009) on the germination of *Ceratonia siliqua* seeds, collected from 20 different individual trees in the population. The final germination rates of control seeds from 17 different individual trees ranged from 7 to 50% and Mean Germination Time (MGT) varied from 14 to 19 days in 17 different individuals. These findings indicate the high variability among seeds.

The germination fraction of small, elongated seeds-those with more surface area-was higher at high elevations: short growing seasons, low temperatures, and higher abiotic stress all contribute to fast germination in seeds from high elevation (Wang *et al*., 2020). The high variations in germination responses identified in alpine plants have been considered as a survival strategy to face unpredictable environmental conditions. Seeds of alpine habitat form persistent soil seed bank providing an ecological advantage by providing favorable environmental conditions for seedling establishment (Ooi, 2012). Knowledge and research on seed traits provides a relevant option for making decision regarding management which allowed such traits to be integrated into conservation practices (Turner *et al*., 2018).

2.5 Research Gap

Different studies and work on seed germination was focused only on frequently used and highly known medicinal plants of Himalaya. Several studies were carried out on molecular mechanisms, extraction of secondary metabolites i.e. aconitine and other bioactive compounds in *Aconitum* species (Shyaula, 2012; Rana and Sreenivasulu, 2013). There is lack of information on germination pattern of alpine medicinal plants especially of genera *Aconitum*. This is the first approach on study on germination of *A. spicatum*.

Study about the effect of environmental conditions is lacking, that may be water stress, climate change which can provide important insights regarding further survival and adaptation of these species when there is global change. There is limited research and information regarding seed germination under different light and temperature environment. The information regarding germination on the basis of seed storage potential is lacking. *Aconitum spicatum*, being one of the traded medicinal plants (Pyakurel *et al*., 2019; Rana *et al*., 2020; Shrestha *et al*., 2022) and important from the economic and medicinal perspective, therefore study on their germination behavior is important.

CHAPTER 3. MATERIALS AND METHODS

3.1 Seed collection site

Three different sites were considered for seed collection within the Annapurna Rural Municipality of Gandaki Province. Study area was designed according to the occurrence of *Aconitum spicatum* populations from three different elevations i.e. along Annapurna Conservation Area (ACA) in North-Central Nepal (Fig. 1). Annapurna Conservation Area is a category VI protected area as per the designation of IUCN. It covered 5 districts: Manang, Mustang, Kaski, Myagdi and Lamjung (Bhatt and Pickering, 2022).

Geographically, the ACA spans an area of 7,629 square kilometers and is situated between 83°34' and 84°25' E longitude and 28°15' to 28°50' N latitude (NTNC, 2016). It is dispersed across Nepal's four physiographic areas, and is bounded to the east by the Marsyangdi Valley, to the west by the Kali Gandaki River, to the north by the arid alpine desert of the Dolpo and Tibet (China), and to the south by the valleys and foothills of Pokhara. The ACA, which is found in Nepal's north-central region, is the country's largest protected area. According to this conservation area harbours, flowering plants of 1,264 species (monocot-210, dicot-1,054), which is rich in biodiversity (NTNC, 2016).

3.1.1 Climate

The climatic zones from Tropical to Nival were included in the ACA. The country's highest precipitation ranges are in the southern Annapurna region. ACA extends from Modi Valley at 950 m to Annapurna I at 8,091 m above sea level (m asl) with average annual temperature of 14°C (maximum 35°C, minimum −0.3°C) and rainfall average 193-2987 mm annually (NTNC, 2016).

Due to the lack of meteorological station in the study area, nearest meteorological station of Lumle (Lat: 28.29654°N and Long: 83.81791°E) with elevation 1738 m asl was taken as a reference point. The climatic data past 11 years (2012-2022) was recorded in Lumle station of Kaski, Nepal where mean annual min temperature: 12.05°C, and max temperature: 20.57°C; annual precipitation: 5254 mm (Fig. 2) (Department of Hydrology and Meteorology).

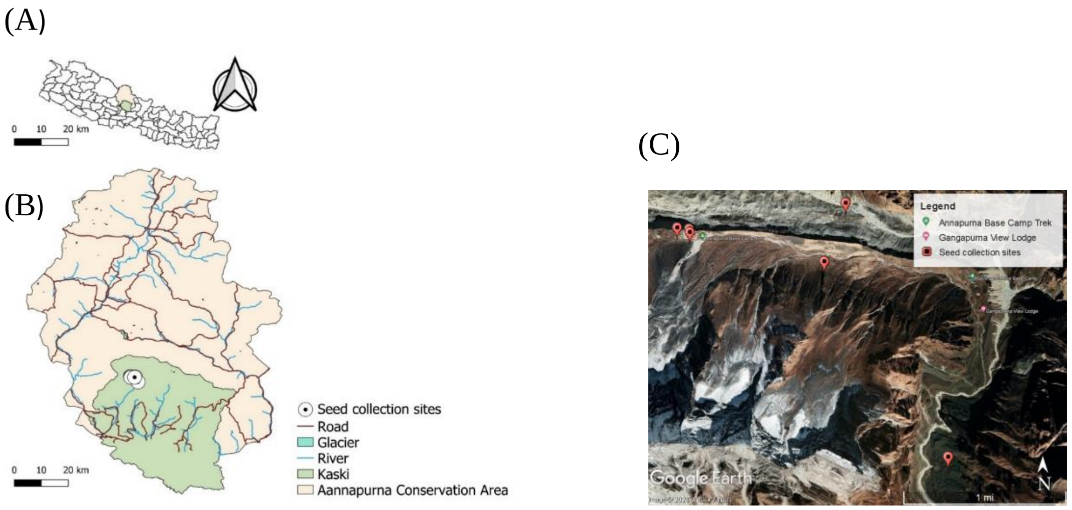


Figure 1. (A) Map of Nepal showing Annapurna Conservation Area (B) Map of Annapurna Base Camp (C) Seed collection sites

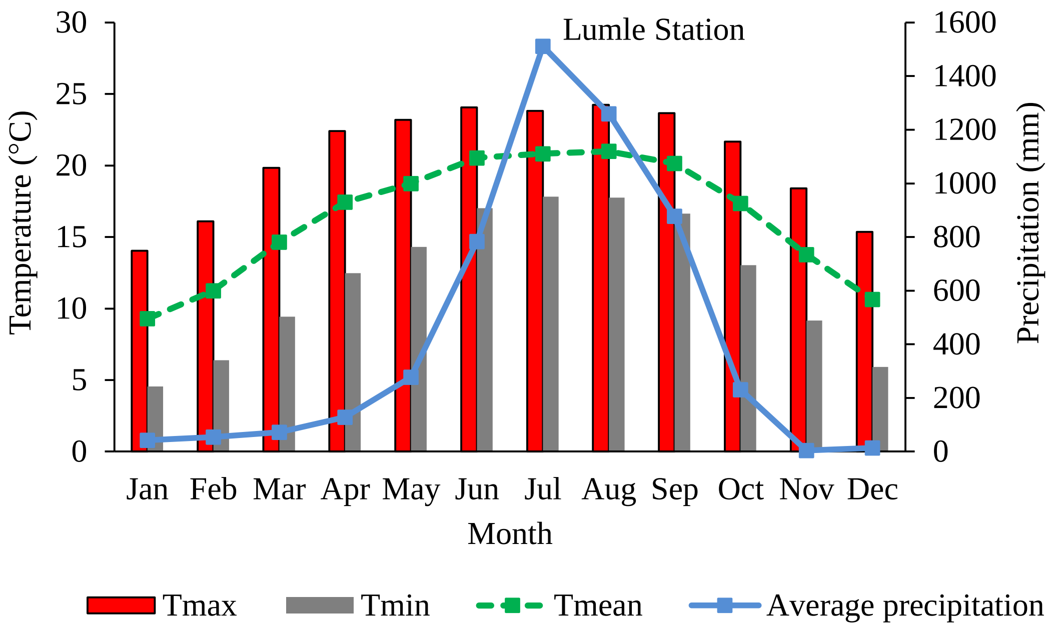


Figure 2. Mean of maximum and minimum temperature as well as precipitation recorded between 2012 and 2022 at Lumle station of Kaski, Nepal (Department of Hydrology and Meteorology)

3.2 Study species

3.2.1 *Aconitum spicatum* (Brühl) Stapf

Aconitum spicatum (Brühl) Stapf (Fam.: Ranunculaceae), also known as monkshood is a herbaceous, perennial, medicinal plants of the Himalayas with an erect stem up to two meter in height and purplish flower in open dry/moist region (Ghimire *et al*., 1999). It is distributed along the Himalayas of Nepal, India, and Bhutan at elevation between 1800-4800 m (Shrestha *et al*., 2022). Each plant produces about 1-110 fruits and each fruit produces an average of 41 seeds (Chapagain *et al*., 2019) (Fig. 3).

Aconitum spicatum is one of the highly valuable and traded Medicinal Plants (Pyakurel *et al*., 2019; Rana *et al*., 2020; Shrestha *et al*., 2022) listed in vulnerable category of Conservation Assessment Management Plan (CAMP) and threatened category of International Union for Conservation of Nature and Natural Resources -IUCN Red List (Gurung and Pyakurel, 2017). Being one of the ten most trafficked medicinal plants from Nepal's Himalayan region, *Aconitum spicatum* (Olsen and Larsen, 2003) had a trade volume of >300 kg/year for the rhizome (DoF, 2017).

In Traditional Chinese Medicine (TCM) specifically, *Aconitum* is a herb of tremendous significance, due to its cytotoxic, anti-microbial, anti-arrhythmic and anti-epileptiform properties (Nyirimigabo *et al*., 2014). *Aconitum's* toxicity can be lessened utilizing a variety of methods so that its therapeutic effects can be obtained (Nyirimigabo *et al*., 2014). Although diester diterpene alkaloids are the main contributors to the toxicity of *Aconitum* (Shyaula, 2012), different Chinese medical systems have devised methods, such as pressure steam processing, which reduces the toxicity of aconitine (Nyirimigabo *et al*., 2014).

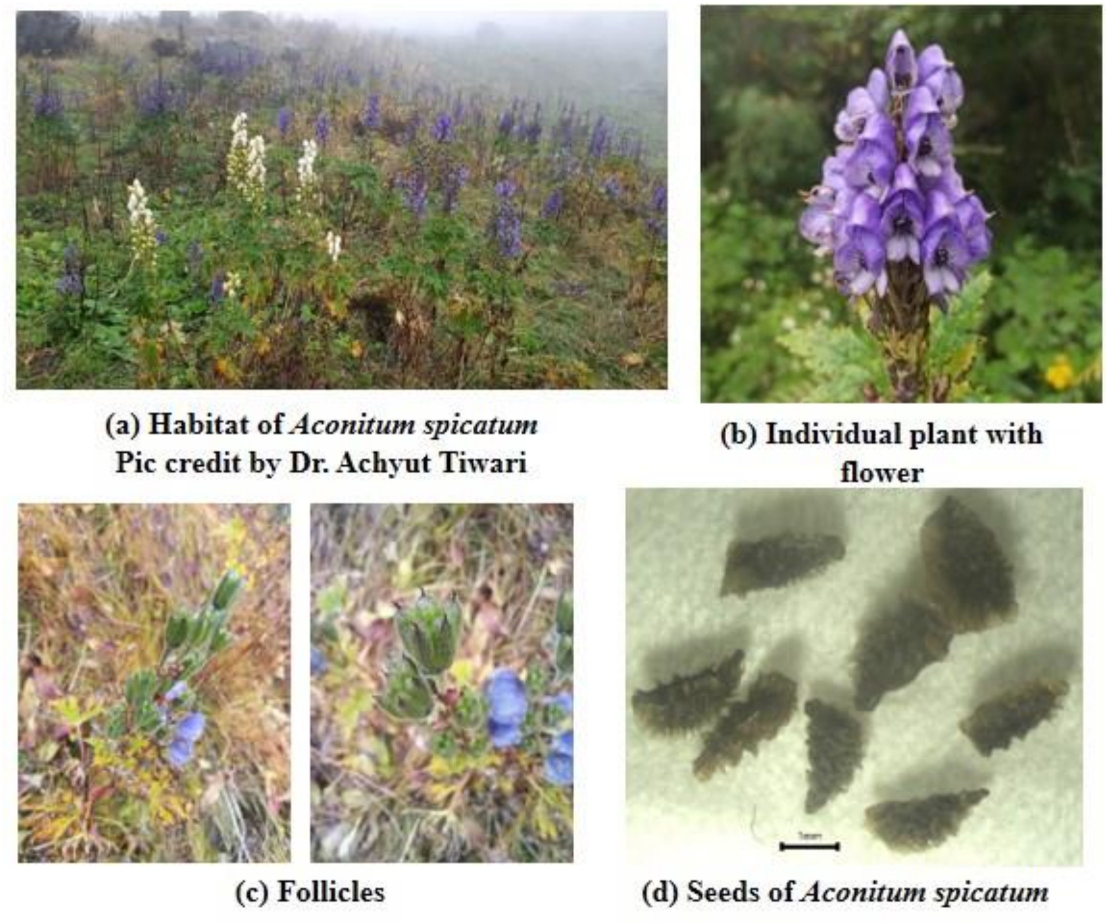


Figure 3. *Aconitum spicatum*

3.3 Seed collection and storage

Mature and healthy seeds of *Aconitum spicatum* were collected from three different elevations along Modi river valley in the Annapurna Base Camp area in 2021 and 2022 (Table 1). The seed collection sites ranged from subalpine to alpine regions. Fruits with mature seeds were collected in cloth bags (Fig. 4).

Seeds were air-dried for one week and cleaned with the help of winnowing basket and sieve (mesh strainer). Prior to use seeds, all seeds were kept separately according to elevation wise in an air-tight plastic bottles inside refrigerator at 4°C until needed (Langhu and Deb, 2014; Bajwa *et al*., 2016) (Fig. 4). Silica gel was also used for moisture absorption in the seeds kept in bottles (particle size: 6-20 mesh).

Table 1: Seed collection details of *Aconitum spicatum*

Year	Seed collection sites		Date	Latitude (°N)	Longitude (°E)	Habitat
	Locality	Elevation (m asl)				
2021	Deurali	3540	Nov 11	28.527154	83.892083	Sheepfold and river trial
	Between Machhapuchre Base Camp and Annapurna Base Camp	3935	Nov 11	28.526679	83.877684	Sheepfold and grassland
	Annapurna Base Camp	4260	Nov 12	28.529882	83.87777	Sloppy and rocky
2022	Deurali	3315	Nov 14	28.50935	83.90481	Sheepfold and river trial
	Between Machhapuchre Base Camp and Annapurna Base Camp	3910	Nov 15	28.52886	83.89433	Sheepfold and grassland
	Annapurna Base Camp	4200	Nov 15	28.53037	83.87641	Sloppy and rocky

3.4 Seed mass

In order to determine the seed mass, 50 seeds collected in the year 2022 from each elevation were weighed after oven dry. Prior to oven dry, seeds of same size were packed in small paper pockets and oven dried at 60°C for 72 hours (Baskin and Baskin, 2014). For each elevation three replicates were taken. Seed mass was measured using digit weighing balance (0.0001g) (Model: MG214Ai BEL ENGINEERING SRL VIA CARLO CARRA 5 MONZA 20900 *ITALIA*).

3.5 Germination Experiments

Germination experiments were conducted in a growth chamber (Model: GC- 300TLH, Jeio Tech, Korea) (Fig. 4).

3.5.1 Preliminary germination test

Prior to germination experiment preliminary test was carried out and germination started after 10th days from the test. Germination was carried out for 20 days and germinated seeds were counted for 20 days.

3.5.2 Germination under different environmental conditions

Germination experiment were carried out under different light (12h photoperiod and complete dark, water stress and temperature conditions (low: 25/15°C and high 30/20°C) within the growth chamber at Ecology Lab of Central Department of Botany for fresh seeds that were collected during 2022 from elevation (3315 m, 3910 m and 4200 m asl). Petri dishes (9 cm-diameter) were used for seeds placement (Vandelook *et al*., 2009; Bhatt *et al*., 2021). For each elevation 35 Petri dishes and 1050 seeds were used. Each Petri dish containing 30 healthy and intact seeds were uniformly placed on the 2 layered Whattman No.1 filter paper (Joshi and Dhar, 2003; Prakash *et al*., 2011; Baskin and Baskin 2014). Petri dishes were sealed with transparent parafilm to minimize moisture loss. White fluorescent light of 3000 lux intensity was maintained inside growth chamber with 70% humidity. Price tag was used with proper labeling on each Petri dish. For each treatment, five replicates were used. For control and complete dark, only distilled water was used to wet the filter paper containing seeds for each elevation and about three ml distilled water was used for each Petri dish. Amount of distilled water for moistening filter paper depend on the absorption capacity of water by the seeds and filter paper.

Aluminium foil was used to wrap the Petri dish for complete dark (Fig. 5) and data of germinating seeds were recorded on 20th days from the date of seed incubation for complete dark (Herranz *et al*., 2010; Bhatt *et al*., 2021). Seeds were observed daily for recording daily germination data only for complete control and different concentration of osmotic stress. Except for the seeds under complete dark, all the other treatment data were daily recorded. Germinated seeds were removed during each observation on the basis of visible radicle protrusion as the criterion for germination. Seeds were considered germinated when radical about (≥1) mm emerged (Meiado *et al*., 2010; Bhatt *et al*., 2021).

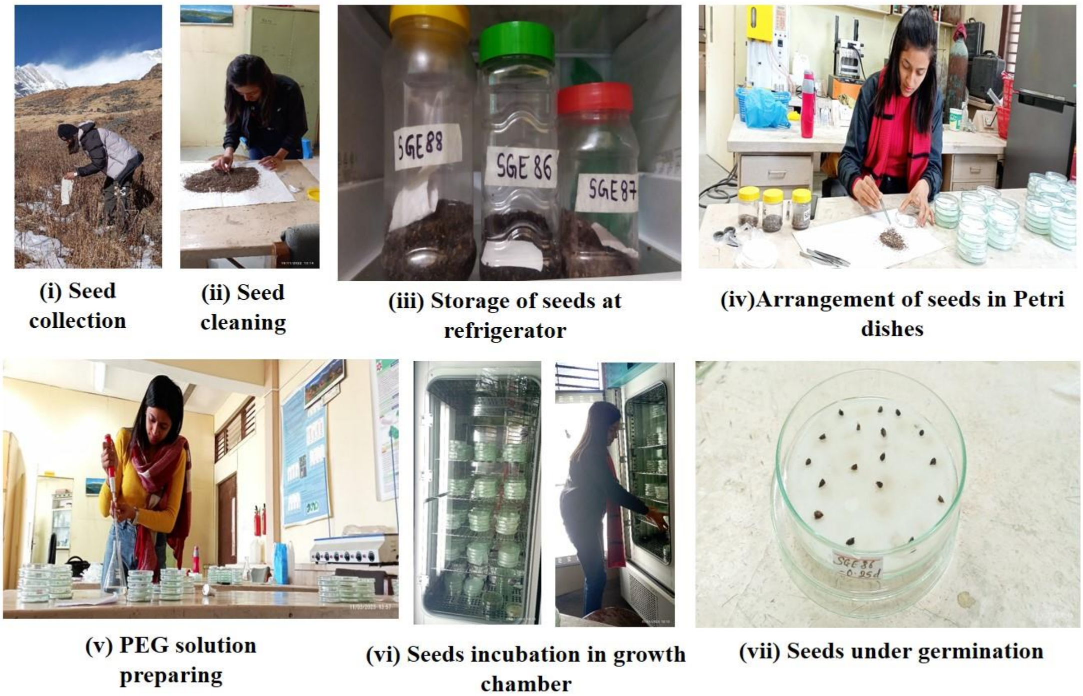


Figure 4(i-vii). Different stages of seed germination experiment

A. Light

The effect of light on germination was investigated for fresh seeds collected during 2022. For this, seeds were subjected at two different light regimes of photoperiod [12hr/12hr] (light/dark) and complete dark [24hr] (Cristaudo *et al*., 2014; Bhatt *et al*., 2021). One was wrapped with double-layer aluminum foil (dark treatment) (Fig. 5) and the next was left uncovered for light treatment (12 h photoperiod).

Effect of Far-red light

For the effect of far-red light on germination, seeds collected in the year 2021 from the three different elevation (3540 m, 3935 m and 4260 m) were wrapped with blue and red colored cellophane papers and exposed to white and far-red light (Sharma and Sen, 1975). The regulation of several ecophysiological features of plants, including seed germination, is greatly influenced by light. Red and far-red light regulate seed germination through the phytochrome system, which is a well-known process (Kendrick and Nagatani, 1991). Five replicates each containing 30 seeds of three elevation were prepared. Each Petri dish was covered with single layered each blue and red colored cellophane and seeds were kept for experiment under low temperature (25/15°C) (Fig. 6). Germination count was done on the 20th days of seed incubation. Cellophane paper was removed and germinated seeds were counted.

Figure 5. Petri dish wrapped under aluminium foil

Figure 6. Germinated seeds under blue and red cellophane paper

B. Temperature

The two different temperature regimes were designed, one was low temperature (25/15°C) which was the average mean temperature of Kathmandu and to imitate the effect of climate change, another high temperature (30/20°C) was maintained. A standard growth conditions was maintained of alternating day/night temperature and 70% humidity (Prakash *et al*., 2011; Langhu and Dev, 2014) and the experiment was carried out for fresh seeds collected during 2022. Germination counting started from 10th days of seed subjected.

C. Water stress

Aqueous solutions of −0.1, −0.25, −0.5, −0.75 and −1MPa were prepared using polyethylene glycol (PEG 6000) solution (Kandari *et al*., 2008; Santo *et al*., 2013; MousaviNik *et al*., 2016). PEG solution was prepared according to the required amount for different concentrations where, 296 g of PEG was dissolved in distilled water to prepare 1 liter of PEG solution of −1MPa (Michel and Kaufmann, 1973; Meiado *et al*., 2010). The solution then diluted to prepare PEG solution of other concentrations. About three ml of PEG solution was used for each Petri dish for water stress. The effect of water stress was carried out for fresh seeds collected during 2022 in low temperature as described previously. The germination rate was determined after counting number of germinated seeds after 10th days of seed incubation.

And, in the end of experiment, non-germinated intact seeds that collapsed when pinched with forceps were removed and seeds that were firm and did not collapse and had white embryo were considered viable. Distilled water was used for viability test where seeds were rinsed in distilled water and kept for further germination procedure after the termination date. The cycle was completed in the 20th days from the date of seed incubation. Germination data was recorded only up to −0.5 MPa water potential coz, there was no germination at –0.75 and −1MPa water potential.

D. Effect of different storage time period

Seeds that were collected during 2021, from the three different elevation (3540 m, 3935 m and 4260 m) were incubated under low temperature (25/15°C) with 70% humidity. Seeds that were stored for 26, 34 and 73 weeks were incubated for experiment for different storage time period. For 24 h dark, 12 h photoperiod, seeds that were stored for 26, 34 and 73 weeks were used and for different level of water potential, seeds that were stored for 34 and 73 weeks were used and experiment terminated after 20 days. 1st experiment was carried out after 26 weeks from the date of collection for seeds that were stored at 4°C in refrigerator, 2nd after 34 weeks and after 73 weeks of duration of storage, 3rd experiment was carried out for the same seeds under same temperature.

The daily recorded germination data were used to calculate different germination parameters such as Germination Percentage (GP), Mean Germination Time (MGT) and Timson's Index (TI).

3.6 Data Analysis

3.6.1 Seed germination parameters

Seed germination percentage, mean germination time and the Timson's Index were calculated using following formulae (Baskin and Baskin, 2014):

Germination percentage = (Number of germinated seeds/ Total number of seeds) ×100

Mean germination time (MGT) is the time required for the maximum number of seeds to germinate and was calculated as:

Mean Germination Time (MGT) = $\sum_{i=1}^{k} niti / \sum_{i=1}^{k} ni$

Where, *ni*= number of seeds germinated at time *t*

ti= time from the day of experiment start

Timson's Index of germination measures seed germination rate

Timson's Index (TI) $\sum k = \sum_{i=1}^{k} Gi$

Where, *Gi*= cumulative daily germination percentage in time interval *i* and *k* is total number of intervals

3.6.2 Statistical Analysis

Data were tested for normality and homogeneity of variance prior to the parametric test. Germination percentage were first square root transformed and then Arcsine transformed (Ahrens *et al*., 1990). To analyze effect of temperature, light (far-red) and dark within elevation and the effect of two different storage time period within elevation on germination and effect of water stress within elevation, independent sample t-test was performed. To analyze effect of temperature, dark and light (far-red) among three different elevation and effect of three different storage time period, one-way Analysis of Variance (ANOVA) (Post-hoc test) was performed. To analyze effect of interactions of elevation and water potential (MPa) on GP, MGT and TI, two-way ANOVA was performed. All the analyses were done using MS Excel 2013 and IBM Statistics 25 (IBM Corp. 2017).

CHAPTER 4. RESULTS

4.1 Seed Mass

Among the seeds of *Aconitum spicatum* collected from the three different elevations, seed mass collected from the low elevation (3315 m) was the highest, followed by the high and mid elevation sites (Table 2).

Table 2: Mean (±S.D.), Seed mass of *Aconitum spicatum* (n=3) from different elevation

Elevation(m asl)	Seed mass (mg/seed)
Low (3315)	$1.2 \pm 7.9 \times 10^{-5}$
Mid (3910)	$0.6 \pm 5.6 \times 10^{-5}$
High (4200)	$1 \pm 6.2 \times 10^{-5}$

4.2 Germination pattern

4.2.1 Effect of temperature

Germination percentage

Germination was observed at both low (25/15°C) and high temperature (30/20°C). Within each elevation, germination was significantly higher at low temperature than high temperature in both photoperiod and complete dark ($p \leq 0.05$) (Fig. 7). Among three elevation, germination did not vary significantly in photoperiod at low temperature. Germination was significantly higher in mid elevation at high temperature (Fig. 7a). In complete dark, among three elevations, germination did not vary significantly in both high and low temperature (Fig. 7b).

Mean Germination Time and Timson's Index

Within each elevation, Mean Germination Time was the longest at high temperature while it was shortest at low temperature (Fig. 8a). Among three elevation MGT did not vary significantly at both high and low temperature.

Timson's Index was significantly higher at low temperature compared to the high temperature in seeds from all the three elevation (Fig. 8b). Among three elevations, seeds of mid elevation had significantly higher TI at high temperature while at low temperature, TI did not vary significantly.

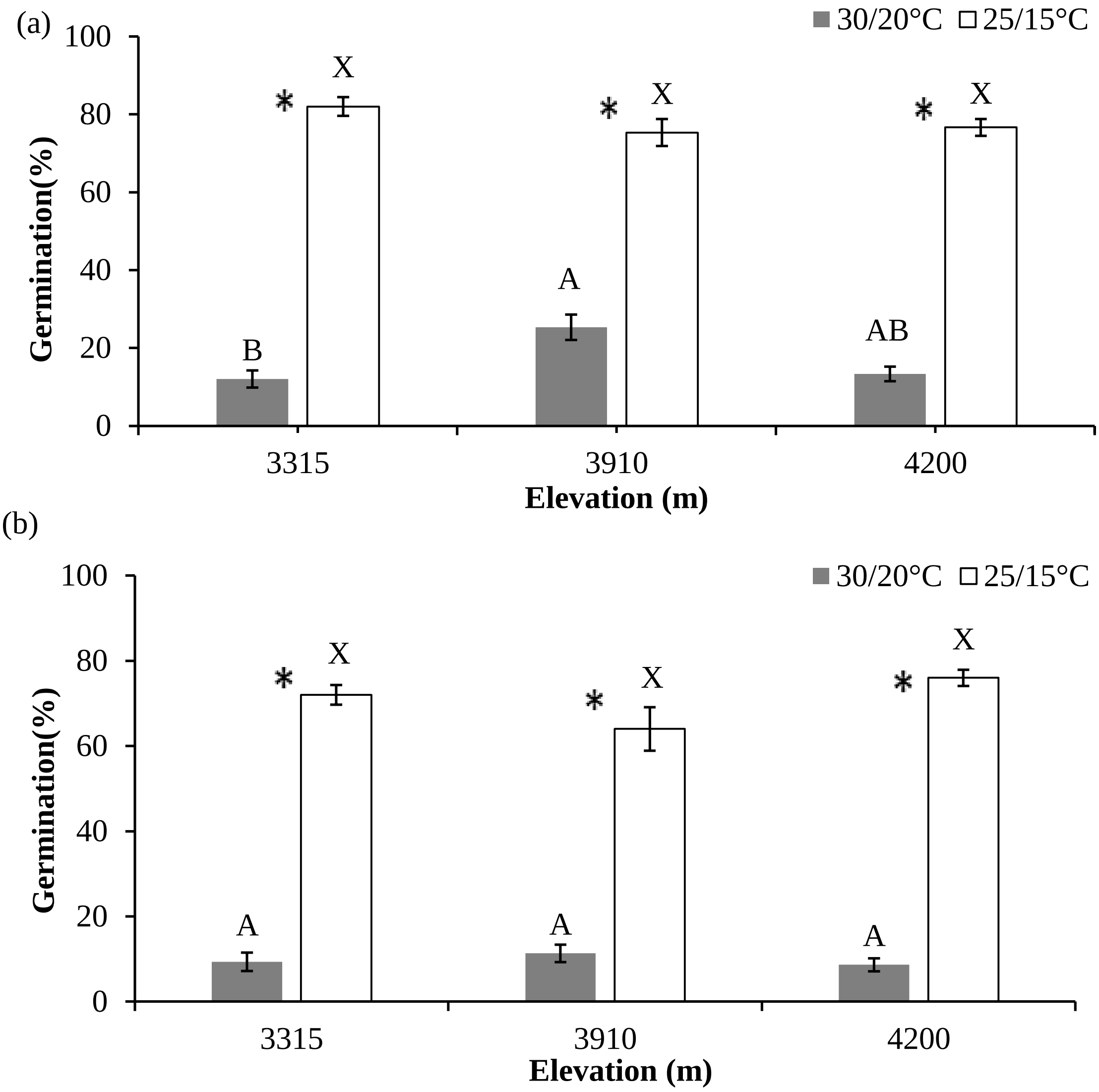
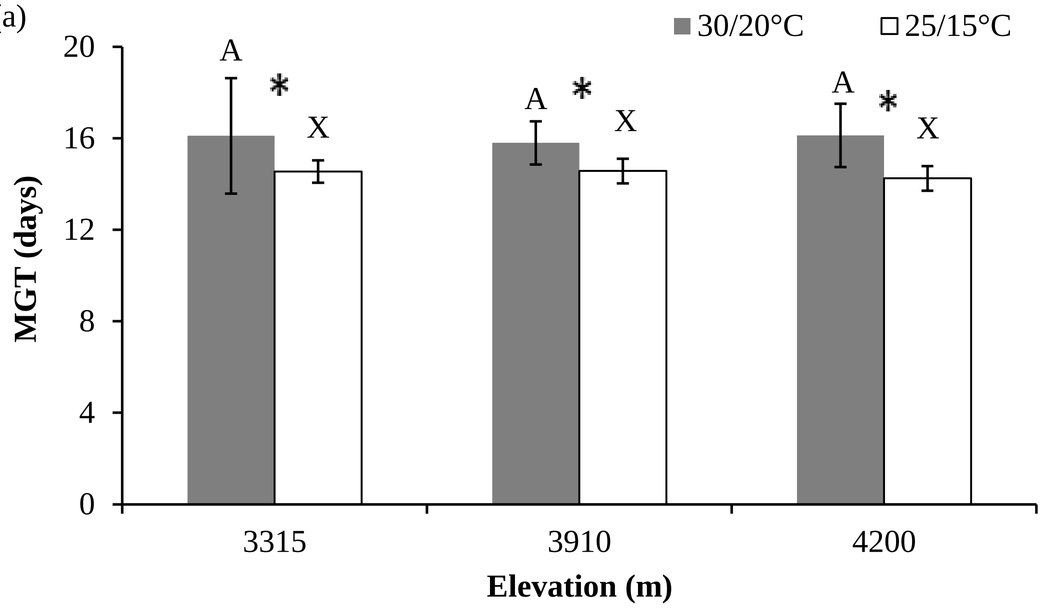


Figure 7. Effect of temperature on Germination percentage (GP) at (a) Photoperiod and (b) Dark. Error bars represent (±S.D.) of the mean (n=5). Different capital letters (A/B/X) above bars indicate significant difference ($p \leq 0.05$) among elevation and '*'represents significant difference ($p \leq 0.05$) within elevation.

4.2.2 Effect of far-red light

Within all elevation, germination was significantly higher in white light compared to the far-red light except mid elevation, where seed germination did not vary significantly. Among the three elevation (3540 m, 3935 m and 4260 m), germination was significantly higher at low and high elevation than mid elevation (Fig. 9). Among elevation, there was no significant difference in GP in far-red light while there was significant difference in GP in white light.

(a)



(b)

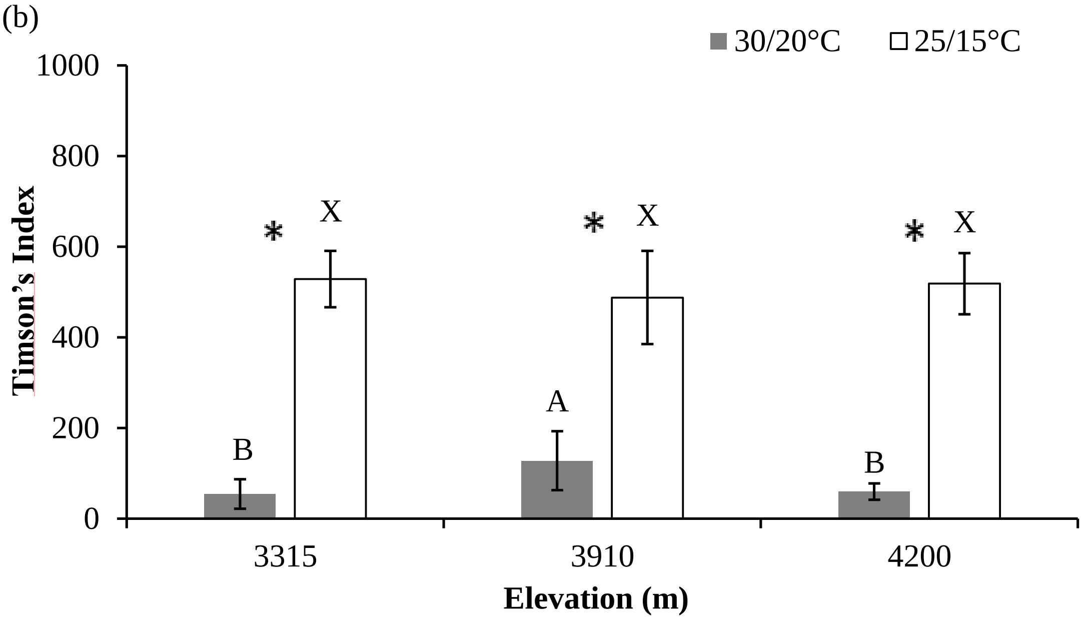


Figure 8. Effect of temperature on (a) Mean Germination Time (MGT) and (b) Timson's Index at photoperiod. Error bars represent (±S.D.) of the mean (n=5). Different capital letters (A/B/X) above bars indicate significant difference ($p \le 0.05$) among elevation and '*'represents significant difference ($p \le 0.05$) within elevation.

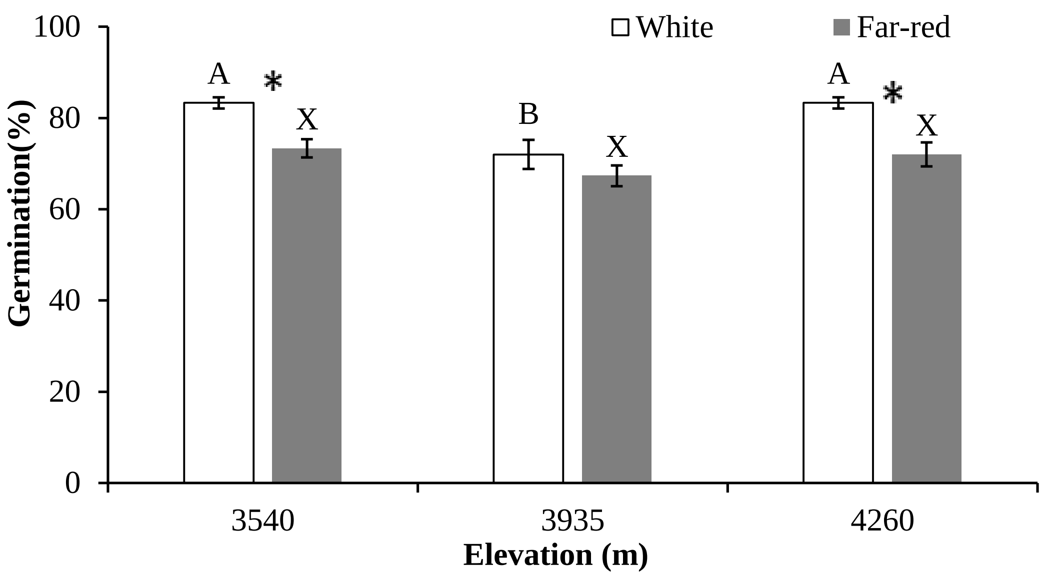


Figure 9. Effect of white and far-red light on Germination percentage (GP) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5). Different capital letters (A/B/X) above bars indicate significant difference ($p \leq 0.05$) among elevation and '*'represents significant difference ($p \leq 0.05$) within elevation.

4.2.3 Effect of water stress

Germination percentage

Germination was only up to −0.5 MPa water potential. There was no germination at −0.75 MPa and −1 MPa water potential. For seeds of all elevation, decrease in level of water potential resulted in significant decrease in germination percentage. When compared within elevation, seeds of all elevation exhibited significantly higher germination percentage at control (0) and −0.1 MPa water potential but beyond that it decreased significantly (Fig. 10).

When compared among different level of water potentials, germination percentage in low and mid elevation did not vary significantly at water potential of control (0), −0.1, −0.25 MPa except at−0.5 MPa while in high elevation there was significant difference at −0.25 MPa and −0.5 MPa water potential.

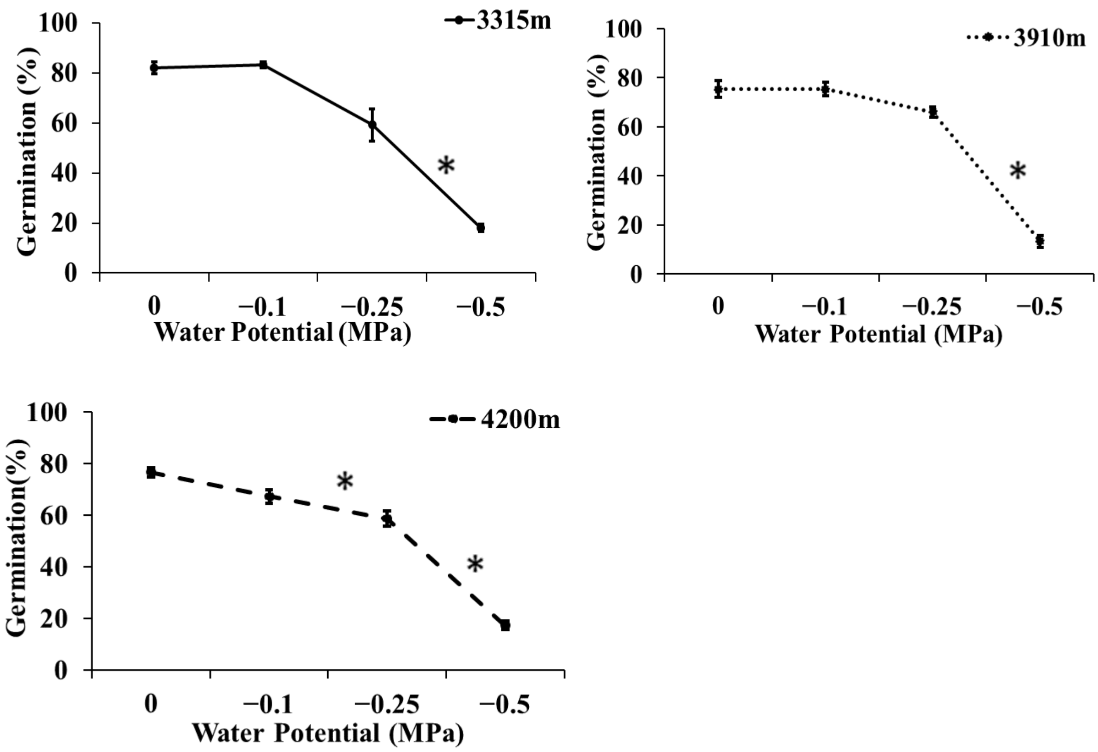


Figure 10. Effect of water stress on Germination percentage (GP) of three elevations under different water potential (MPa) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and '*'represents significant difference ($p \leq 0.05$) among different level of water potential.

Mean Germination Time

Mean Germination Time (MGT) was the longest at −0.5 MPa water potential while it was shortest at control and −0.1 MPa water potential when compared among different level of water potentials. For seeds of all elevation, MGT increased significantly with decrease in water potential (Fig. 11).

When compared within elevation, there was no significant difference in the MGT of low and high elevation at control and −0.1 MPa water potentials except at mid elevation. In low and high elevation, there was significant difference in MGT at −0.25 and −0.5 MPa water potential.

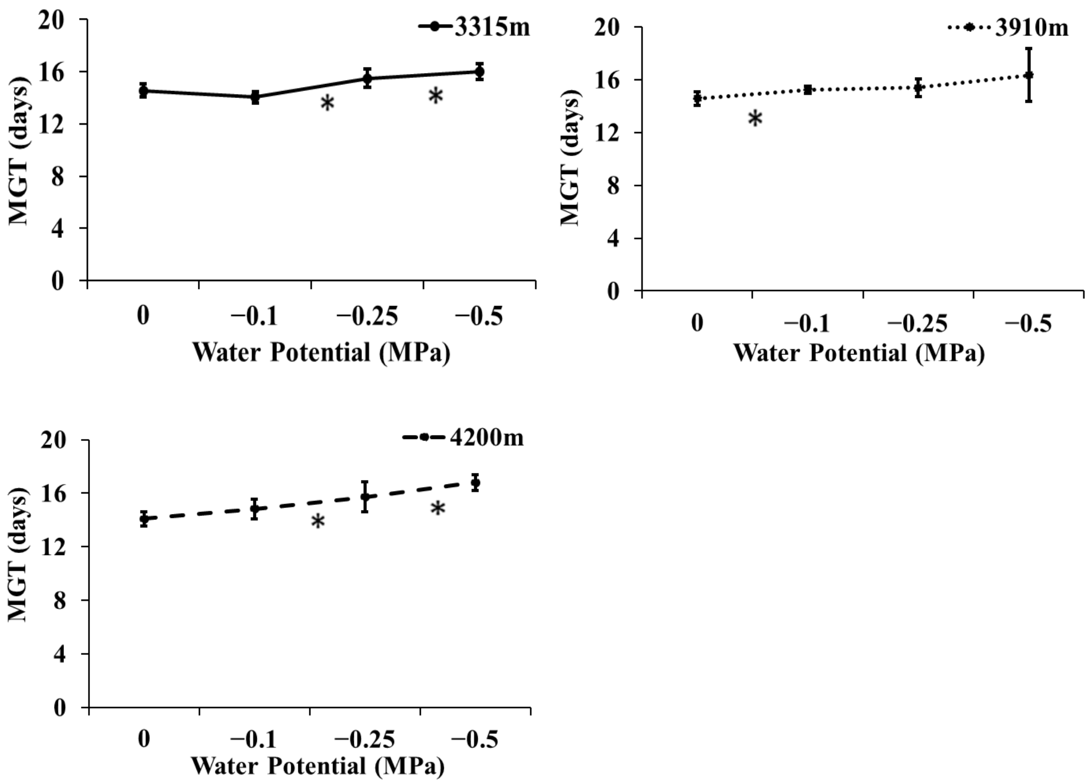


Figure 11. Effect of water stress on Mean Germination Time (MGT) of three elevations under different water potential (MPa) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and '*'represents significant difference (p≤0.05) among different level of water potential

Timson's Index

Within elevation, Timson's Index (TI) was significantly higher at control and −0.1 MPa water potential but beyond that it decreased significantly when compared among different level of water potentials (Fig. 12).

While comparing among elevation, TI was significantly higher at low elevation followed by high and mid elevation. In low and high elevation there was significant difference in TI at −0.25 MPa and −0.5 MPa water potential while in mid elevation, there was significant difference in TI at −0.5 MPa water potential.

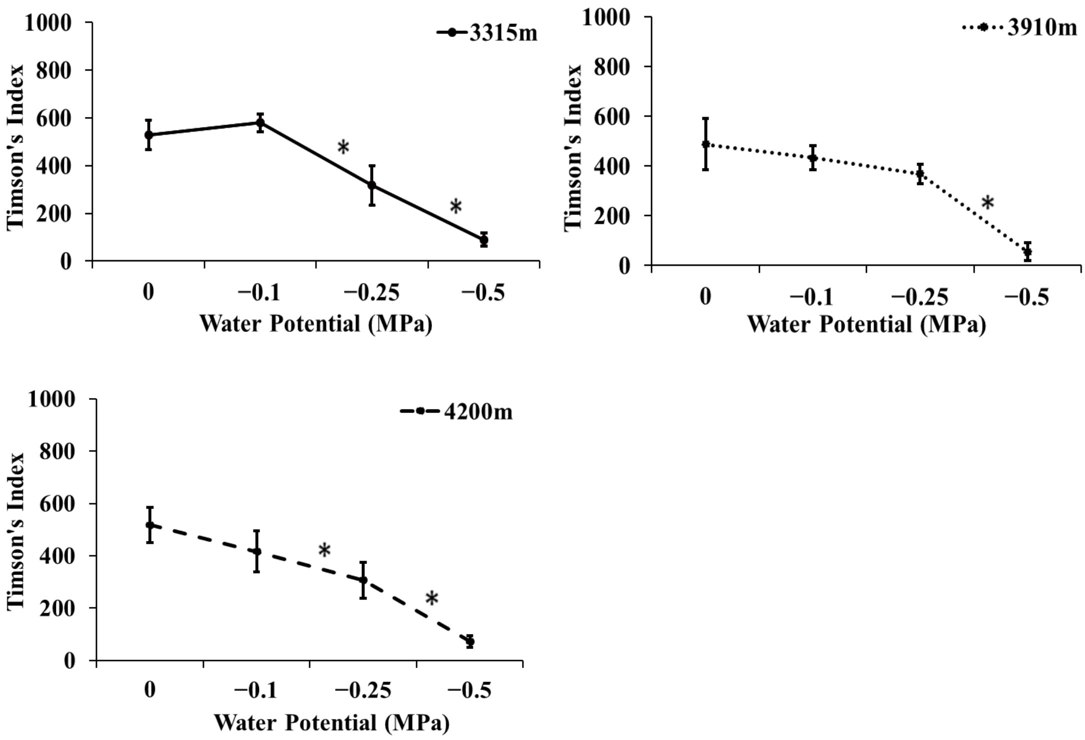


Figure 12. Effect of water stress on Timson's Index (TI) of three elevations under different water potential (MPa) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and '*'represents significant difference (p≤0.05) among different level of water potential.

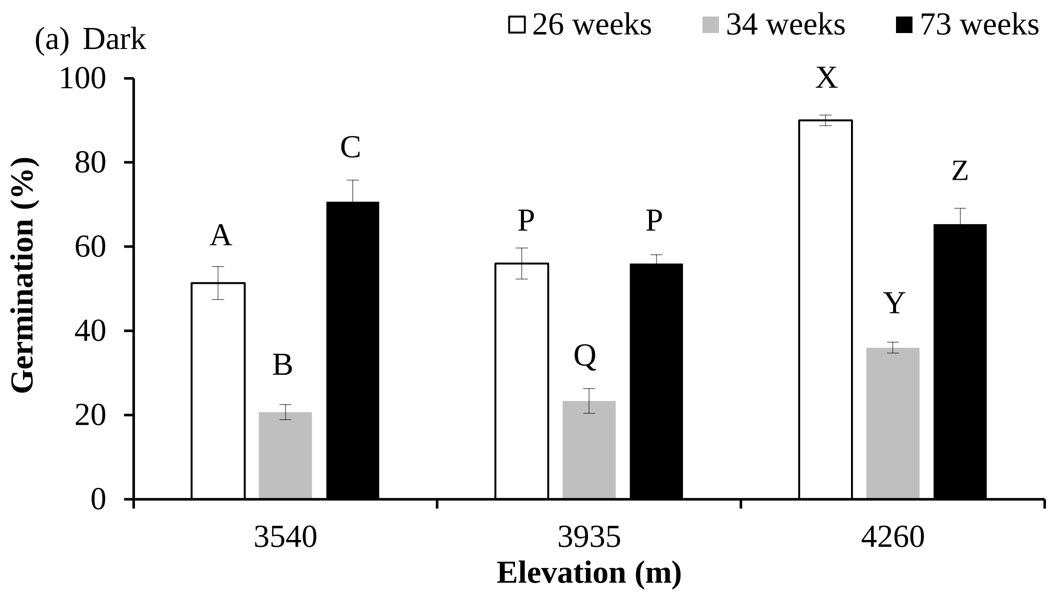
4.2.4 Effect of different storage time period

A. Effect of dark and photoperiod

Germination percentage

In 24 h dark (Fig. 13 a) there was significant difference in GP between 26, 34 and 73 weeks of duration of storage in low (3540 m) and high elevation (4260 m) but in mid elevation (3935 m) there was significant difference between 26 and 34 weeks and between 34 and 73 weeks of duration of storage.

In 12 h photoperiod, (Fig. 13b) there was significant difference between 26 and 34 weeks and between 34 and 73 weeks of duration of storage in all elevation but no significant difference between 26 and 73 weeks of duration of storage.



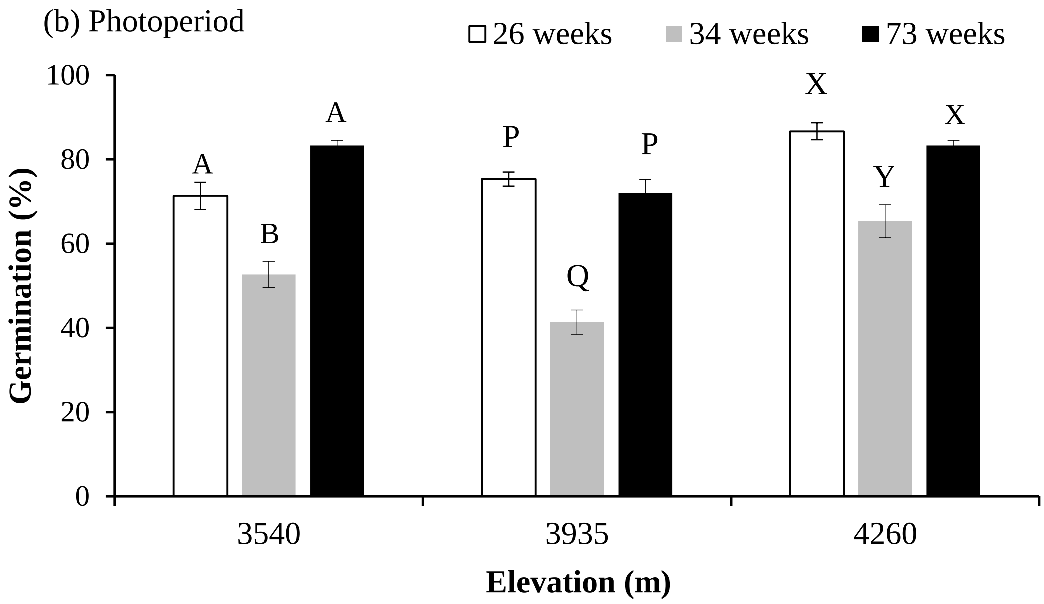


Figure 13. Effect of storage time period on Germination percentage at (a) dark and (b) photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and different letters (A/B/C), (P/Q) and (X/Y/Z) above bars represents significant difference ($p \leq 0.05$) within different storage time period within each elevation.

Mean Germination Time and Timson's Index

There was significant difference in MGT between 26, 34 and 73 weeks of duration of storage in mid elevation but no significant difference in high elevation. In low elevation there was significant difference between 26 and 34 weeks and between 26 and 73 weeks of duration of storage (Fig. 14a).

There was significant difference in Timson's Index between 26 and 34 weeks and between 34 and 73 weeks of duration of storage while there was no significant difference between 26 and 73 weeks of duration of storage (Fig. 14b).

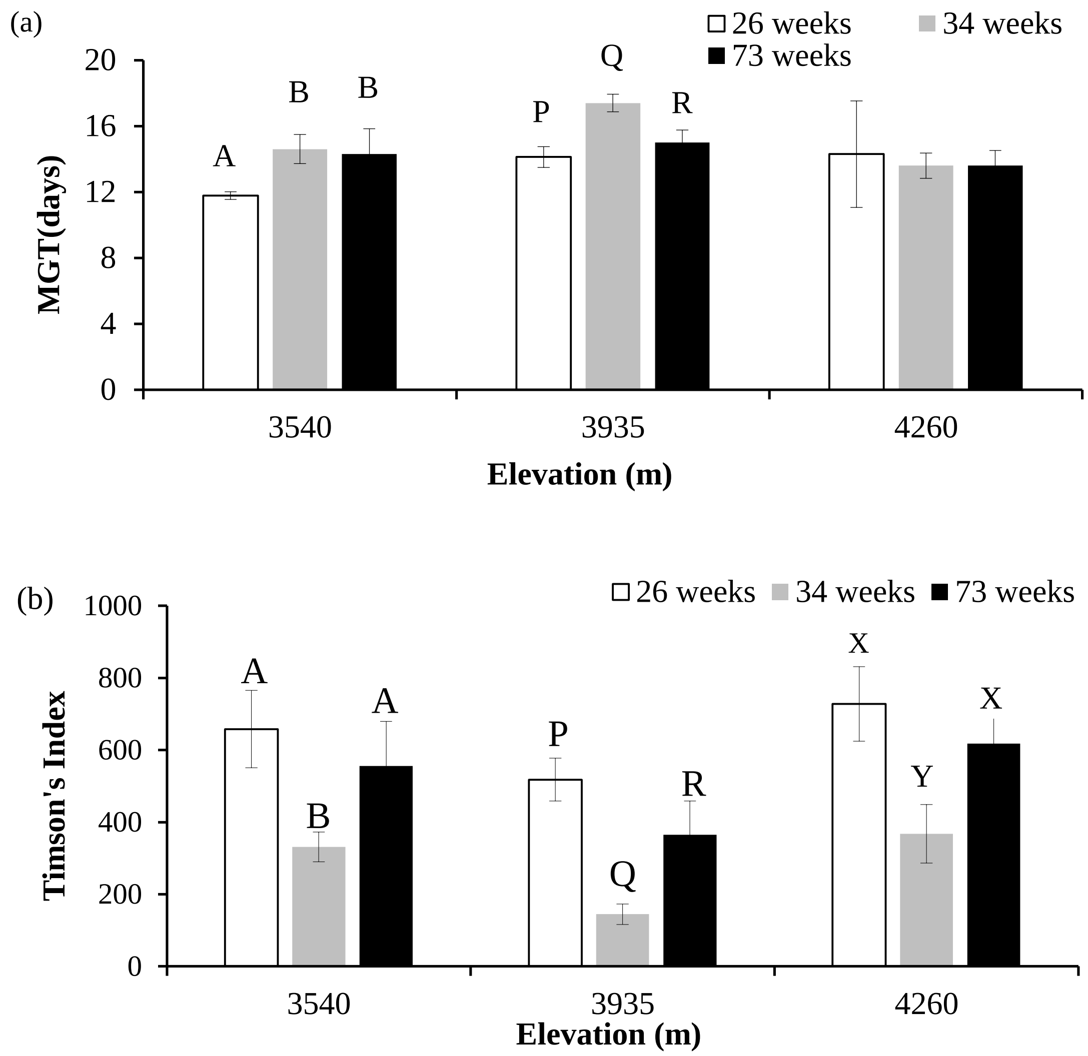


Figure 14. Effect of different storage time period on (a) Mean Germination Time (MGT) (b) Timson's Index (TI) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and different letters (A/B), (P/Q/R) and (X/Y) above bars represents significant difference ($p \leq 0.05$) within different storage time period within each elevation.

B. Effect of water stress

(i) Low elevation (3540 m)

Germination was significantly higher in seeds stored for 73 weeks at control and −0.1 MPa water potential. While comparing germination percentage within different level of water potential, there was no significant difference in germination percentage at control, −0.1 and −0.25 MPa water potential in seeds stored for 34 weeks except at −0.5 MPa water potential. There was significant difference in germination percentage at all level of water potentials in seeds stored for 73 weeks (Fig. 15). MGT was longest at −0.5 MPa water potential for seeds stored for 34 weeks and shortest at control, −0.1 MPa and −0.25 MPa water potential. There was no significant difference in MGT at all level of water potentials (Fig. 15). Timson's Index was significantly higher for seeds stored for 73 weeks (Fig. 15). While comparing TI within different level of water potential, there was no significant difference in TI at control, −0.1 MPa and −0.25 MPa water potential for seeds stored for 73 weeks except at −0.5 MPa water potential. While there was significant difference in TI at control, −0.1 MPa and −0.5 MPa water potential except at −0.25 MPa water potential for seeds stored for 34 weeks.

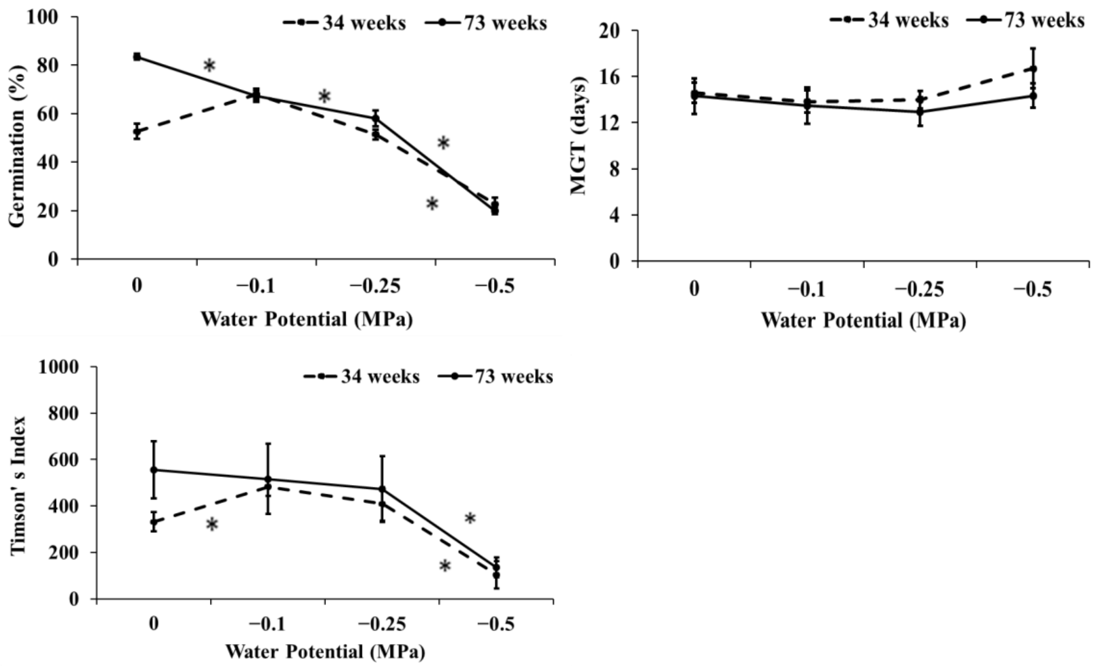


Figure 15. Effect of water stress on Germination percentage (GP), Mean Germination (MGT) and Timson's Index (TI) in (i) low elevation (3540 m) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and '*'represents significant difference ($p \leq 0.05$) within different storage time period at different level of water potential.

(ii) Mid elevation (3935 m)

Germination was significantly higher in seeds stored for 73 weeks. While comparing germination percentage within different level of water potential, there was significant difference in germination percentage at all level of water potentials for seeds stored for 34 weeks. There was significant difference in germination percentage at −0.25 MPa and −0.5 MPa water potential for seeds stored for 73 weeks (Fig. 16). MGT was longest at −0.5 MPa water potential for seeds stored for 73 weeks compared to that MGT was shortest for seeds stored for 34 weeks. There was no significant difference in MGT at all level of water potentials except at −0.5 MPa for seeds stored for 34 weeks (Fig. 16). Timson's Index was significantly higher at control and −0.1 MPa water potential for seeds stored for 73 weeks (Fig. 16). While comparing TI within different level of water potential, there was no significant difference in TI at control, −0.1 MPa water potential for seeds stored for 73 weeks except at −0.25 and −0.5 MPa water potential. There was significant difference in TI at all level of water potentials for seeds stored for 34 weeks.

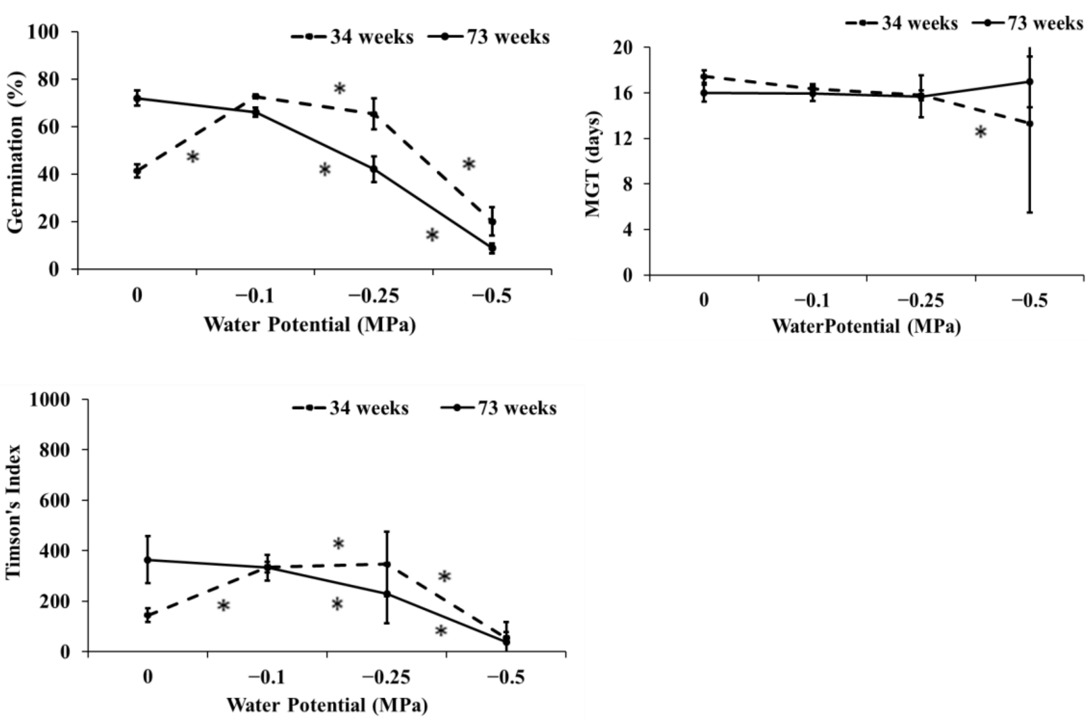


Figure 16. Effect of water stress on Germination percentage (GP), Mean Germination Time (MGT) and Timson's Index (TI) in (ii) mid elevation (3935 m) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and '*'represents significant difference ($p \leq 0.05$) within different storage time period at different level of water potential.

(iii) High elevation (4260 m)

Germination was significantly higher in seeds that were stored for 73 weeks (Fig. 17). While comparing germination percentage within different level of water potential, there was no significant difference in germination percentage in seeds stored for 34 and 73 weeks at all level of water potentials except at −0.5 MPa water potential.

MGT was longest at −0.5 MPa water potential for seeds stored for 34 weeks and shortest at control, −0.1 MPa and −0.25 MPa water potential. There was no significant difference in MGT at all level of water potentials for both storage duration (Fig. 17).

Timson’s Index was significantly higher for seeds stored for 73 weeks (Fig. 17). While comparing TI within different level of water potential, there was no significant difference in TI at all level of water potential for seeds stored for 34 weeks except at −0.5 MPa water potential. While there was significant difference at all level of water potential for seed stored for 73weeks except at −0.25 MPa water potential.

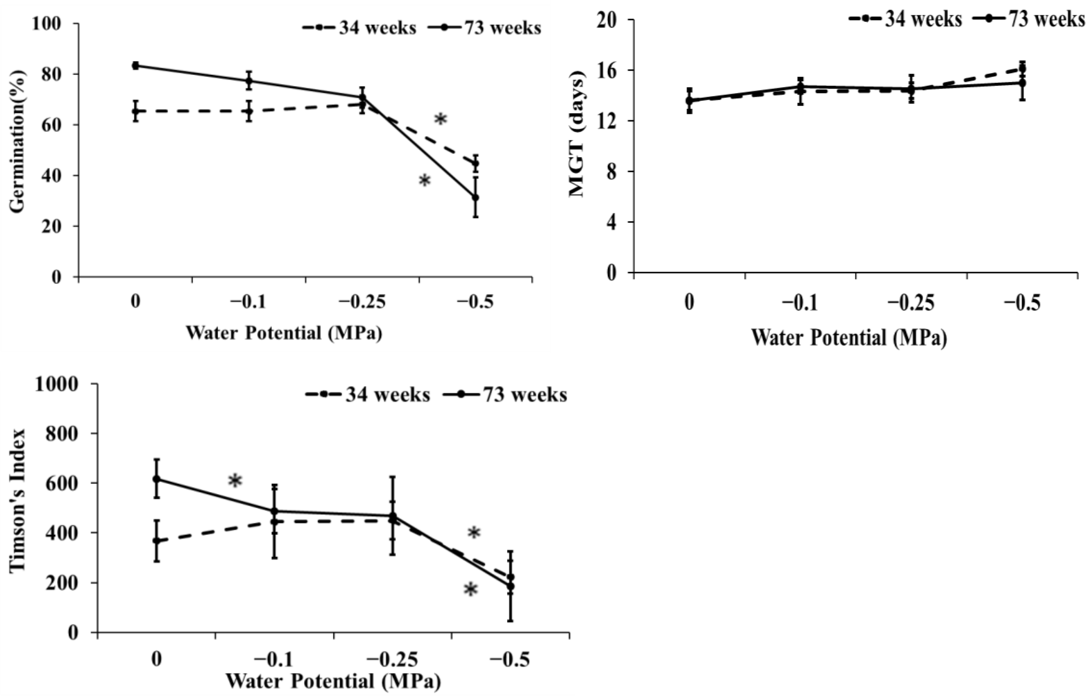


Figure 17. Effect of water stress on Germination percentage (GP), Mean Germination Time (MGT) and Timson’s Index (TI) in (iii) high elevation (4260 m) at photoperiod at low temperature (25/15°C). Error bars represent (±S.D.) of the mean (n=5) and ‘*’represents significant difference ($p \leq 0.05$) within different storage time period at different level of water potential.

Germination percentage, Mean Germination Time, Timson's Index varied significantly with elevation, water potential as well as their interaction (Table 3). The effect of elevation and water potential alone was significantly higher compared to their combined effect on Germination percentage, MGT and TI.

Table 3: Result of two-way ANOVA showing the effect of interactions between elevation (3315 m, 3910 m and 4200 m) and water potential (MPa) on Germination percentage (GP), Mean Germination Time (MGT) and Timson's Index (TI).

	Variables	**df**	**F-value**	**Sig.**
Germination Percentage	Elevation	2	2.03	0.142
	Water Potential	3	114.93	0.000
	Elevation × Water Potential	6	1.14	0.355
Mean Germination Time	Elevation	2	1.40	0.256
	Water Potential	3	15.52	0.000
	Elevation × Water Potential	6	0.94	0.474
Timson's Index	Elevation	2	3.861	0.028
	Water Potential	3	146.79	0.000
	Elevation × Water Potential	6	3.13	0.011

CHAPTER 5. DISCUSSION

The approach of studying germination pattern along different elevation of the seeds of *Aconitum spicatum* shows different germination behavior under different environmental conditions. This gives better knowledge about the adaptation strategy and survival potentiality of the species. Compared to the high temperature, germination was significantly higher at low temperature. Since, seeds shows germination both in photoperiod and dark, we could determine the non-photoblasticity nature of seeds.

5.1 Seed mass

Seed mass is regarded as a vital fitness parameter and a crucial characteristic that affects plant population dynamics and community organization. (Dainese, 2011). Seed mass is influenced by variations in maternal environmental factors like as climate, soil moisture and soil nutrient concentrations (Tremayne and Richards, 2000). In the present study it was found that seed mass of low elevation (3315 m) was higher in comparison to high elevation (4200 m) and mid elevation (3910 m). Species at high elevation face resource limitation for seed production and seed filling as well as temperature determines their reproductive output (Pluess *et al*., 2005). Earlier studies in alpine regions found that seed mass has decreasing pattern at high elevation i.e. at 2040 m-3020 m range (Pluess *et al*., 2005) due to the low temperature, short growing season, low photosynthetic rate, long snow cover that reduces average seed mass.

There is variation amongst populations of *Haloxylon salicornicum* in seed characteristics related to size, dormancy and germination in response to abiotic circumstances (Bhatt *et al*., 2021). This variation aids in the persistence of the species in environments where the timing of favorable conditions for seedling survival is variable (Bhatt *et al*., 2021). Mountain ecosystems are considered most powerful natural ecosystem that exhibits changes in environmental conditions which becomes more severe with increasing elevation (Körner, 2007). Such unfavorable environmental conditions might constrain seed development, reduce seed mass (Pluess *et al*., 2005). Breen and Richards, (2008) showed that seed mass considerably increased with availability of nutrients, but when elevation increases, there are resource restrictions that cause seed mass to decrease.

5.2 Germination pattern along different environmental conditions

Successful germination of any plant species that is under high selection pressure from environmental conditions is important to determine the survival capacity of the plant in a particular area (Bhatt *et al*., 2021; Klupczyńska and Pawłowski, 2021. This will make it easier to understand how the species will act both before and after germination (Bhatt *et al*., 2021; Klupczyńska and Pawłowski, 2021). Several studies on germination experiments indicate that the small seeds of *Panicum racemosum*, *Spartina ciliata* and *Blutaparon portulacoides* have lower germination percentage than larger seeds which demonstrate positive effect of seed mass on germination (Cordazzo, 2002).

In the present study, seeds germinates significantly higher under low temperature regime (25/15°C) than high temperature (30/20°C) and in photoperiod compared to dark. Mean Germination Time (MGT) was longest for high temperature (30/20°C). This explains a wide variation in germination pattern along different environmental conditions. White fluorescent light stimulation on germination was higher than far-red stimulation, which is consistent with a study by Devkota and Jha, (2010). Seeds of *Centella asiatica* whose seeds germinated significantly better in white light than in far-red or blue light indicated inhibitory role of different colors of light on germination (Devkota and Jha, 2010). The results of this finding were in accordance with the present study which showed that seeds of *Aconitum spicatum* showed better germination in white light compared to far-red light. During seed development, phytochrome mediate germination response on the basis of red/far red ratio (Hayes and Klein, 1974).

The present experiment showed that with increasing temperature, the beginning of germination was delayed as well as germination percentage was significantly lower in all the three elevation (Walder and Erschbamer, 2015). In the result of present study, germination of seeds of *Aconitum spicatum* was maximum in light compared to the dark. Kawatani *et al*., (1977), also found that light was preferable to darkness for the growth of *Stevia* seeds. Therefore, light is necessary for the germination of seeds. Žutić and Dudai, (2008) studied the germination of *Salvia officinalis* seed at temperatures of 20°C, 25°C and 30°C under continuous light or in the dark. This result in that the temperature with the highest germination rate (63%) was at 25°C with continuous light. This result was in accordance with the present study where germination was maximum at low temperature (25/15°C) and photoperiod.

In the present study, higher temperature (30/20°C) result in lower germination rates. According to a study by Ter Borg (2005), the germination rates of the high elevation (2500 m) species *Rhinanthus minor, R. alectorolophus* and *R. glacialus* were highest at 4°C and 8°C and lowest at 20°C. This may be due to the changing environmental conditions that could be the disadvantage for alpine species resulting their weaker growth and delay in germination (Walder and Erschbamer, 2015).

The sensitivity of germination also depends on moisture availability i.e. soil water content (Evans and Etherignton, 1990). Drought stress is considered an important environmental factor affecting germination (Channaoui *et al*., 2017). In the present study, all the elevation exhibited significantly higher germination percentage at −0.1 MPa water potential followed by −0.25 and −0.5 MPa. 75% of seeds were able to germinate at −0.1 and −0.25 MPa level of water potential. There was no germination at all in −0.75 and −1 MPa level of water potential. There is variation in germination under different level of water potential. Seeds of all the three elevation shows declination in germination percentage with the increasing level of water stress. Germination decreased significantly with decreasing level of water potential (control > −0.1 > −0.25 > −0.5 MPa). Study on one of the Iranian medicinal plant, *Nepeta persica* explained that germination percentage decreased from 60%-5%, under polyethylene glycol (PEG) solution. This indicates low water potential inhibited germination and growth of that species (Mohammadizad *et al*., 2013). According to the findings by Rohamare *et al*., (2014), water stress (−0.2 MPa) considerably decreased germination of seeds of *Trachyspermum ammi* which concluded that the species is moderately drought-tolerant.

With decreasing level of water potential (increasing PEG concentration), germination of seeds of *Aconitum spicatum* decreased. PEG had a very significant impact on the germination of *Lavandula mairei* seeds, according to the study by Hamdaoui *et al*., (2021). The seeds of *L. mairei* were prevented from germinating by gradually decreasing osmotic potential, by raising the content of PEG. Germination was, in fact, significantly decreased (11.11%) at the concentration of −0.53 MPa. This indicates the low germination potential of these species under drought conditions. Therefore, it suggests the low adaptation and survival potentiality of these species under water stress conditions (Srivastava *et al*., 2010) and the sensitivity of alpine species to drought could have major implications for the future if extreme weather events like drought increases (Zubler *et al*., 2014).

5.3 Germination pattern along elevation gradients

Knowing how intra and inter-population variation affects seed germination can help us better understand these mechanisms and how they relate to seed provenance (Bhatt *et al*., 2021). According to the hypothesis set, in the present study, germination of same species varied among elevation under different environmental conditions which gives explanation about variation along elevation gradients. This might be due to the variable environmental condition of mother plant during seed production and the maternal effect (Giménez-Benavides and Milla, 2012). In the present result, seeds germinated equally in photoperiod and dark. *Juniperus procera* seeds collected from various locations showed better germination performance for seeds incubated under continuous light (Mamo *et al*., 2006). However, seeds of certain populations germinated equally well in both light and darkness, indicating variation in the level of photo-dormancy in *Juniperus procera* seeds by population. As much as 59–61% of the overall difference in the germinability of *Juniperus procera* seeds collected throughout its distribution area was related to population impact, particularly whether incubated in light or darkness (Mamo *et al*., 2006).

The variation in seed mass, light requirement, temperature and drought tolerance during germination in the present study may be caused by differences in the habitat (Table 1), whether maternal or degree of disturbance (Bhatt *et al*., 2021). According to Roach and Wulff, (1987), maternal factors have a significant role in promoting the germination of *Miconia albicans* seeds. Mendes-Rodrigues *et al*., (2010) explained this by pointing out that developing seeds in mother plants are subject to situations that have a significant impact on the result of their germination. Similar to the present study, *Gymnocalycium monvillei* seed germination also responded well to environmental factors such as 12 h photoperiod and temperatures approaching 25°C, with the best germination occurring in the seeds of populations growing at the highest elevation. According to Bauk *et al*., (2017), these findings emphasize the significance of reproductive characteristics in explaining the extensive elevational distribution of this species.

The result of present study revealed the variation in germination among different elevation in alternating temperatures, light and water stress. The low elevation had highest seed mass value and germination percentage followed by high and mid elevation. This specified the hypothesis that was set that there is variation in germination pattern along different elevation under different environmental conditions. Elevation caused a greater initial loss of seed mass during the imbibition process (Mendes-Rodrigues *et al*., 2010). Since Mean Germination Time (MGT) lowers with increasing elevation and germinability similarly rises, it is conceivable to hypothesize that the loss of mass is caused by a loss of phenolic chemicals, shattering the chemical dormancy (Mendes-Rodrigues *et al*., 2010).

In the present study, seeds incubated at low temperature (25/15°C) showed maximum and fastest germination compared to the high temperature (30/20°C). One of the study at the highest elevation sites along the Tunisian ridge, which are home to the coldest *Quercus ilex* populations, had the quickest germination rates at low temperature (Amimi *et al*., 2023). The observed opposing change of these features is suggestive of population-specific ecological strategies and germination niches that are adapted to regional Tunisian climates (Amimi *et al*., 2023).

5.4 Germination pattern along different storage time period

Detail study has been done on systemic storage methods, facilities and seed preservation processes (Koo *et al*., 2003). Longevity of seeds is not universal, it is species specific (Langhu and Deb, 2014). In the present study, seeds stored for long duration (73 weeks) at low temperature (4°C) of low (3540 m), mid (3935 m) and high elevation (4260 m) had shown increment in germination than seeds stored for short duration (26 and 34 weeks). Seed viability was not lost even after long duration of storage at 4°C. The viability of seeds of most of the species declines with time after harvest and seeds exhibit germination differently (Walters, 2004). So, for some species fresh seeds give better germination than stored seeds. This is contrasting outcome in case of this present study, where with increasing storage time period, stored seeds gave better germination. Data collected in the present study showed increment in germination after 73 weeks of storage which is contrasting with the findings of Langhu and Deb, (2014) in case of *Aconitum nagarum* where seeds lost its viability after 6 months of storage.

One of the main factors that affect storage of seeds is initial moisture content of seeds when stored. After reducing the moisture content at specific temperature if seeds are stored and the moisture condition provide information to predict the storability of seeds for further uses. Similar to the present study, in the findings of Dagnachew *et al*., (2023) there was no reduction in germination percentage during storage duration at 4°C for 12 months in 3 species *Euryops pinifolius*, *Kniphofia foliosa* and *Lobelia rhynchopetalum* which demonstrated that storage at low temperature and seed moisture content maintains seed viability (Nyo *et al*., 2019).

In the present experiment, seeds stored at low temperature for long duration showed better germination (73 weeks). Aghilian *et al*., (2014), studied seed storage potential in medicinal plant species and outcome after research was that in *Satureja hortensis* after nine months and twelve months storage germination increased significantly. In *Cynara scolymus*, *Malva sylvestris*, *Plantago psyllium* and *Rudbeckia hirta* also, germination percentage increased after nine months and twelve months of storage period. Increasing germination in these species during storage period showed that their seed dormancy was possibly broken with passing time (Aghilian *et al*., 2014).

CHAPTER 6. CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Among the three elevation, seeds of low and high elevation comparatively showed a better performance in terms of seed germination. Seed mass was also higher in low elevation. In addition, under different environmental conditions, photoperiod, dark, high temperature (30/20°C), low temperature (25/15°C), far-red and white light, higher germination percentage was at low temperature, photoperiod as well in white light compared to far-red. In case of water stress among different level of water potential, there was germination at −0.1, −0.25 and −0.5 MPa and no germination at −0.75 and −1 MPa. The variation in germination under different environmental conditions among three elevation gives explanation about variation along elevational gradients. The findings from this study provides a step ahead information and understanding of the possible impacts of climate change on species at alpine regions.

The interaction of temperature, moisture, seed quality results in the variability on viability of seeds. Here, in the present study, seeds even after several weeks of storage showed increment in germination which concluded that using appropriate storage procedures we can sustain viability of seeds following better germination even after long time. The same species living at different elevations produce seeds with varied germination and recovery responses after alleviating the water stress, which may be essential for species survival in this hostile environment. In this study, we found that germination responses varied widely among elevational provenances and that there isn't always a consistent pattern related to elevation. Another significant finding of the current study is that characterizing the germination response of a species by looking at just one population may not be sufficient since, as it showed that surrounding environmental factors may have an impact on seeds. Therefore, the observed variation in how seeds respond to changes in temperature, water potential and light appears to have adaptive value.

6.2 Recommendation

The seed germination and seed characteristics of himalayan medicinal plants might be taken into account when assessing how vulnerable climate change may be for its potential impact on the survival of the species like *Aconitum spicatum*.

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APPENDICES

Appendix 1: Effect of temperature on Germination Percentage (GP) at photoperiod (Mean±S.D.)

Elevation (m asl)	High temperature (30/20°C)	Low temperature (25/15°C)
3315	$12±2.19^{*B}$	$82±2.41^{*X}$
3910	$25.33±3.29^{*A}$	$75.33±3.44^{*X}$
4200	$13.33±1.87^{*AB}$	$76.67±1.87^{*X}$

'*' represents significance level at $p≤0.05$ (t-test). Alphabets 'X' represents significant difference for low temperature (25/15°C) and (A/B) for high temperature (30/20°C) (one-way ANOVA).

Appendix 2: Effect of temperature on Germination Percentage (GP) at complete dark (Mean±S.D.)

Elevation (m asl)	High temperature (30/20°C)	Low temperature (25/15°C)
3315	$9.3±2.17^{*A}$	$72±2.30^{*X}$
3910	$11.3 ±1.85^{*A}$	$64±5.12^{*X}$
4200	$8.7±1.52^{*A}$	$76±2.17^{*X}$

'*' represents significance level at $p≤0.05$ (t-test). Alphabets 'X' represents significant difference for low temperature (25/15°C) and 'A' for high temperature (30/20°C) (one-way ANOVA).

Appendix 3: Effect of temperature on Mean Germination Time (MGT) and Timson's Index (TI) at photoperiod (Mean ± S.D.)

Elevation (m asl)	Low temperature (25/15°C)		High temperature (30/20°C)	
	MGT	TI	MGT	TI
3315	$14.55±0.49^{*X}$	$528.67±6.12^{*X}$	$16.11±2.52^{*A}$	$54.67±32.37^{*B}$
3910	$14.57±0.54^{*X}$	$488±67.44^{*X}$	$15.8±0.95^{*A}$	$128±65.26^{*A}$
4200	$14.25±0.54^{*X}$	$518.67±67.44^{*X}$	$16.13±1.39^{*A}$	$60±17.79^{*B}$

'*' represents significance level at $p≤0.05$ (t-test). Alphabets 'X' represents significant difference for low temperature (25/15°C) and 'A/B' for high temperature (30/20°C) (one-way ANOVA).

Appendix 4: Effect of far-red light on Germination Percentage (GP) at photoperiod at low temperature (25/15°C) (Mean ± S.D.)

Elevation (m asl)	Low temperature (25/15°C)	
	White light	Far-red
3540	83.33±1.22^{*A}	73.33±2^{*X}
3935	72±3.21^{B}	67.33±2.28^{X}
4260	83.33±1.22^{*A}	72±2.61^{*X}

'*' represents significance level at $p \leq 0.05$ (t-test). Alphabets 'X' represents significant difference for far-red and 'A/B' for white light (one-way ANOVA).

Appendix 5: Effect of water stress on Germination Percentage (GP), Mean Germination Time (MGT) and Timson's Index (TI) at low temperature (25/15°C) at different water potentials (Mean ± S.D.)

Elevation (m asl)	Parameters	0	−0.1	−0.25	−0.5
3315	GP	82±2.41	83.33±1.22	59.33±6.42	18±1.52*
	MGT	14.55±0.5	12.46±0.4	15.48±1*	16±0.61*
	TI	528.67±62	579.33±37	316.67±83*	90±28.9*
3910	GP	75.33±3.44	75.33±3.4	66±2.2	13.33±2.55*
	MGT	14.57±0.5	15.24±0.3*	15.39±1	16.35±2
	TI	488±103	433.33±47	368.67±39	55.4±36*
4200	GP	76.67±1.87	67.33±2.59	58.7±3*	17.33±1.6*
	MGT	14.25±0.5	14.82±0.7	15.72±1.11*	16.79±1*
	TI	518.67±67	416.67±77	307.33±68*	72.67±2*

'*' represents significance level at $p \leq 0.05$ (t-test).

Appendix 6: Effect of different storage time period on Germination Percentage (GP) at photoperiod at low temperature (25/15°C) (Mean ± S.D.)

Elevation (m asl)	Storage time period	GP
3540	26 weeks	71.33 ± 3.21^{A}
	34 weeks	52.67 ± 3.11^{B}
	73 weeks	83.33 ± 1.2^{A}
3935	26 weeks	75.33 ± 1.7^{P}
	34 weeks	41.33 ± 2.9^{Q}
	73 weeks	72 ± 3.2^{P}
4260	26 weeks	86.67 ± 2^{X}
	34 weeks	65.33 ± 3.9^{Y}
	73 weeks	83.33 ± 1.2^{X}

Different letters (A/B), (P/Q) and (X/Y) above bars represents significant difference ($p \leq 0.05$) (one-way ANOVA).

Appendix 7: Effect of different storage time period on Germination Percentage (GP) at complete dark at low temperature (25/15°C) (Mean ± S.D.)

Elevation (m asl)	Storage time period	GP
3540	26 weeks	51.33 ± 3.9^{A}
	34 weeks	20.67 ± 1.8^{B}
	73 weeks	70.67 ± 5.1^{C}
3935	26 weeks	56 ± 3.7^{P}
	34 weeks	23.33 ± 2.9^{Q}
	73 weeks	56 ± 2.1^{P}
4260	26 weeks	90 ± 1.2^{X}
	34 weeks	36 ± 1.3^{Y}
	73 weeks	65.33 ± 3.8^{Z}

Different letters (A/B/C), (P/Q) and (X/Y/Z) above bars represents significant difference ($p \leq 0.05$) (one-way ANOVA).

Appendix 8: Effect of different storage time period on Mean Germination Time (MGT) at photoperiod at low temperature (25/15°C) (Mean ± S.D.)

Elevation (m asl)	Storage time period	MGT
3540	26 weeks	11.79±0.23^{A}
	34 weeks	14.6±0.9^{B}
	73 weeks	14.3±1.5^{B}
3935	26 weeks	14.13±0.6^{P}
	34 weeks	17.4±0.5^{Q}
	73 weeks	15±0.8^{R}
4260	26 weeks	14.3±3.2
	34 weeks	13.6±0.8
	73 weeks	13.6±1

Different letters (A/B) and (P/Q/R) above bars represents significant difference ($p \leq 0.05$) (one-way ANOVA).

Appendix 9: Effect of different storage time period on Timson's Index (TI) at photoperiod at low temperature (25/15°C) (Mean ± S.D.)

Elevation (m asl)	Storage time period	TI
3540	26 weeks	658±107.3^{A}
	34 weeks	331.33±41.3^{B}
	73 weeks	556±123.3^{A}
3935	26 weeks	518±59.5^{P}
	34 weeks	144.67±28.2^{Q}
	73 weeks	364.67±93.9^{R}
4260	26 weeks	728±103.4^{X}
	34 weeks	367.33±81.3^{Y}
	73 weeks	618±78.1^{X}

Different letters (A/B), (P/Q/R) and (X/Y) above bars represents significant difference ($p \leq 0.05$) (one-way ANOVA).

Appendix 10: Effect of different storage time period on Germination Percentage (GP) at photoperiod at low temperature (25/15°C) under different water potential (MPa) (Mean ± S.D.)

Elevation (m asl)	Storage time period	0	−0.1	−0.25	−0.5
3540	34 weeks	52.7±3.11	68±2.2	51.33±1.95	22.7±2.59*
	73 weeks	83.3±1.22	67.3±2.49*	58±3.36*	20±1.41*
3935	34 weeks	41.3±2.88	72.7±0.84*	65.3±6.58*	20±6.04*
	73 weeks	72±3.21	66±1.92	42±5.37*	8.7±2.1*
4260	34 weeks	65.3±3.91	65.3±3.91	68±3.51	44.7±3.2*
	73 weeks	83.3±1.22	73.3±1.39	70.7±4.02	31.3±7.76*

'*' represents significance level at $p \leq 0.05$ (t-test).

Appendix 11: Effect of different storage time period on Mean Germination Time (MGT) at photoperiod at low temperature (25/15°C) under different water potential (MPa) (Mean ± S.D.)

Elevation (m asl)	Storage time period	0	−0.1	−0.25	−0.5
3540	34 weeks	14.59±0.88	13.84±0.48	13.98±0.76	16.69±1.72
	73 weeks	14.30±1.54	13.47±1.59	12.95±1.23	14.34±1.04
3935	34 weeks	17.44±0.54	16.37±0.42	15.78±0.45	13.92±7.8*
	73 weeks	15.98±0.75	15.96±0.66	15.69±1.86	16.97±2.23
4260	34 weeks	15.21±0.76	14.32±1.03	14.37±0.64	16.08±0.56
	73 weeks	13.58±0.93	14.7±0.51	14.51±1.04	14.98±1.34

'*' represents significance level at $p \leq 0.05$ (t-test).

Appendix 12: Effect of different storage time period on Timson's Index (TI) at photoperiod at low temperature (25/15°C) under different water potential (MPa) (Mean ± S.D.)

Elevation (m asl)	Storage time period	0	−0.1	−0.25	−0.5
3540	34 weeks	331.3±41	482.6±39*	408.6±72.9	103.3±58*
	73 weeks	556±123	516.7±151	472.7±141.7	134.7±42.8*
3935	34 weeks	144.7±28	335.3±21*	346.7±128.5*	69.9±62.7*
	73 weeks	364.7±94	332.7±50.1	230±116.8*	38±40.2*
4260	34 weeks	367.3±81	445.3±146.5	448.7±75.5	222±66.9*
	73 weeks	618±78	488±89.3*	468.7±155.4	185.3±139*

'*' represents significance level at $p \leq 0.05$ (t-test).

Appendix 13: Poster presented at International Conference on Biodiversity and Bioprospecting organized by Department of Plant Resources (DPR), Thapathali, Kathmandu.

Germination responses of *Aconitum spicatum* to different Environmental Gradients

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Introduction

•Seed germination- useful parameter comprising a complex phenomenon of biophysiological and chemical changes leading to activation of embryo (Parihar *et al.*, 2014).
•Seed ecology and germination patterns determine range limits, species diversity, adaptation to environmental variation and community responses to climate change (Huang *et al.*, 2016).
•Seed traits i.e. seed size, seed coat permeability, light and temperature requirement determines their establishment and persistence in environment (Saatkamp *et al.*, 2018).

Objectives

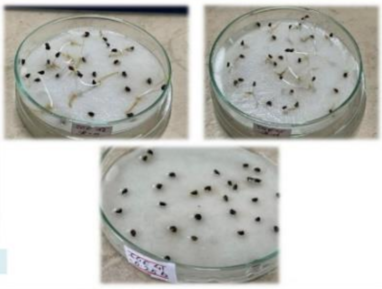
•To evaluate the germination rate under a wide range of environmental conditions.
•To develop appropriate conservation strategies for Aconitum of Annapurna Conservation Area.
•To understand the impacts of different environmental changes to this species.

Materials and Methods

Study Species
▪*Aconitum spicatum*: Herbaceous perennial, vulnerable mountainous medicinal plants restricted to the Himalayas of Nepal, China, and India at elevation 2900-4200m above sea level (Ghimire *et al.*, 1999).
▪Fruit, an aggregate of five to six follicles with average of 41 seeds; an individual plant with about 574 seeds.

Methods
•Seeds of *Aconitum spicatum* were collected from three different elevation i.e.4260m, 3935m and 3540m.
•Germination was examined under different environmental conditions complete dark and light conditions (Prakash *et al.*, 2011) low (25/15˚c) and high temperature (30/20 ˚c) and varied osmotic stress (-0.1, -0.25, -0.5, -0.75 and -1Mpa).
•Germination Percentage (GP), Mean Germination Time (MGT) (Kandari 2008), Timson Index (TGI), Coefficient of Velocity from Germination (CVG) were calculated from experimental data showed variation in germination patterns in three different elevations.

Aconitum spicatum



Seeds of *A. spicatum* during germination period

Result & Discussion

▪Germination percent was highest (86.67%) in the seeds collected from the highest elevation under control condition.
▪Seed germination percent was slightly higher at low temperature (78.67%) than at high temperature (64.22%).
▪Seeds showed fairly high germination (50.22%) even under dark that indicates its wide range of growing conditions (Vandelook *et al.*, 2009).
▪Seeds subjected to mild water stress (0.75MPa) showed germination but there was no germination at high water stress(-1MPa).
▪Comparatively, even under water stress (-0.75MPa) seeds showed better germination than low water stress(-0.5MPa) (Santo *et al.*, 2014).

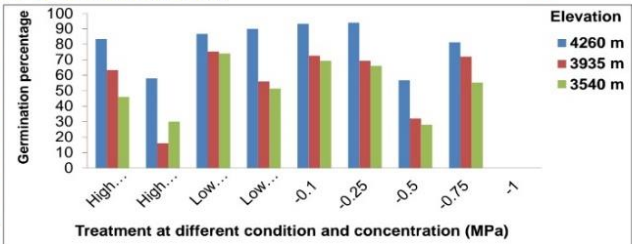


Fig:1 Average Germination Percentage

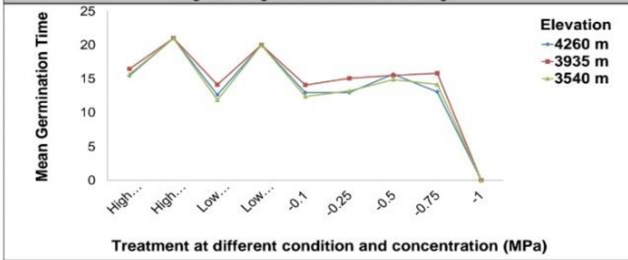


Fig:2 Average Mean Germination Time

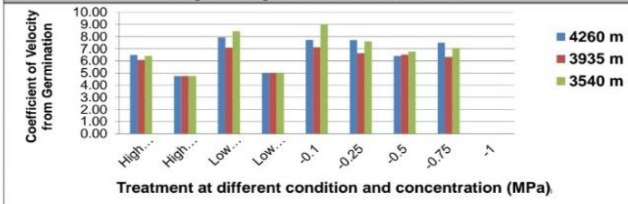


Fig:3 Average Coefficient of velocity from Germination

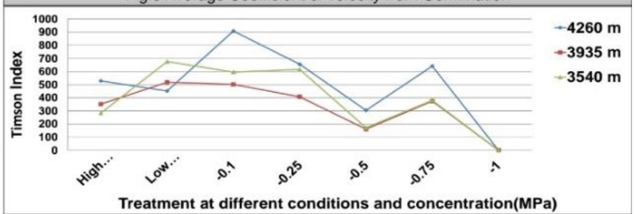


Fig:4 Average Timson Index

Conclusion

•*A. spicatum* seeds showed fair germination under a wide range of temperature, light and water stress conditions which suggests that the species may grow under a wide range of environmental conditions.
• Further field experiment and laboratory analysis can be more efficient and effective

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PHOTOPLATES

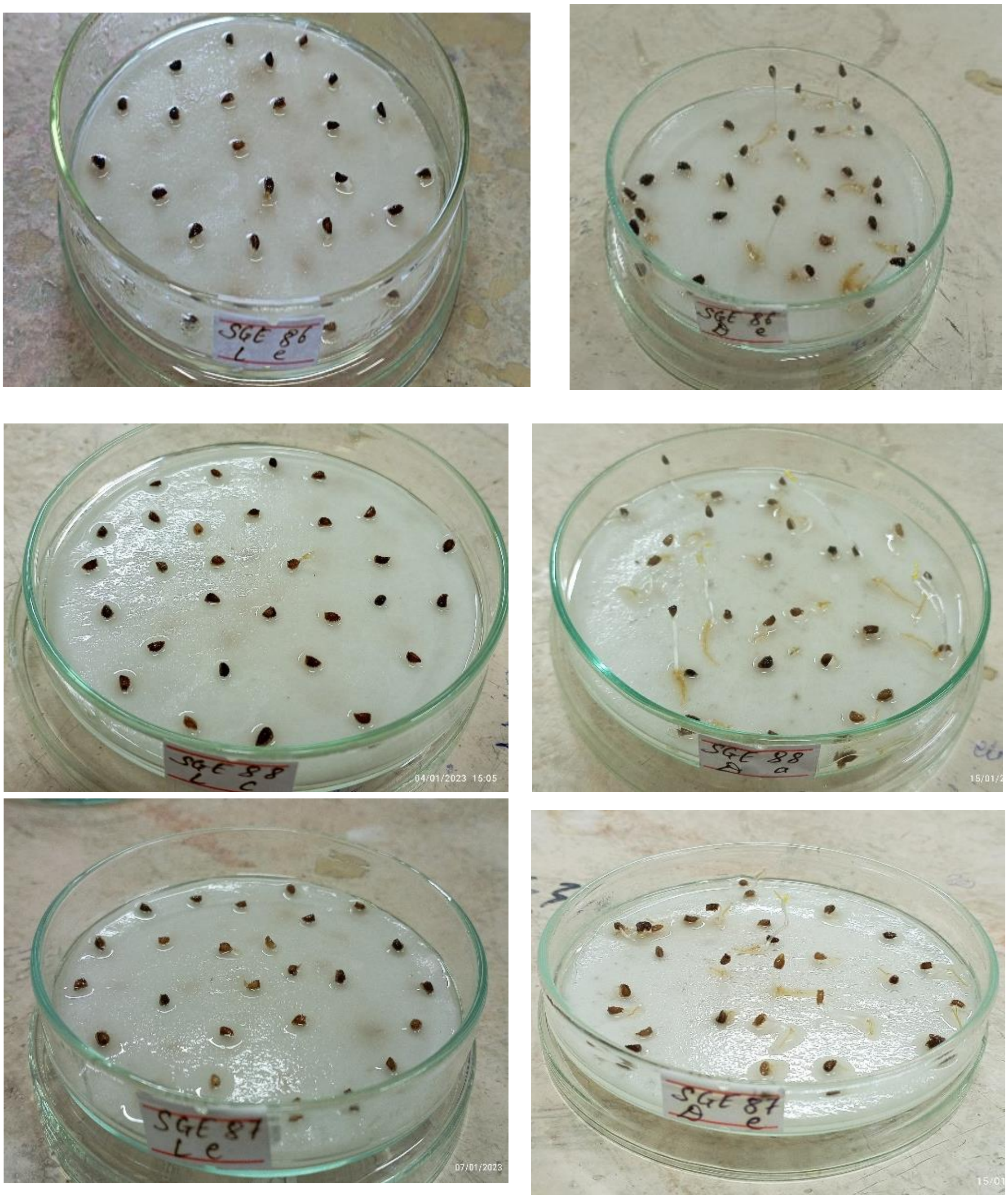

Photo plate 1: Effect of photoperiod and dark

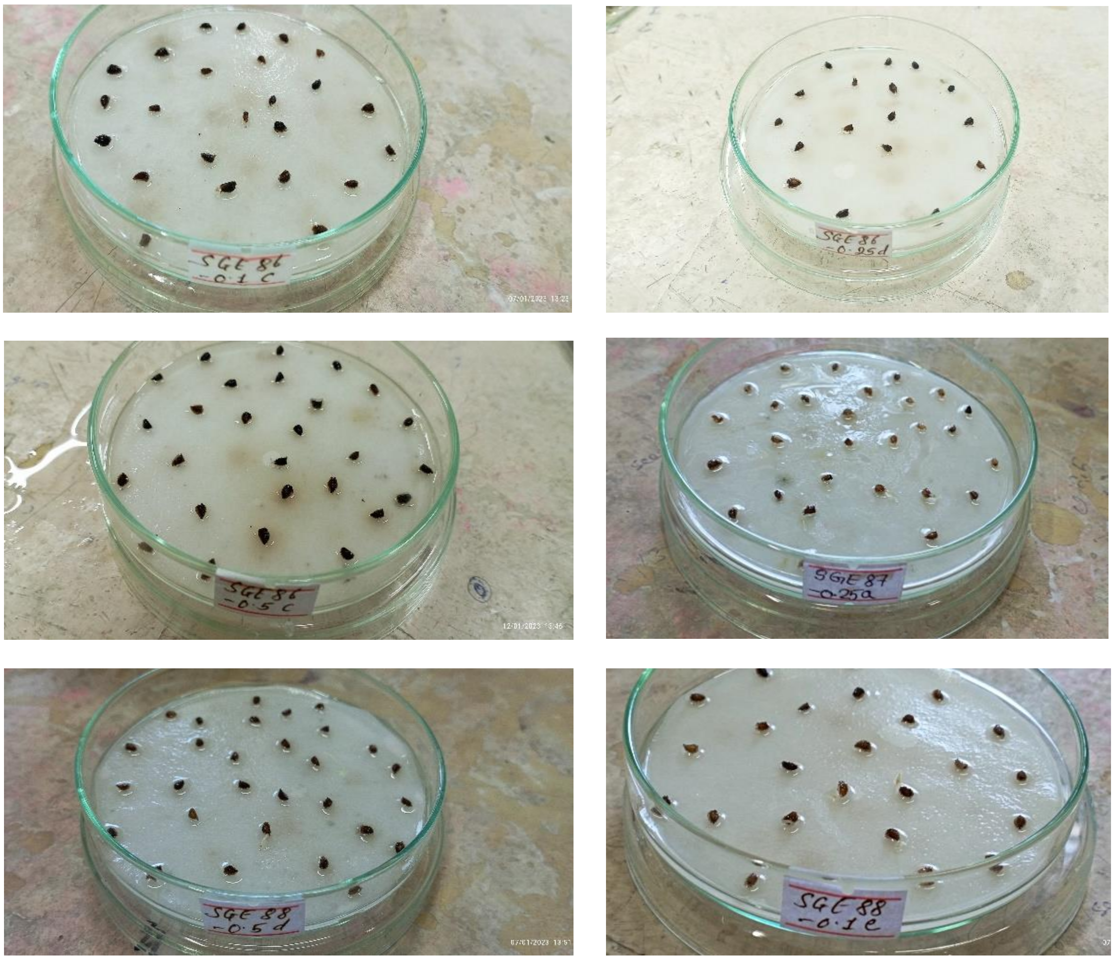

Photo plate 2: Effect of water stress

Photo plate 3: Effect of far-red light

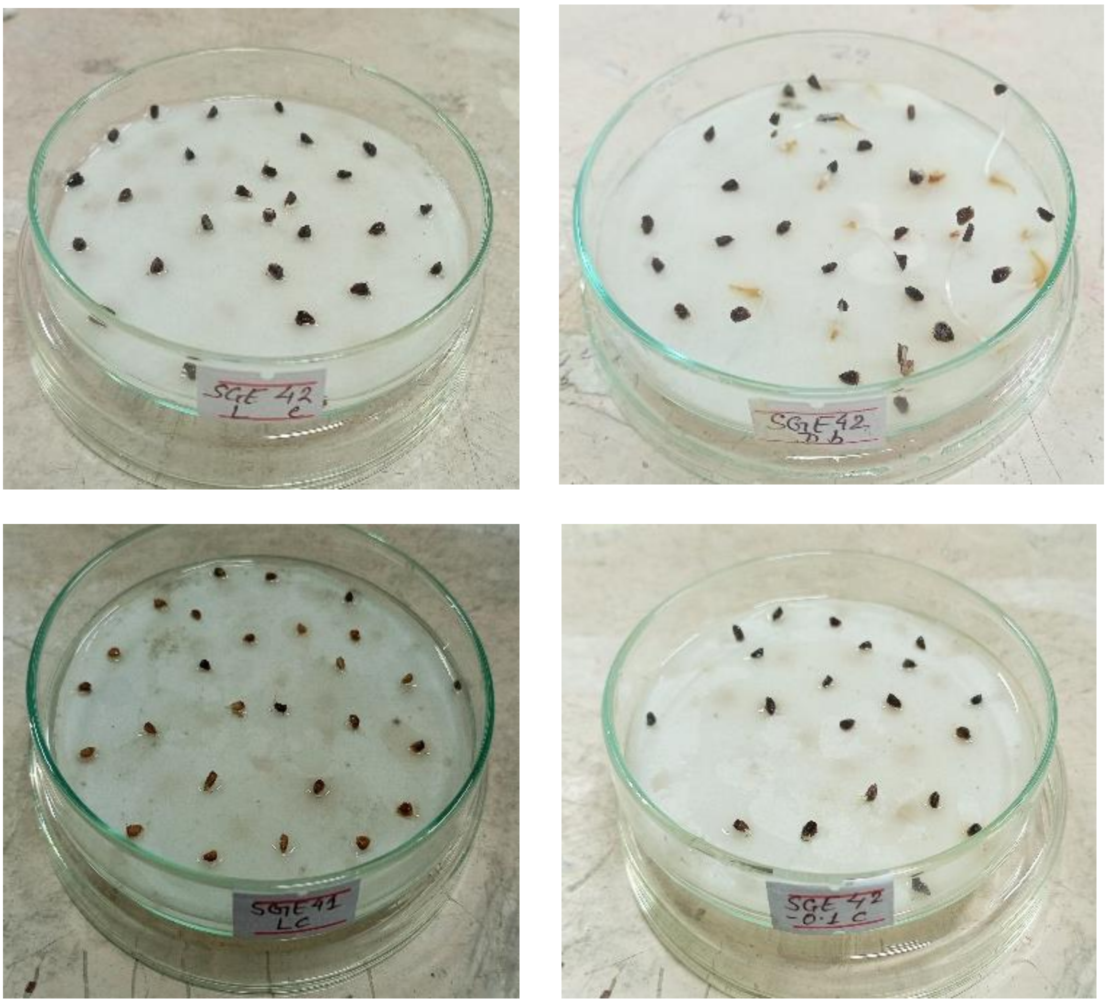

Photo plate 4: Effect of different storage time period

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Ref: 90 /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:
NTNC-ACAP Unit Conservation Office
Ghandruk / Lwang / Manang / Jomsom / Lomanthang

Central Office : P.O. Box 3712
Khumaltar, Lalitpur, Nepal
Tel. No. : 00977-1-5526571, 5526573
Fax : 00977-1-5526570
Website: www.ntnc.org.np

Headquarters : P.O. Box 183
Pokhara, Kaski, Nepal
Tel. No. : 00977-61-431102, 430802, 432288
Fax No. : 00977-61-431203
E-mail : info@acap.org.np

नेपाल सरकार

वन तथा वातावरण मन्त्रालय

राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण विभाग

(.. शाखा)

फोन नं. : ४२२०८५०, ४२२०९१२, ४२२७९२६

फ्याक्स नं. ४२२७६७५

पो. ब. नं. - ८६०

बबरमहल, काठमाडौं

Email: info@dnpwc.gov.np

http//:www.dnpwc.gov.np

पत्र संख्या :- 32
०७८/७९ इको

चलानी नं :- 280

मिति: २०७८/५/२०

विषय: 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	इमेल: **Shresthabb@gmail.com**	फोन नं. ९८४१२४१४८४
सम्बद्ध संस्था	वनस्पतिशास्त्र केन्द्रीय विभाग, त्रिभुवन विश्वविद्यालय, किर्तिपुर, काठमाण्डौं ।		
अनुसन्धानको प्रकृती	व्यक्तिगत		
पद	सह-प्राध्यापक		
अनुसन्धानको तह	राष्ट्रिय स्तर		
अनुसन्धानको शिर्षक	**Impact of climate change on germination, growth, polliantion and distribution of Himalayan medicinal herbs *Aconitum spicatum* and *A. naviculare***		
अनुसन्धान विधि	Transplant Experiment in field	नमुना संकलन गर्ने	नमुना परिक्षण कहाँ गर्ने नेपालमा
अनुसन्धानको अवधि	२० भाद्र २०७८ देखि १९ भाद्र २०८० (दुई वर्ष)		

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 रोहवरमा 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 इलेक्ट्रोनिक 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 विधुतीय प्रतिवेदन सम्बन्धित कार्यालय र विभागमा बुझाउनु हुन अनुरोध छ ।

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

वनस्पतिशास्त्र केन्द्रीय विभाग, त्रि.वि: जानकारीको लागि अनुरोध छ ।