Studies on Certain Reproductive Behaviours of *Aconitum nagarum* Stapf and *Dioscorea villosa* L. and Their *In Vitro* Propagation

By

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Contents

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DECLARATION

I, Ms. Tabitha Langhu, bearing Ph. D. Registration number 471/2012 dated October 14, 2011 hereby declare that, the subject matter of my thesis entitle **'Studies on certain reproductive behaviours of *Aconitum nagarum* Stapf and *Dioscorea villosa* L and their *in vitro* propagation'** is the record of original work done by me, and that the contents of this thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. The thesis has not been submitted by me for any Research Degree in any other University/Institute.

This thesis is being submitted to the Nagaland University for the Degree of **'Doctor of Philosophy' in Botany**.

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General Introduction

Biodiversity is the variety and variability of flora and fauna in an ecosystem (Roy and Sameer, 2004). Thus biodiversity is the sum total of all species of plants and animals and generally, is greatest in the oldest ecosystem (Roy and Behera, 2002). The term biodiversity was first coined by Walter in 1985 while planning the '*National Forum on Biological Diversity'* organized by the National Research Council (NRC), Washington which was held in 1986. Biodiversity is concentrated in the tropics, around the equator and decrease toward the Polar Regions. The concept of biodiversity cuts across the disciplinary boundaries. It is an environmental issue with sub-division for the quality of human life and yet there remains a great deal waiting to be discovered for human use. The earth's biodiversity is the result of 4 billion years of evolution and it is estimated that the species diversity living in our planet is about 5 to 50 million, out of which only 14,35,662 species have been described so far. But the rate of species lost greatly exceeds compared to origin of new species due to various factors. The human population has witnessed a three-fold increase in the last century and the rate of fossil

fuel consumption has increased by 12 fold during the period, by which the carrying capacity of earth would saturate by the middle of this 21st century (Myers, 1990). According to IUCN (IUCN 2000, *Red List of Threatened Species*, Switzerland: The World Conservation Union), plant species are declining in South and Central America, Central and West Africa and Southeast Asia. Malaysia has the most threatened species (681) followed by Indonesia (384). Globally, the number of threatened plants listed is 5,611, but this is based on an assessment of only 4% of the world's described plants, which suggest that the percentage of threatened plants may be much higher. The recent report of IUCN brings out a list of 34 biodiversity hotspot regions of the world. Biodiversity hotspots are geographical regions which are extremely rich in species, have high endemism, and are under constant threat. There has been a rapid decline in the biodiversity of the world. Biodiversity losses have been alarming in the developing countries in tropics. Therefore, indicating an alarming situation the world is faced with, in terms of biodiversity resource vis-à-vis future of mankind.

Among the 34 hotspot of the world, India is a home for 4 of them extending into neighbouring countries-the Western Ghats/Sri Lanka, the Himalaya, the North-East and the Nicobar Island (MoEF, 2014) and they figure in hottest hotspots. India is also one of the 17 mega biodiversity countries and has 26 recognized endemic centre that account for nearly a third of the flowering plants, though it constitute only 2.4% of land mass. India harbours about 45,000 species of plant which include 15,000 flowering plants, 5000 species of algae, 2700 of bryophytes, 1600 of lichens, 20,000 of fungi, 600 of pteridophytes. It has also 75,000 species of animals including 50,000 insects, 2000 fish, 4000 molluscs, 140 amphibians, 1200 birds and mammals, 420 reptile accounting for 6.5% of the world known biodiversity (Kushwaha and Behera, 2002). Therefore, biological diversity must be treated more seriously as a national as well as a global

resource, to be induced, used and above all preserved because the greater the biodiversity in ecosystem, species and individual leads to greater stability. For example high population adapted to a wide variety of conditions is more able to face weather disturbances, diseases and climatic change. Greater biodiversity also enrich us with more varieties of foods and medicine especially plants product and have been used in the traditional health care system from the time immemorial, particularly among tribal communities. The World Health Organization (WHO) has listed over 20,000 medicinal plants globally (Laloo *et. al.*, 2006) of which India's contribution are ~20%. According to an estimate of WHO about 80% of the population in the developing countries depends directly on plants for their sole source of medicine (Deb *et. al.*, 2009). In India, about 2000 drugs used are of plant origin (Dikshit, 1999). Though a vast ethnobotanical knowledge exists in India from ancient times, very few plants used by locals for medicine are subjected to scientific investigation. The need for conservation of medicinal plants and traditional knowledge, particularly in developing countries like India, taking into account the socio-cultural and economic conditions is urgent (Misra, 1999).

Northeast India is a centre of mega-biodiversity which is equally rich in flora and fauna; contains more than one-third of the country's total biodiversity. It lies between 22º9' - 29º6' N latitude and 87º7' E longitude. North-East India is the most important floristic region owing to its rich biodiversity and high endemism. It is known for its diverse and extensive lush forest cover and species composition, but is one of the major regions facing severe deforestation. The region is one of the 17 hottest hotspots of the world, having at least 7,500 flowering plants. Out of 315 families of angiosperms in India, more than 200 are represented in Northeast India; thus, accounting for nearly 50% of the total number of plant species in India as a whole. The region is considered a

meeting region of temperate east Himalayan flora, paleo-artic flora of Tibetan highland and wet evergreen flora of South East Asia and Yunnan, forming a bowl of biodiversity. For this rich biodiversity, Takhtajan (1969) named the North-East region *'The Cradle of Flowering Plants'*. The region also host a number of plant species which are endemic to the region. Though most of the species are of economic importance, some of the species plays a vital role in rural economy and used as medicines, vegetables, construction materials, as dye etc. Some of the important economically plant species are: *Sapria himalayana, Nepenthes khasiana, Rhododendron, Aloe vera, Dioscorea* sps., *Aconitum nagarum., Malaxis acuminate, Panax pseudoginseng, Strobilanthes flaccifolious, Taxus bacata, Berberis* sps., *kiwi, Lassia spinosa, Livistona jenkinsiana, Distemon indicum, Acorus calamus, Tricopus zeylanicus* etc. But the population of these economically important plant species are done sized in the natural habitat due to various anthropogenic activities which is often related to the developmental projects like land conversion, construction of dams, over harvesting, pollution, inappropriate and often accidental introduction of exotic plants and animals, etc.

Biodiversity is also lost due to sudden natural calamities like floods, cyclones, hurricanes, earthquakes, reproductive bottlenecks etc. Reproductive bottlenecks includes failure of pollination, pre or post fertilizer barriers leading to no or poor seed set, poor reproductive vigour due to inbreeding depression and very low germination rates imposed constraints on the multiplication and survival of the species. Therefore, any conservation approach has to be based on an in-depth study of plant reproductive biology which provides information on seed germination capacity, survival rate of seedlings and adults, age at flowering, reproductive lifespan and number of flowers and seeds, seed biology. Such studies would prove to be fruitful in planning various

programmes specific to different habitats. The knowledge combined with *in vitro* propagation/micropropagation will also help in developing strategies to preserve the genetic potential of rare species crucial for reintroduction.

Reproductive biology

Understanding the reproductive behaviour of plant is essential for developing effective strategies for their sustainable utilization and conservation. The knowledge of reproductive study is a prerequisite for attempting any breeding programmed. This recognition has made reproductive study a frontline area of research. There has been a profound change in this area of research from the traditional method with the incorporation of techniques of cell and tissue culture, physiology, molecular biology, biochemistry etc. Therefore reproductive studies is an in-depth study of plant phenology, floral biology, floral morphology, mode of pollination, reproductive output (seed ovule ratio), seed biology (seed germination and seed viability) and seed to seed cycle. These help in pinpointing the bottlenecks, if any, in the life cycle of a species, which reduce its reproductive capacity and ultimately threaten its survival.

Studies on the phenology of medicinal plants are the basic knowledge to be obtained for the right season for collecting the medicinal parts and propagules and for establishing the appropriate growth environment for propagation purposes (Abera *et. al.*, 2008). It also helps in formulating conservation strategy of the species. The knowledge of reproductive biology is one of the most important aspects that should be considered in plant species selection for habitat restoration. Reproductive biology also helps in providing information on life form, rate of flowering, plant pollinator interaction, fruit and seed output, overall fitness and survival of the species (Singh *et. al.*, 2010).

The reproduction of plants largely relies on their interaction with pollinators, fruit and seed dispersers. The morphological and physiological features of the species thus evolve in order to fit into pollinator's interest (Silva and Silingardi, 2001); hence any change in the ecological parameters can affect breeding cycle and flowering (Moza and Bhatnagar, 2007). As failure of reproductive processes to cope with environmental changes is often the fundamental reason for species loss as sexual reproduction is the only natural process that incorporates variability and ensures survival of species under adverse conditions. Successful fertilization is also depending on effective pollination because pollination is the pre-requisite for the survival of plants species in natural communities.

Temperature and light also has various effects on seed germination and dormancy of economically plants (Okagami and Kawai, 1982; Shehbaz and Schubert, 1989; Terui and Okagami, 1993; Walek *et. al.,* 2001; Ren *et. al*., 2005; Albrecht, 2006; Onen, 2006; Deb *et. al.*, 2012). The knowledge of plant species phenology, pollination, seed dispersal can managed natural and disturbed areas for conservation and restoration (Ramirez, 2006). Understanding of plant reproduction is also important for biotechnology, the conservation of biodiversity (Barrett, 2010). Many of the micro propagated plants in culture that entails positive result in the laboratories fails to take off in the field due to lack of information about the reproductive features (Moza and Bhatnagar, 2007).

Seed biology

The natural lifespan of a seed is influence by several factors including: permeability of the seed coat, dormancy, seed physiology and storage environment. Inside each seed is a living plant embryo that even in a state of dormancy breathes

through the exchange of gasses and is consistently undergoing metabolic (aging) process. Seeds of many of our native plants and weedy alien species have dormant embryos and hard seed coats, a condition that retards germination and consequently enhance longevity. The presence and degree of seed dormancy and subsequent metabolic rates varies considerably between and thus influences their lifespan. For most species from temperate and arid climates reducing and maintaining a low seed moisture content, storing seeds at low to moderate temperatures, and taking precaution not to damage seeds during cleaning and handling, slows down the metabolic process and thereby increases their longevity in storage.

Seeds are generally categorized into three types:

1. **Orthodox seeds**: The seeds that can be dried, without damage, to low moisture contents, usually much lower than those they would normally achieve in nature. Their longevity increases with reduction in both moisture content and temperature over a wide range of storage environments.

2. **Recalcitrant seeds**: Those seeds that do not survive drying to any large degree, and are thus not amenable to long term storage.

3. **Intermediate seeds**: Those seeds that are more tolerant of desiccation than recalcitrant, though that tolerant is more limited than is the case with orthodox seeds, and they generally lose viability more rapidly at lower temperature.

Recalcitrant seeds are not only desiccation-sensitive, but also metabolically active. In contrast, orthodox seeds, owing to their dry state, are metabolically quiescent (Berjak, 2005). One can estimate a species natural potential for storage tolerance by:

1. **Seed size**: Seeds that are often large have high moisture content and are generally recalcitrant in their storage behaviour.

2. **Climate and habitat conditions in which the species grow**: Seeds from plants adapted to tropical or riparian habitats, due to a semi to permanent water source and/ or consistently mild and reliable growing conditions, may not require long term seed viability for survival. On the other hand, plants from desert, temperate climates, where environmental conditions suitable for germination are often infrequent, are more likely to produce seeds capable of surviving for long periods.

3. **Seeds physiology**: A heavy impervious seed coat even on large seeds, as is often found on dessert legumes and lupines, promotes long term seed viability.

4. **Life cycle**: Annual and perennials are more dependent on a persistent soil seed bank than woody and long-lived shrub and tree species.

5. **Ecological association**: Plants that are early succession colonizer species that may occur only after disturbances and species that depend on other plants for their development must maintain viability until a suitable host plant is available.

Recruitment of tree species on the forest floor or in the field is governed by various factors including the seed traits and microhabitat conditions (Kitajima, 2007). Likewise, germination of seeds in nature is strongly influenced by internal as well as external environmental factors (Baskin and Baskin, 1998). The germinating seeds and seedlings are most vulnerable to predation, desiccation, and developmental stage of embryos, competition and damage as the seeds and seedlings constitute important resource for the herbivores and pathogen. Seedling predation and disturbance in the form of trampling and microhabitat alternation have direct implications for recruitment and multiplication of species. The survival in seedling and plantlet growth is enhances

by consistency in microhabitat condition, right desiccation of seeds, right developmental stage of the embryos in the harvested seeds and duration of post harvest storage of seeds. Germinating seeds, depending upon their state such as inherent properties, developmental stage, reserve food materials, moisture content, and nature of pericarp greatly influence the initial seed germination and establishment of the seedling in the seed bed.

The intolerance to desiccation leads to difficulties in conserving the seeds sensitive to desiccation (Barbedo and Cicero, 2000). Many forests have a high number of species with recalcitrant seeds. Therefore many programmes that involve the rational and economic use of the tree species are hampered due to the lack of storage methods of recalcitrant seeds for longer periods. The methods in use preserve such seeds for periods varying from days to months. Thus, inclusion of several species in those programmes is limited. Identification and conservation of recalcitrant seeds as well as studies increasing their tolerance to desiccation were performed and pointed out respectively by several authors (Berjak *et. al.*, 1990; Terui and Okagami, 1993; Neves, 1994; Pammenter and Berjak, 1999; Pammenter *et. al*., 1998; Albrecht, 2006; Onen, 2006; Deb *et. al.*, 2012). There is also an agreement about the desiccation intolerance of recalcitrant seeds and their short longevity. The major differences between recalcitrant and orthodox seeds at the end of the process of seed formation is that the orthodox seeds undergo a nearly essential process of desiccation, at the end of maturation and this process allow these seeds to change their metabolism from development to germinate (Kermode, 1990). These changes are not observed in recalcitrant seeds due to several factors including hormonal balance, protein and sugar contents and water physical properties, among others (Kermode, 1990; Barbedo and Filho, 1998).

A new approach on seed germination and storage of the recalcitrant seeds is a matter of investigation as the available methods of seed drying are not suitable for the recalcitrant seeds. Although it is not possible to define unequivocally critical moisture content for viability loss in recalcitrant seeds because they show differential response to dehydration at different drying rates, it is necessary to maintain their water level above critical levels (Pammenter *et. al*., 1998). However, under these conditions, seed may initiate germination and or undergo microorganism infection, which could lead to seed deterioration. High humidity favours the initial steps of seeds germination processes but seed is damage if water is not supply in sufficiently. Therefore, storage treatments maintaining high moisture of the seeds are often associated with low temperatures (Cunha *et. al*., 1995). Nevertheless, many of these seeds have little tolerance to cold conditions. Therefore, studies on these factors will give a better understanding on seed storage, seed germination and seedling establishment of the species produces recalcitrant seeds.

In vitro propagation

India is one of the 17 mega biodiversity regions of the world and nurtures enormous plant diversity. As many as 45,000 species of plants have so far been recorded in India of which 5285 species of angiosperms belonging to 140 genera are endemic to the country (Moza and Bhatnagar, 2007). At least 10% of India's recorded wild flora are on the list of threatened species many on the verged of extinction (Singh, 2004). A number of biologically rich areas like North-Eastern regions have not been fully explore and studied even though it encompasses a broad range of ecological habitats (Kushwaha and Behera, 2002). About 50% of total higher plant species of India occur in this region (Roy and Sameer, 2004). However as time passed by, this

plant genetic resources are getting eroded significantly due to over exploitation of the plant, Jhum cultivation and other developmental activities like industrialization and unplanned human activities. Therefore it is necessary to develop protocols for mass multiplication and *in vitro* conservation of these threatened plant species. *In vitro* propagation of plants through Tissue culture technique is a boon by providing an efficient means of producing many economical plant products. Plant tissue culture is a technique of growing plant cells, tissue and organs in an artificial prepared liquid medium or semi solid medium under aseptic condition.

One of the most important problems that stand on the way of propagating is the difficulty of raising the plant through seeds, because when the seeds are shed from the plant the embryos inside is undifferentiated, but plant tissue culture techniques has proved to be a great boon to combat these problems. Through this technique, the vegetative parts can be induced to form callus on a defined nutritive medium containing appropriate quantity of auxins and cytokinins and can be differentiated into large number of plants or as a direct organogenesis within a short period of time. Whereas in nature, the clonal propagation takes place through rhizomes, cuttings, corm, bulbs, suckers, offshoot, etc, but it is not possible to meet the markets demand. Therefore, plant tissue culture technique has become extremely important in medicinal plants species. As medicinal plants continue to be the key role in the treatment of number of diseases, and they are the only source of medicines in the treatment of people in the developing world. Moreover the toxicity and health hazard associated with the use of synthetic drugs and antibiotics have renewed the interest in the use of plants and plant based drugs which increase the demand of medicinal plants, but only a small percentage of medicinal plants traded in India are solely cultivated. The remaining huge raw material of medicinal plants is met from their wild population. Over exploitation

and indiscriminate collection to meet the market demand of medicinal plants supply in fact threatened the survival of many rare species. Thus, plant tissue culture can solved the problem and provides many production advantages like-

1. Large number of plantlets can be produced inexpensively.

2. Quick and easy scale-up can be achieved.

3. Short to medium term storage via slow growth of cultures.

Many plant species have been propagated successfully through plant tissue culture technique particularly the rare, endanger and threatened plant species and reintroduce into the wild ameliorating their status in nature. Different explants sources like seeds, foliar explants, rhizome, roots, inflorescence, etc, have been used for *in vitro* propagation of different species of plants by many workers for conservation programme. Following are some works done by various workers: *Aconitum heterophyllum* (seeds- Srivastava *et. al.,* 2010, Solanki and Siwach, 2012, nodal segment- Srivastava *et. al*., 2010, Jabeen *et. al*., 2006, leaf and petiol- Giri *et. al.*, 1993); *Aconitum napellus* (shoot tip- Watad *et. al*., 1995); *Aconitum carmichaeli* Debx (anther- Hatano *et. al*., 1987, shoot tip- Shipping *et. al*., 1998); *Aconitum balfourii* Stapf. (leaf- Ruchi *et. al*., 2011, Pandey *et. al*., 2004, root- Sharma *et. al*., 2012); *Aconitum smomontanum* (leaf bud- Ji *et. al*., 2008); *Dioscorea cayenensis* (micro tuber- Ovono *et. al*., 2010); *Dioscorea zingiberensis* (stem- Chen *et. al*., 2003, Shu *et. al*., 2005, leaf petiol- Shu *et. al*., 2005, floral bud- Huang *et. al*., 2009); *Dioscorea rotundata* (petiole- Felicia *et. al.*, 2012, nodal segment- Polzin *et. al*., 2013*); Dioscorea fordii* Prain et Burk (nodal segment- Yan *et. al*., 2011); *Dioscorea alata* (nodal segment- Heena and Lele, 2012, Borges *et. al*., 2004, leaves and tuber- Heena and Lele, 2012); *Dioscorea remotiflora* (nodal segment- Antonia *et. al*., 2012); *Dioscorea*

nipponica Makiro (nodal segment- Chen *et. al*., 2007); *Dioscorea composite* Hemsl (nodal segment- Alizadeh *et. al*., 1998); *Dioscorea bulbifera* L. (nodal segment- Chu and Ribeiro, 2002); *Dioscorea oppositifolia* (nodal segment- Pornima and Ravishankar, 2007, Maheswari *et. al*., 2012); *Dioscorea pentaphylla* (nodal segment- Asha and Nair, 2010, Poornima and Ravishankar, 2007); *Dioscorea floribunda* (male inflorescence- Borthakur and Singh, 2002, nodal segment- Chaturvedi, 1975; leaf- Chaturvedi, 1979); *Dioscorea wightii* (nodal segment- Mahesh *et. al*., 2010); *Strobilanthes flaccidifolius* (foliar explants – Deb and Aremnongla, 2011; Nodal explants – Deb and Arenmongla, 2012); *Cinnamomum tamala* (foliar explants – Deb *et. al.,* 2013; nodal segments – Deb *et. al.* (In-press); zygotic embryo – Deb *et. al.* (In press).

A wide range of endangered plants of medicinal value have now been successfully propagated using *in vitro* techniques. There are many reports on *in vitro* multiplication of different medicinal plant species. Different workers have reported regeneration of plants in cultures using different explants sources like shoots, nodal segment, leaf, rhizome, seeds.

Seed/embryo culture

The technique of seed culture is referred to as embryo culture, ensures a better germination frequency and favours the production of virus free seedlings at a faster rate. For establishment of culture from the seed/embryos, it is important to identify the right development stage of the embryos. Seeds of many plant species undergo dormancy after maturation and at times fail to give response under *in vitro* condition. Because of this for many species it is necessary to harvest the immature seeds/embryos for successful culture establishment. The earliest stage at which the embryos can be cultured successful varies with the plant species and the local conditions. *Doritis* ovules

from pollinated ovaries germinated readily after getting fertilized *in vitro* (Yasugi, 1984) suggesting that fertilization is a pre-requisite for germination. Yam and Weatherhead (1988) also noted that immature embryo germinates better than the mature ones due to their distended testa cells and metabolically awakened embryos; they also lack dormancy or inhibitory factors. Whereas, in orchids very young ovules do not form suitable explants because the embryo sac development is post pollination phenomenon and fertilization a prerequisite for obtaining seedlings. *Arachnis labrosa* and *Cleisostoma racemiferum* embryos obtained between 16 and 18, and 16 weeks after pollination (WAP) respectively (Temjensangba and Deb, 2005a, b, c, 2006) readily germinate but their germination frequency declines sharply, when obtained from beyond this window period. Likewise, in *Satyrium nepalense*, *Nephalaphyllum cordifolium*, *Phaius tankervilliae* and *Cymbidium* species, germination frequency shows sharp decline when the embryos are collected 3-4 weeks prior to fruit dehiscence.

Meristem culture

The technique of using resident meristem (shoot-tips, axillary bud) has opened new vistas in micropropagation (Arditti and Ernst, 1993; Deb and Temjensangba, 2005, 2006a). Through this technique, up to 200,000 plants can be regenerated from a single resident meristem within a year. Though, the plant source, genetic constitution and physiological age of the explants are some of the important factors for regeneration. The juvenile tissues from greenhouse grown plants respond better than the mature ones grown outdoors. Generally, the proliferative loci get activated in the sub-epidermal cells and soon develop into somatic embryos and or shoot buds. Somatic embryogenesis is either direct or callus mediated development, and multiplication and

differentiation of plantlets is influenced by the chemical stimulus present in the nutrient pool (Vij and Pathak, 1990; Seeni and Latha, 1992).

The advantages of leaf, nodal segment culture are apparent for more than one reason: they are easy to obtain, easier to disinfect, and their excision does not endanger the mother plant. Furthermore, as the regeneration occurs in the dermal cells, which is cytologically more stable, mass production of genetically uniform plant from this is within the realm of reality (Vij, 2002).

The growth and development for different species exhibit different specific needs in respect to their nutritional requirement and treatment with plant growth regulator (PGR). So, no standard media formulation can be prescribed for all the species. Most commonly employed basal media for tissue culture are Knudson 'C' (1946) for orchids; Vacin and Went (1949); Murashige and Skoog (MS) (1962) for different types of plant; Nitsch and Nitsch (1969); Mitra *et. al.* (Mitra *et. al*., 1976) for orchids. The use of α-naphthalene acetic acid (NAA) and one of the cytokinins like Benzyladenine (BA) and Kinetin (Kn) yields a rich crop of propagules.

The phenolic exudation by the explants into the medium is a serious problem in tissue culture as it impairs the growth of plant/regenerates considerably. But this can be offset by the use of activated charcoal (AC) in the medium. Activated charcoal favours better health of the cultures because of its ability to absorbed exudates/growth inhibitors, enhance medium aeration, and absorbed light and provide enhanced quantum of energy per unit plant material.

Objectives of the present study

Northeast India is blessed with a wide range of physiography and ecoclimatic conditions, lying between 21º34'N to 29º50'N latitude and 87º32'E to 97º52'E longitude

and covers an area of ca 2, 62,060 sq km. Northeast India represents the transition zone between the Indian, Indo-Malayan and Indo-Chinese biogeographic regions and a meeting place of the Himalayan Mountains and Peninsular India. Northeast India is thus the geographical gateway for much of India's flora and fauna, and as a consequence, the region is one of the richest in biological values. The vegetation of the Northeast regions accounts for approximately 7500 species of angiosperms. Out of 315 families of angiosperms in India, more than 200 are represented in Northeast India and this region accounts for nearly 50% of the total number of plant species in India as a whole. It is of interest to note that about one third of the flora of Northeast India is endemic to this region.

The primary forest of Northeast India is losing day by day due to *'Slash and Burn'/*Jhum Cultivation practice by the local people, fragmentation of forest, indiscriminate collection of the plant species, unplanned developmental activities, natural calamities like land slide, drought, forest fire etc. As a result many important plant species are facing threat or on the verge of extinction in their natural habitat.

Studies on the reproductive biology will help in formulating conservation strategy by removing the reproductive bottlenecks such as pollination barriers face by the plant, pre or post fertilization failure leading to no or poor seed set, very low germination rate which imposed constraint on the multiplication of the plant. But, the conventional method of plant propagation is slow therefore tissue culture opens up an area for conserving threatened species as small explants can generate large number of disease free propagules which can again be introduced in the nature. Therefore keeping the above point in mind, I have researched on, Studies on certain reproductive behaviour with *in vitro* propagation for conserving the two economical and threatened

medicinal plant species- ***Aconitum nagarum* Stapf**. and ***Dioscorea villosa* L.** for my Ph.D. degree, with the following objectives:

1. Studies on certain reproductive behaviours which hinder in their multiplication and regeneration of the selected species

2. Studies on seed biology like the effect of stratification, temperature on the seed germination

3. Initiation of *in vitro* culture from various explants like the nodal segment, seeds and leaf.

4. Optimization of physico-chemical factors for *in vitro* culture initiation and culture proliferation.

5. Hardening of *in vitro* raised regenerates.

6. Introduction of the regenerates in the poly-house and wild.

A Brief about the selected species

A. *Aconitum nagarum* Stapf.

The genus *Aconitum* belongs to the family Ranunculaceae with about 300 species distributed in the temperate regions of the Northern Hemisphere of the world (Shu *et. al.*, 2001). Out of these, 33 species in total are found in the great Himalayan all along the Hindu-Kush and Himalayan ranges from Afghanistan in the west to Myanmar in the east (Shah, 2005) and is widely distributed in the alpine and sub-alpine regions of tropical parts of Northern hemisphere.

Aconitum nagarum is endemic to the North Eastern part of India (Nagaland, Manipur, and Arunachal) (Sharma *et. al.*, 2003) and found in Yunnan province of china

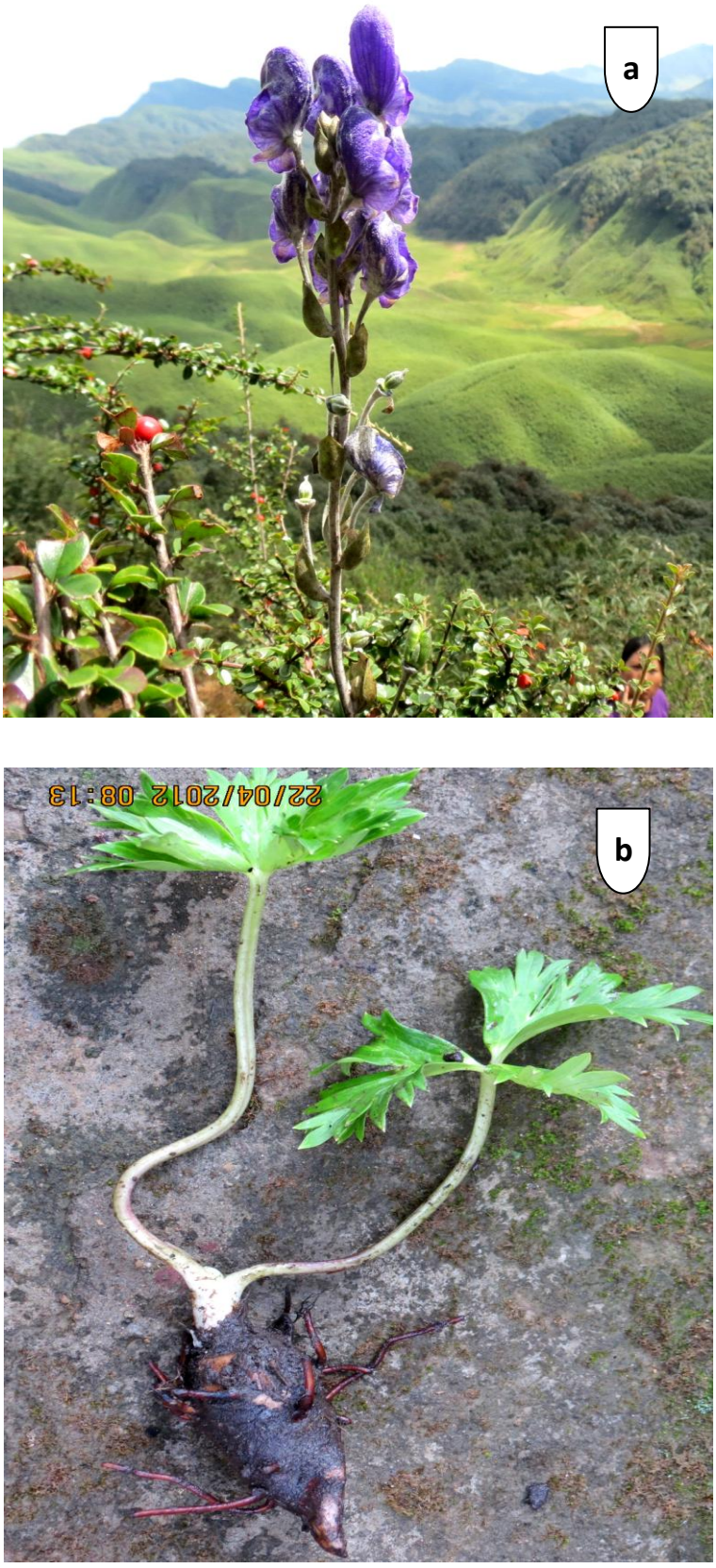


Figure 1: a. *Aconitum nagarum* habitat and flower; b. *A. nagarum* plant with rhizome

among the grassy sloppy mountain (**Fig. 1a**). The species has 3 varieties (Shu *et. al*., 2001). The plant is erect, roots paired, biennial, slightly over 2cm long, tuberous (Stapf, 1905). Leaves scattered, the lowest decayed at the time of flowering, upper few and very remote, lowest petioles up to 60 cm long, widened, sulcate and amplixicant at the base. Flowers are large, pale blue, helmet shaped (**Fig. 1b**). Seeds are obpyramidal, brown, 3mm long transversely lamellate. It flowers from October to November.

They are mainly cultivated for their tubers. The alkaloid produce from the tubers are used in curing wide range of diseases and also used as arrow poison. The diterpenoid alkaloids from *A. nagarum* have been isolated by different workers (Dong *et. al*., 2000; Zhang *et. al*., 2005; Ji and Wang, 2006). Seven known diterpenoid alkaloids: denudatine, songorine, songoramine, virescenine, neotine, 14- acetylneoline and flaconitine have been isolated from the roots of *Aconitum nagarum*. In china the plant is used to treat pain (Dong *et. al.,* 2000; Zhang *et. al.,* 2005). It is also used in treatment of antipyretic, anti rheumatic, paralysis and snake bite (Srivastava *et. al.*, 2010). The plant also has an antibacterial activity against *Staphylococcus aureus*, *Sallmonella typhimurium*, *Escherichia coli* and *Bacillus subtilis* (Sinam *et. al.*, 2012). The vegetative propagation takes 3-4 years. Due to the habitat destruction, collection for ornamental, as well as for drugs, etc, the plant is facing a decline in the population. Moreover, decrease in the population size is also attributed to failure in the reproductive behaviours like seed germination, climatic factors etc. Therefore there is an urgent need to conserve the plant *ex-situ* and *in situ*.

B. *Dioscorea villosa* L.

The genus *Dioscorea* (Dioscoreaceae) includes over 650 species and is grown throughout the tropics for their stem tubers. They are important food for millions of

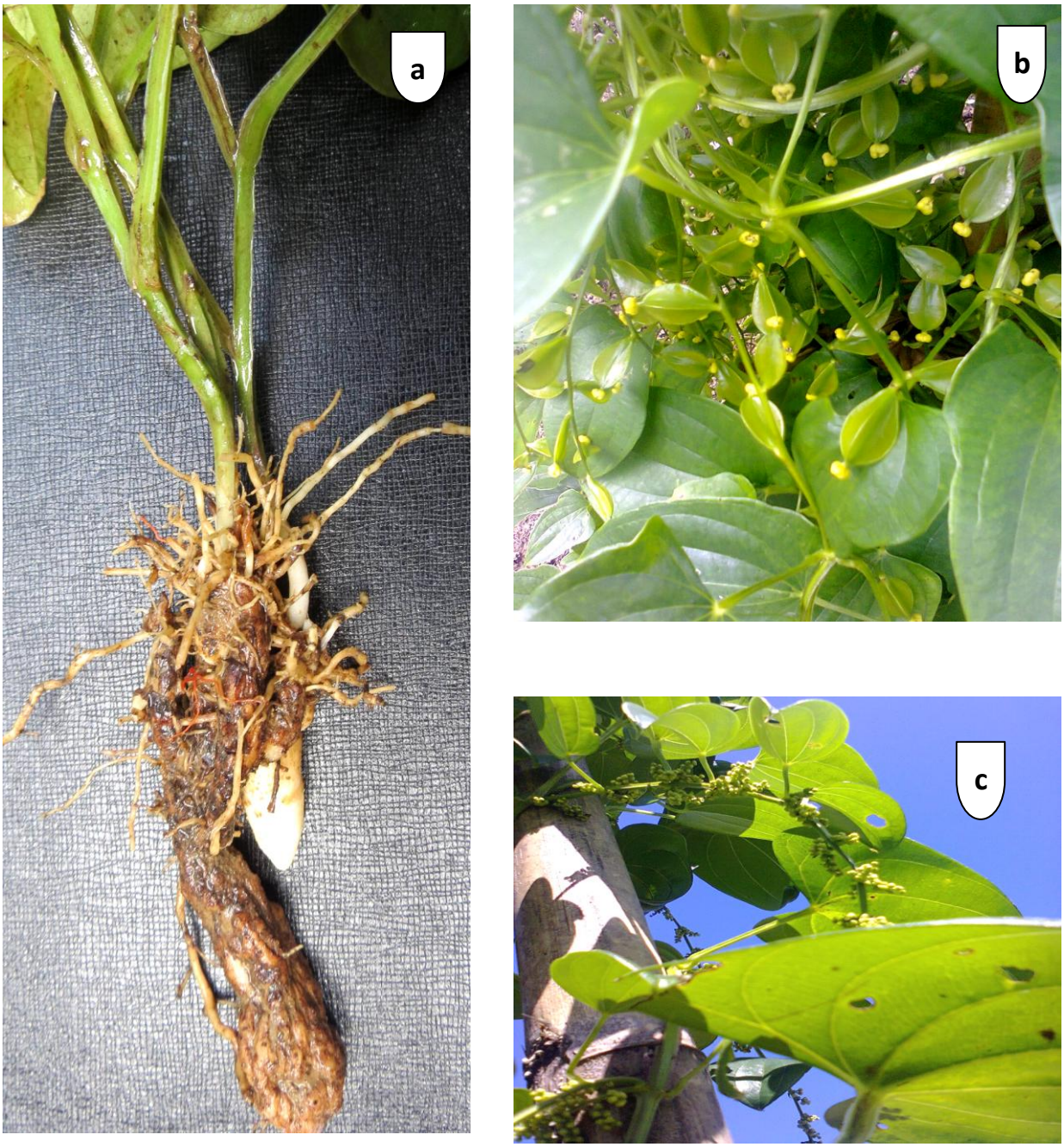


Figure 2: a. *Dioscorea villosa* plant with tuber; b. Female plant with flower and fruits; c. Male plant with flower.

people in the tropics. Annual world production of yams is estimated to be about 19 million tonnes. In India, yams are cultivated mostly as a garden crop or as subordinate crops. They are propagated vegetatively by cutting the tip or crown of the tuber containing buds or eyes. The crop matures within 7-10 months. The yam is a high source of carbohydrate. But in the recent years, the wild yam is extracted discriminately for the steroidal sapogenins which can be converted into cortisone, into the testosterone and the oestrogens as well as progesterone. Among the wild species sought after for its steroidal sapogenins presence, *Dioscorea villosa* is also one of them.

Dioscorea villosa L. is a perennial, herbaceous vine with a tuberous root (**Fig. 2a**). The plant is unisexual (dioecious plants) (**Fig. 2b, c**) with a height of about ten feet with alternate, verticillate lower leaves. The stem is narrowly winged, polygonal. Staminate inflorescence bracteolate.The inflorescence is single and in the axils of upper leaves (Shehbaz and Schubert, 1989). The species grows in moderate moisture, woods, roadsides, river bottom forest. The species is use as a source of saponins for preparation of steroids in the pharmaceutical (Dweck, 2002). The rhizome extract is a medicinal herb used to treat symptoms of rheumatoid arthritis and menopause, colic (Kaimal and Kemper, 1999; Wojcikowski, 2008). As skin cream – diosgenin as a source of natural hormones, antifungal, use in urinary tract problems in Chinese traditional medicine, treatment of asthma and cardiovascular (Kaimal and Kemper, 1999). The species also possess anti carcinogenic potential (Chao-chin *et. al.*, 2007). Due to the many fold uses, the plant is facing problems as conventional method of propagation is slow and could be cultivated only once a year. To overcome such problem *in vitro* propagation of the species and its studies on certain reproductive behaviours are taken into account.

Chapter – 2

Reproductive Biology and Seed Biology of *Aconitum nagarum* Stapf.

The genus *Aconitum* includes approximately 300 species belonging to the family Ranunculaceae and 33 species are found in India (Shah, 2005). The word '*Aconitum*' comes from the word 'Akon' meaning 'rocky' which is the type of area where the Aconite plant grows. The word is also believed to originate from the Greek word 'Akwan' meaning a dart. Aconites are widely recognized for their medicinal importance and it is known since the ancient times and mention in old Hindu medicine as '*Ativisa*' meaning a deadly poison that neutralize the effect of another poison. These are herbaceous perennial or biennial plants growing in moisture retentive but well draining soils of mountain meadows. The Aconite plants are mainly cultivated for their tubers which contain a diterpenoid alkaloid and flavanoids which is used for curing many diseases.

Aconitum nagarum is a biennial herbs growing at an altitude of 1600-3800m ASL. They are mainly cultivated for their tubers (**Fig. 1b**). The alkaloid produce from the tubers are used in curing wide range of diseases and also used as arrow poison. The diterpenoid alkaloids from *A. nagarum* have been isolated by different person (Dong *et. al*., 2000; Zhang *et. al*., 2005; Ji and Wang, 2006). In China the plant is used to treat pain (Dong *et. al.*, 2000; Zhang *et. al.*, 2005). It is also used in treatment of antipyretic, antirheumatic, paralysis and snake bite (Srivastava *et. al.*, 2010). The plant also has an antibacterial activity against *Staphylococcus aureus*, *Sallmonella typhimurium*, *Escherichia coli* and *Bacillus subtilis* (Sinam *et. al.*, 2012). The vegetative propagation takes 3-4 years. Due to the habitat destruction, collection for ornamental, as well as for drugs, etc, the plant is facing a decline in the population. Moreover, decrease in the population size is also attributed to failure in the reproductive behaviours like seed germination, climatic factors etc. Therefore there is an urgent need to conserve the plant *ex situ* and *in situ*.

Material and Methods

Floral biology

The study was conducted in Dzuku valley at Khonoma village, Nagaland, at an elevation of 2684 m ASL 25º36'44.8′ N latitude and 094º00'03.4′ E longitude and Sirui hills of Urkhrul district, Manipur at an elevation of 2427 m ASL, 25º 06' 39. 6" N latitude and 094º 27' 13.3" E longitude among the grassy bamboo slope. The reproductive phenology and floral morphology viz, time of budding, time of anthesis and stigma receptivity, different stages of anther development, anther dehiscence, fruit and seed setting etc. were studied. Floral phenology of *A. nagarum* at Dzuku valley, Nagaland and Sirui hills, Manipur was comparatively studied. In order to estimate

flower production, total number of flower per plant was counted manually in the selected plants. Seeds per pod were counted to quantify production of pods. Anther counts were done on randomly selected flowers. Pollen counts were made on 20 anthers from different flowers. The anther lobe was removed from the style with the help of forceps and blade. The removed anther lobe was put on a slide. The anther was smashed uniformly by adding a drop of glycerine and spread evenly. The slide was covered with the cover slip. The slide was kept under the microscope and counting of the pollen was done.

Distribution pattern of the plant and its associated species

The distribution of *Aconitum nagarum* in North-Eastern part of India in Nagaland and Manipur was also taken into account for comparative study of the plant distribution pattern in these two states. A study was conducted to understand the rule of associated species on the growth, reproduction and survival of *A. nagarum*.

Seed biology

The plant and mature fruits were collected from the forest of Dzuku valley, Khonoma, Nagaland, India at an elevation of 2648 m ASL from the grassy bamboo slope. *Aconitum nagarum* reached peak flowering from first week of October and seed setting starts from second week of October. Therefore harvesting of seeds can be done from third week of October. On the contrary, in Sirui hills, Manipur, at an elevation of 2427m ASL, peak flowering starts from first week of November and seed was setting from second week of November. During the second week of November few flowers were seen but very rare. Matured dried seeds can be collected from third week of November.

Seed collection and processing

Method of seed collection, processing and post harvest storage of seeds for experimental purpose may materially affect the study results. In the present study, mature seeds were harvested randomly from the natural habitat during 2011-2013 along with the plant stalk. The stalks were wrapped in newspapers and covered with polythene bag and transported to the laboratory within 1-2 days. The collected fruits were dried by spreading uniformly over the old newspaper for 1-2 days in the laboratory at 25^{o}C. The dried fruits were removed from the stalk and seeds are taken out of the carpel. The seeds were then stored in poly bag in the laboratory for further experiments. The processed seeds were washed with 'Labolene' (1:100, v/v) (a commercial laboratory detergent) and rinsed under running tap water and finally with distilled water. The seeds were made into different groups for germination experiment.

Preparation of potting mix

The potting mix for the experimental purpose was made by mixing soil and chopped coconut coir at 1:1 ratio. The garden soil was crushed into find powder, sun dried and mixed with the coconut coir in the ratio of 1:1 and put in a plastic pot and transparent poly bag. The poly bag and plastic pot were made perforated for better aeration. They were kept moist before sowing the seeds for germination.

Experimental process

A part of the seeds were processed and sowed immediately after harvest while others were treated differentially at 4^{o}C in a refrigerator for 0, 24, 48, 72, 96 hours and sowed as described below:

1. A set of stratified seeds were sowed in filter paper in a humidity chamber of 90 mm in diameter and kept in a laboratory (25^{o}C).

2. Another set of processed seeds (stratified seeds) were sowed in potting mix and kept in an incubator at constant temperature of 30ºC.

3. While another set of stratified seeds were sowed in seed bed (poly bag) and maintained in a poly house.

4. To test the post harvest tolerance of the seed for various periods, the processed seeds were stored at 25ºC (in the laboratory) in sealed poly bags before they are sowed in the seed bed for seed tolerance experiment.

5. The seedling morphology and seedling mortality rate was also studied.

To study the emergence, survival and growth of seedlings of *Aconitum nagarum* under each condition, 4 replicates of 13 seeds each (for filter paper test, N=52 seeds/test) and 20 seeds each (for seed bed germination, N=80 seeds/test) were used. In each polybag the soil mixture was packed. In each poly bag 20 seeds were sowed. In filter paper test 13 seeds were sowed. The seed beds were watered at regular interval. The experimental design was completely randomized. The data was collected daily basis for seed germination, seedling morphology; seedling mortality; percent response etc.

The seedling were maintained in the respective polybag and watered at regular interval and the seedling morphology was studied. Once the seedling showed normal functioning like rooted plantlets, emergence of normal leaves etc., the seedling were transferred to the poly house. Once the seedlings were established in the poly house, seedling mortality was observed.

Filter paper test at room temperature 25ºC

The seeds are treated at 4ºC in a refrigerator for different periods (0, 24, 48, 72, 96 hours). The seeds are then placed on moist filter paper in a humidity chamber of 90 mm diameter and kept for germination at laboratory (25^{o}C). The seeds are kept moist throughout the study period. The germination process is completed by the emergence of radical followed by leaf with seedling formation. A total of 13 seeds were used for each treatment with 4 replicates (N=52 seeds/treatment). The seedlings are transferred to poly house for further seedling growth.

Germination test in incubator

The stratified seeds (stratified at 4º C for 0, 24, 48, 72 and 96 hrs) were placed on potting mix to test the role of stratification on germination. For each treatment consists of four replicate with 20 seeds each (N=80 seeds/treatment). The differently stratified seeds were sowed in the potting mix and incubated at 30°C in the incubator. The seeds were monitored at regular interval for seed germination, seedlings morphology and seedlings mortality etc.

Germination test in seed bed (poly bags)

The differentially stratified seeds were placed on potting mix in a perforated polybag of 150mm in diameter. Each treatment consists of four replicate with 20 seeds (N=80 seeds/treatment). The plants are monitored regularly for germination. Germination percentages were calculated after 8wk of seed culture.

Post harvest storage tolerance test for *Aconitum nagarum*

The seeds are store at a temperature of 25ºC. The seeds were tested for the post harvest storage tolerance. Every month sets of processed seed were sowed in the seed

Table 1: Distribution pattern of *Aconitum nagarum* at different location of Nagaland and Manipur

Site	GPS Coordination	Altitude (mASL)	Distribution	Locality
Nagaland				
Southern Dzuku valley	N25 34 30.4, E 94 02 43.3	2400	Common	Valley and hill slope
Western Dzuku valley	N 25 36 44.8, E 094 00 03.4	2648	Common	-do-
Mount Saramati	N 26 2 26.7, E97 6 97 13	2000-3841	Common	-do-
Japfu Hills	N 25 35 86.3, E 094 04 047	3020	Common	Hill slope
Manipur				
Sirui Hils	N 25 06 39.6, E 094 27 13.3	2427	Less common	Hill slope and top of hills
Dzuku valley	N 25 34.40.5, E 094 04 48.9	2550	Common	Valley and hill slope

Table 2: Associated Species of *Aconitum nagarum* at different places of Manipur and Nagaland.

Associated species of *Aconitum nagarum*	**Nature of association**
***Sinarundinaria rolloana* (Dwarf Bamboo)**	Dominance
***Gaultheria* sp.**	Dominance
***Fragaria* sp.**	Co-dominance
Berberries walliachinia	Co-dominance
Grass	Dominance
***Circium* sp.**	Co-dominance
Fern	-do-

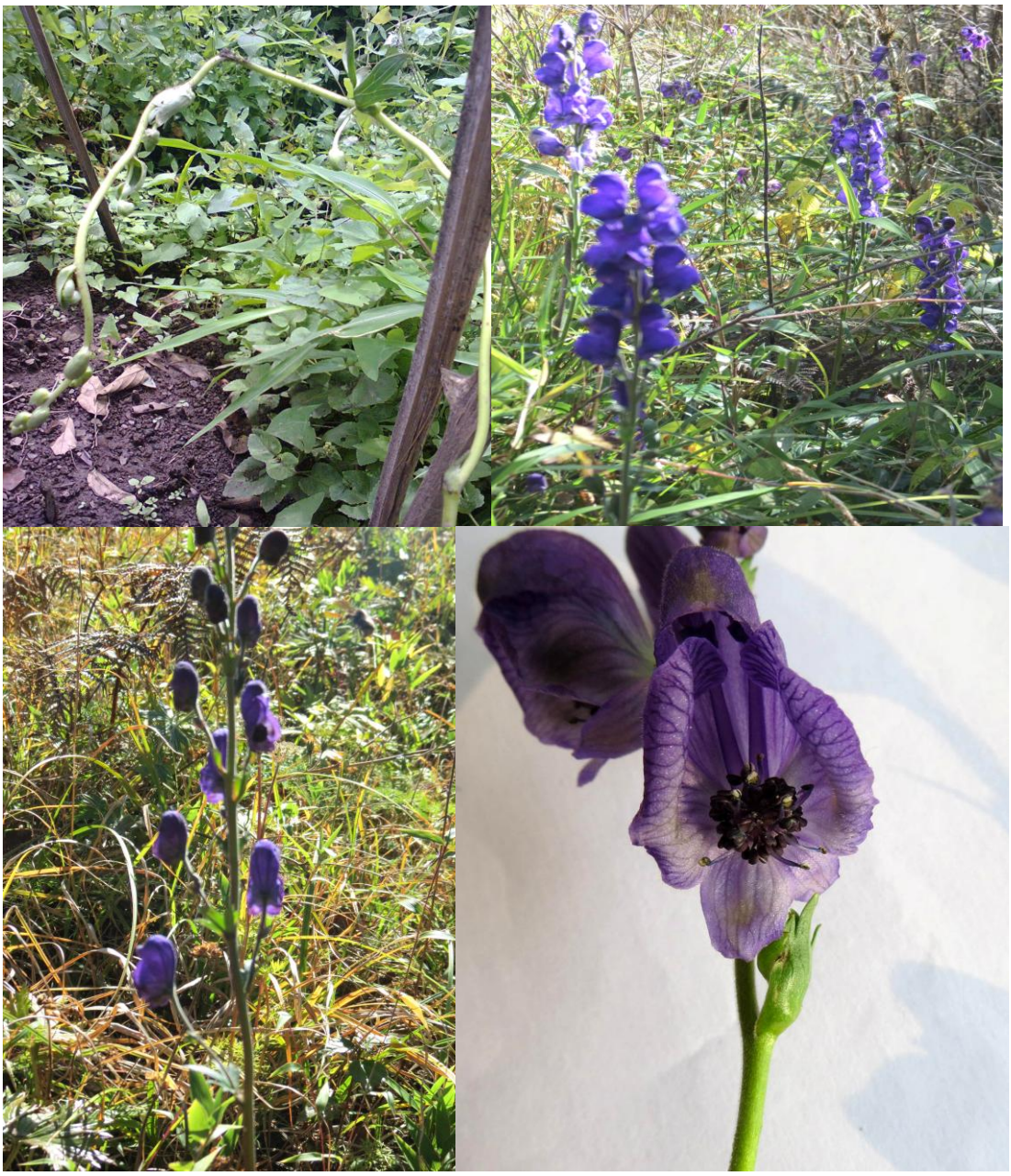


Figure 3: a. *A. nagarum* at budding stage in the experimental plot; b. Population in full bloom in the natural habitat; c. *A. nagarum* inflorescence showing acropetal type of flowering; and d. A flower showing longitudinal dehiscence of anther.

bed. Their germination rate for each experiment was carried out and the results obtained were recorded monthly. The result obtained was compared and checked to see how far *Aconitum nagarum* seeds can be tolerance to storage.

Seedling mortality and seedling morphology

The seedling mortality was observed by transplanting the seedling grown from the differently treated seeds in the poly house and percent seedling survival was recorded till the plant mature or till its survival period which ever was earlier.

The transplanted seedlings in the poly house were also check for any changes/modification in the seedling was observed after the transplantation of the germinated seeds (like modification in the leaf, roots etc).

Results

Associated species, floral biology and morphology

Present study was conducted in Nagaland and Manipur at the altitude between 2000 m to 3841 m above mean sea level (**Table 1**) in 6 different geographical areas. In all the study areas the populations were found in the hill slopes of the valley. In all the study areas one interesting feature was observed and it was associated species. In all the areas there were certain associated species which were common in all the study areas viz. *Sinarundinella* species, *Gaultheria* species, *Fragaria* species etc (**Table 2).** In all the areas *A. nagarum* was growing healthy where these associated species were available. In the present study it was observed that the plants of *Aconitum nagarum* growing at Dzuku valley started budding from September second week (**Fig. 3a**) with peak flowering in October first week (**Fig. 3b**). The flowers are blue, in slender raceme,petals and filaments glabrous, carpel 5, hermaphrodite. The flowers bloom

Table 3: Floral display of *Aconitum nagarum*

Parameters	Observation
Inflorescence	Alternate raceme
Number of inflorescence/plant	8-28 nos.
Flower type	Hermaphrodite
Anthesis	6-6.30A.M
Mode of anther dehiscence	Longitudinal
No. of anther/flower	49
No. of pollen grains/anther	1000-2000
Stigma type	Pentacarpellary
Ovary type	Pentalocular
Seed	Obpyramidal, brown
Seed/plant	270-540 nos.
Root	Hearth shape, dark brown

Data are compiled from successive two years of study/observations.

Figure 4: a. *A. nagarum* fruit development (early stage); b. Mature fruits (dried) ready for storage.

Figure 5: Main pollinator of *A. nagarum* (honey bee).

Table 4: Different stages of anther development and stigma receptivity of *Aconitum nagarum*

Stages of anthers	Anther Development	Stigma Development
Before anthesis	All anthers in group as cluster at the centre of corolla	Stigma lobe is surrounded by the anthers
Anthesis	Anther more towards corolla and increase in size, form gaps with stigma	Style increase in size
After anthesis	Anther movement continues	Stigma lobe start opening and spike formation of the stigmatic
Anther dehiscence	Pollen dehiscence	Stigma lobe open more prominent
4-5 days after dehiscence	Anther shunken and fully dried	Lobes receptivity and pod formation starts

Table 5: Difference in the floral phenology of *Aconitum nagarum* at Dzukou valley, Nagaland and Sirui hills, Manipur

Parameter	**Dzuku valley**	**Sirui hills**
Budding	September	October
Flowering time	October 1st week	November 1st week
Anthesis	6-6.30A.M	6-6.30 A.M
Seed setting	Oct 2nd week	Nov 2nd week
Seed maturity	Oct 3rd week	Nov 3rd week
Sprouting of plants	March/April	April/May
Duration of flowering	4-5 days	5-6 days
Altitude and GPS Coordinates of study area	2684m ASL, N 25º36'44.8 Latitude E094º00'03.4 Longitude	2427 m ASL, N 25º 06' 39. 6" latitude and E 094º 27' 13.3" longitude

Temperature during flowering at Dzuku valley-16°C

Temperature during flowering at Sirui Hills-17°C

Table 6: Fruit setting of *Aconitum nagarum*

Sl. No.	Flowers/inflorescence	Fruits/inflorescence	Difference of flower and fruit setting/inflorescence
1	20	9	11
2	28	20	8
3	29	11	18
4	13	9	4
5	27	18	9
6	28	15	13
7	20	12	8
8	15	9	6
9	20	14	6
10	22	14	8
11	27	21	6
12	23	12	11
13	22	11	11
14	28	27	1
15	24	14	10
16	20	17	3
17	14	8	6
18	18	9	9
19	17	13	4
20	12	10	2
Mean	**427/20 = 21.35**	**273/20 = 13.65**	**154/20 = 7.7**

acropetally i.e. flower starts blooming from the base of the inflorescence to the tip of the inflorescence (**Fig. 3c**). Thus the fruits also mature acropetally. The anthesis was observed between 6.00-6.30A.M. Anther dehisced longitudinally from 7.00 A.M till 9.30 A.M (**Fig. 3d**).The number of anther was 49 per flower. During the study on floral phenology it was found that there is a strong correlation between anther development and the stigma development (**Table 3**). The flowers colour changes as the plant fully dehisced. The flowering duration per flower varies from 4-6 days followed by fruit formation. Fruits mature within 10-15 days. Fruits formation starts from second week of October (**Fig. 4a**) and by third week of October fruits mature and the plant dries up (**Fig. 4b)**. While, in Shirui hills of Manipur at an elevation of 2427 m ASL 25º 06' 39. 6" N latitude and 094 º 27' 13.3" E longitude peak flowering of *Aconitum nagarum* was observed by first week of November and by the second week flowering decreases with the formation of fruits. By the third week, fruits are mature and the plants were all dried up. In local dialect of the Sirui village, it is known as the summer blue. The number of flower per plant varies from 8-28. The most common pollinator was found to be bee (**Fig. 5**). Seeds per carpel varies from 9-13 i.e., mean wised seeds per flower was 45-65 and per plant was 270-540 (**Table 4 & 5**). Pollen per anther varies from 1000-2000 which means an average of 98000 pollen grain per flower. In the present study it was found that all the flowers to fruits ratio was not 1:1, it was 3:2 i.e., a third of the flowers did not support fruit formation. An average of 21.35 flowers developed per inflorescence while of the 21.35 flowers 13.65 flowers ended with fruit formation and remaining flowers did not form any fruits (**Table 6)**.

Seed biology

Table 7: Effect of stratification on the seed germination of *Aconitum nagarum* on filter paper (in a humidity chamber of 90 mm diameter)

Stratification period (hours) at 4°C	% response (±SE)*	Types of plant response
0	**6.7.00 (0.20)**	**Healthy roots**
24	**15.00 (0.25)**	**-do-**
48	**38.00 (0.30)**	**Root healthy, hairy at the zone of maturation**
72	**15.40 (0.20)**	**-do-**
96	**15.00 (0.25)**	**Elongated healthy roots**

* ±SE: Standard error from mean; Data represents the mean of three replicates.

Initiation of the roots was considered as breaking of dormancy.

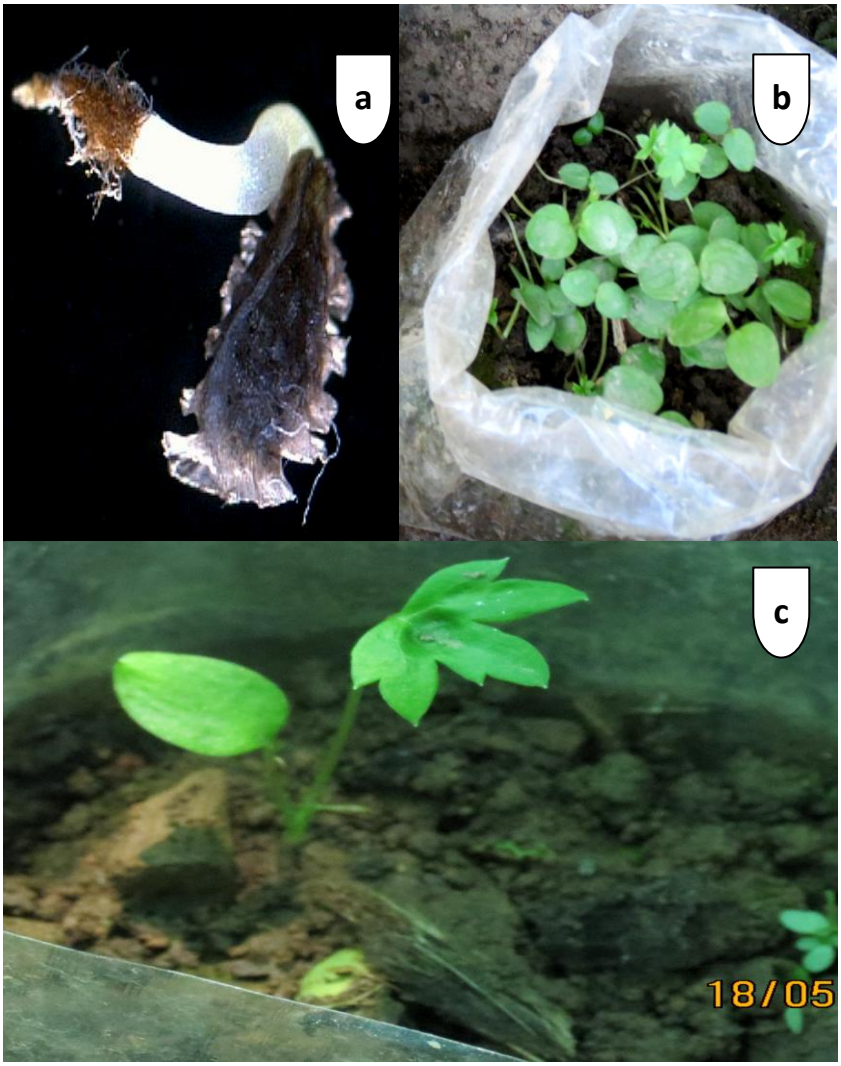


Figure 6: a. *A. nagarum* seed germinated and released radical on filter paper in the laboratory; b. Germinated seeds produced seedlings in the polybag in the seed bed; c. One seedling with secondary leaves.

Table 8: Effect of stratification on the seed germination of *Aconitum nagarum* in seed bed (Polybag)

Treatment type	Avg. time taken to germinate (days)	% response (±SE)*	Types of response
Without stratification	29	6.7 (0.2)	Healthy rooted seedlings
Stratified for 24h at 4°C before sowing	29	10.0 (0.2)	Healthy rooted seedlings with cotyledonary leaf
Stratified for 48h at4°C before sowing	28	3.3 (0.3)	Healthy rooted seedlings
Stratified for 72h at 4°C before sowing	25	3.3 (0.1)	-do-
Stratified for 96h at 4°C before sowing	23	20.0 (0.2)	Healthy rooted seedlings with cotyledonary leaf

* ±SE: Standard error from mean; Data represents the mean of three replicates.

Emergence of the root was considered as breaking of dormancy.

Table 9: Seedling Morphology of *Aconitum nagarum*

Seedling morphology	**No. of days taken**	**Types of plant response**
Germination	29	emergence of the root
Cotyledonary leaf	2-3	emergence of the shoot
True leaf	58-60	cotyledonary leaf shade and true leaf emerged

Data represent the mean of 3 replicates with non-stratified seeds.

Table 10: Effect of post harvest storage (at 25°C) on seed germination and viability of *Aconitum nagarum* on seed bed

Storage duration at 25°C (months)	Time for first sign of germination (days)	% germination (±SE)*	Types of response
0	10-30	6.7 (0.2)	Healthy rooted seedlings
1	30-60	6.5 (0.7)	-do-
2	60-90	6.0 (0.6)	Healthy seedling with stunted growth
3	90-110	5.5 (0.5)	Delayed germination
4	110-130	4.5 (0.6)	-do-
5	130-160	2.2 (0.3)	Delayed germination with stunted growth
6	0	0	No germination

* Standard error from mean.

Data represent the mean of three replicate without any stratification.

In the present study different technique were adapted for seed germination. In the filter paper test, maximum germination was achieved from the seed stratified for 48 hrs at 4ºC followed by 96 hrs when maintained in the laboratory at 25ºC with minimum days taken to germinate. Under this condition 38% and 15.38% seed germination recorded after 13 days and 10 days of sowing respectively (**Table 7 & Fig. 6a**). Seeds without stratification exhibited very poor germination (6.7%). Compare to filter paper test, stratified seeds sowed on seed beds (prepared in poly bags supported better germination).

Seeds sowed in the poly bags exhibited 20% germination within 23 days from the seeds stratified for 96 hrs at 4^{o}C (**Table 8 & Fig. 6b**). But there was no seed germination recorded from the seeds maintained in the incubator at 30ºC across the stratification period. Seed germination was achieved within 29-30 days with emergence of roots with cotyledonary leaves while true leaves formed within 58-60 days (**Table 9 & Fig. 6c**). In the present study it was found that seed germination rate, germination time was greatly influence by pre-treatment of the seeds. There was significant difference in the germination period, germination rate with the stratified and non-stratified seeds.

Post harvest storage tolerance of seeds of *Aconitum nagarum*

During the present study efforts were put into to study the post harvest storage tolerance of the seeds. The seeds were stored at 25^{o}C for 0-6 months and seeds were sowed in the seed beds at one month interval (**Table 10**). Data collected in the present study exhibited gradual decline in germination response after one month of storage and from the third month germination rate declined significantly. The germination rate declined from 6.7% to 4.5% in the fourth month and in the sixth month there was no

germination i.e., seeds lost viability completely. It clearly shows the recalcitrant nature of the seeds of *A. nagarum*.

Seedling morphology and seedling mortality

The first sign of seed germination was the emergence of radical from the seed coat followed by a pseudo cotyledonary leaf formation. The cotyledonary leaf was replaced by a true leaf. The true leaf starts emerging from the base of the pseudo leaf. As the true leaf progress in size, the pseudo leaf turn yellowish in appearance and slowly wither giving way for the true leaf to succeed after 58-60 days of seed germination.

The seedling mortality was observed by transplanting the seedling in the poly house. Monthly basis survey was conducted to access the seedling mortality in the poly house. Seedling survival was very high till the month of April/May and starts decreasing by the last week of June/July. The growth of the plant became stunted. The seedling mortality was very low though seed germination was high in the forest as well as in the poly house.

Discussion

Floral biology

The budding, flowering and seed setting period of the plant varies with different location. The budding and flowering period of the plant differ greatly in Dzuku valley and in the Sirui hills. This difference in the time of flowering, budding and seed setting was influenced by the microclimatic conditions and variation in the geographical condition. As the place was comparatively colder in the Dzuku valley when compare to the Sirui hills. The flower become lighter in colour from rich purple to whitish purple

indicating that the plant had dehisced completely. The dehiscence of the anther was not uniformed and took 3-4 days to completely dehisce.

Distribution pattern and its associated species

The plants are distributed in different places of Manipur and Nagaland at Sirui hills, Dzuku valley, mount Saramati and Japfu hills mainly among the grassy bamboo slope. The plants were found to grow along with the *Frageria* species, *Berberis* species, *Gautheria* species etc.

Seed biology

The time for emergence of radicals from the germinated seeds, germination rate, seedling morphology and seedling establishment is influenced by various factors. The requirement of light for successful seed germination and healthy seedling morphology appears to be species specific. Seedling survival on the seed beds/forest floor is governed by the availability of light, water and nutrients (Kitajima, 2007). Plant species differ greatly in their habit preference, temperature requirement, and post harvest storage, specific pre-treatment for seed germination, seedling emergence and survival. A number of tree species exhibit positive as well as negative correlation between canopy cover/light requirements, temperature etc (Kwit and Platt, 2003; Pages *et. al.*, 2003). Storage containers have also great influence on the germination of seeds (Verma *et. al.*, 2009). Seeds treatment with chemical and low temperature enhances seeds germination (Pandey *et. al.*, 2000; Dosman, 2002; Butola and Badoda, 2004; Srivastava *et. al.*, 2011; Sharma and Gaur, 2012).

Though, the practice of seed preservation is as old as agricultural practices but systemic collection and storage facilities have been a development of the 20th century. At present there is an estimate of over 1500 seed or gene banks around the world

containing 6 million seed accessions. Viability of a seed lot declines over time, though the seed may germinate, the resulting seedlings may have reduced vigour and fail to establish (Walters, 2004). So, for some plant species, using relatively fresh seeds gives superior germination over stored seeds.

During the present study, investigation was carried out on the relationships among rate of cold stratification to determine whether the seeds require pre treatment of low temperature for germination. The finding in the present studies thus support that seeds of *Aconitum nagarum* prefer low temperature for seed germination. The lowest temperature withstood by the recalcitrant seeds seems to vary with the species (Fu *et. al*., 1990; Oliveira and Valio, 1992; Barbedo and Cicero, 2000; Tommasi *et. al*., 2006). *Ginkgo biloba* seeds could be stored at 4°C for one year but when stored at 25°C, seeds died after 6 months (Tommasi *et. al*., 2006). Present study also exhibited a similar response where seeds stratified at 4°C germinated better over seeds stored at 25°C.

Post harvest storage tolerance of seeds of *Aconitum nagarum*

The plant was intolerance to long term storage. As the time of storage period increased, the rate of germination and seedling viability also decreased. Further seeds soaked just after harvest and sowed in the seed bed exhibited better germination. These suggest the recalcitrant nature of the seeds. The seedling mortality was greatly influence by the microclimatic conditions, nutrient availability etc .both in the forest at the natural habitat as well as in the poly house. In natural habitat, the plants though germinated become stunted in growth with yellowish leaves and soon perished.

Seedling morphology and seedling mortality

The seedlings have two phases of growth, the pseudo cotyledonary stage and the true leaf stage. During the cotyledonary stage, plants acclimatized and rhizome

formation started, paving way for the plant to establish. The cotyledonary leaf was replaced by true leaf after a gap of about two months.

Seedling mortality was study in the Dzuku valley, Nagaland and in the poly house in the Botanical garden of the Department of Botany. Seedling mortality was a difficult task to maintain both in the forest and in the poly house. Though the seedling germination was high, seedling survival was a question. In the poly house, the plantlets died because of the water logging during rainy seasons. Therefore great care and efforts was needed to maintain the plant. The plant was also very weak and not hardy which needs extra care while maintaining the plants. The seedling mortality was greatly influenced by the microclimatic conditions, nutrient availability. In the forest plantlets may have died due to micro and macro nutrient deficiency because they grow among the grassy Rocky Mountains.

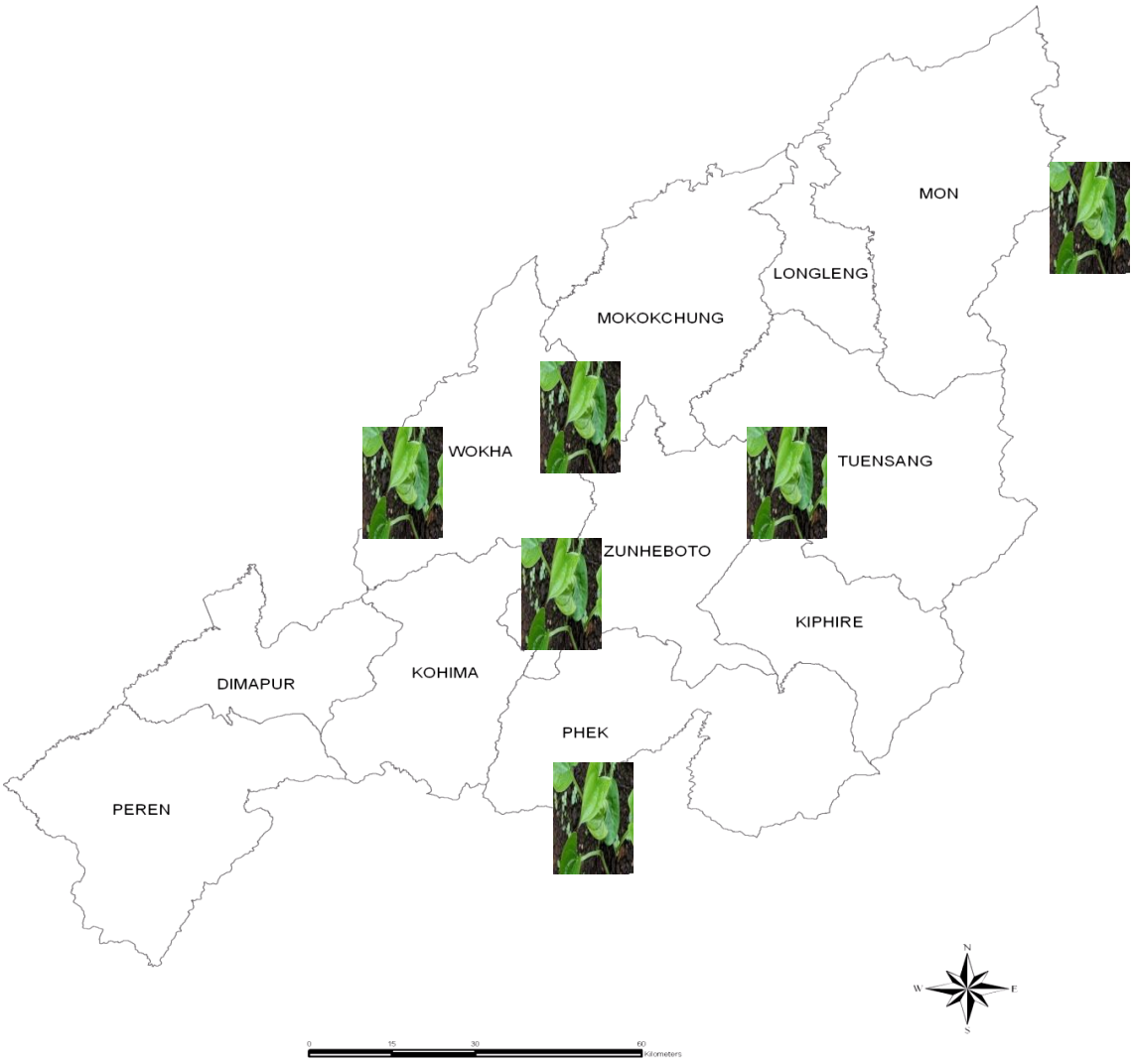
The plant reproductive study is crucial for conservation strategies of this endemic plant. The outcome of the present study will help in working out the conservation strategies and seed propagation of the species. Further studies on reproductive biology are warrants to understand the distribution of this economically important species in nature.

Chapter - 3

Reproductive Biology and Seed Biology of *Dioscorea villosa* L.

The family Dioscoreaceae is represented by about 650 species within ten genera. They possess rhizomes and aerial potato or tubers. *Dioscorea* constitute the staple food in some part of the world. They are rich in carbohydrate and many wild species are believed to contain diosgenin, the precursor in the commercial production of sex hormone and corticosteroids. In India, yams are cultivated as garden crop or as a subordinate crop in some parts. They are propagated through tuber cutting, small tuber or bulbils. The crop matures within 7-10 months. They are grown on well drain, deep soil. Because of the many fold uses as food or as drugs, some of the species are under threat. To understand the reproductive behaviours of one of the important species of

Dioscorea i.e *Dioscorea villosa,* the present work was undertaken which will help in conservation strategies.



Map of Nagaland

Figure 7: Map of Nagaland showing the distribution of *Dioscorea villosa* in Nagaland and adjoining states.

Dioscorea villosa L. (Dioscoreaceae) is a perennial herbaceous vine with a tuberous root. Among the different species of *Dioscorea*, *D. villosa* is the species which do not produce any air potato. The plants sprout out during the month of March-April. The plants are unisexual (dioecious plants) with a height of about ten foot with alternate, verticellate lower leaves (**Fig. 2b & c**). The leaves is 7-9 veined, ovate-cordate, glabrous, leave margins entire, apex acuminate petiolate, reticulate venation .The stem is narrowly winged, polygonal species. The species is use as a source of saponins for preparation of steroids in the pharmaceutical (Dweck, 2002). The rhizome extract is a medicinal herb used to treat symptoms of rheumatoid arthritis and menopause, colic (Kaimal and Kemper, 1999; Wojcikowski, 2008). As skin cream – diosgenin as a source of natural hormones, antifungal, use in urinary tract problems in chiness traditional medicine, treatment of asthma and cardiovascular (Kaimal and Kemper, 1999). The species also possess anti carcinogenic potential (Chao-chin *et. al.,* 2007). Due to the many fold uses, the plant is under threat in the natural habitat. Therefore, with the objective to understand the reproductive phenology, floral morphology, seed biology and pollination of this species present study was undertaken with conservation and propagation perspectives.

Material and Methods

Plant material

The plant was collected from Mopunchuket village of Mokokchung District, 26^{o}24'02.1" N latitude and 94^{o}31'17.6" E longitude at an elevation of 1044 mASL and Lumami village of Zunheboto District, 26^{o}12'37" N latitude and 95^{o}29'28" E longitude, Nagaland (**Fig. 7**). The plantlet with the rhizome was grown in the Botanical garden, Department of Botany, Nagaland University, Lumami located in Zunheboto district of

Figure 8: a. Yellowish green mature fruits of *Dioscorea villosa* ready for harvest; b. Collected mature dried fruits ready for harvest of seeds in the laboratory.

Nagaland (26º12'37" N latitude and 95º29'28" E longitude; altitude 1150-1200 m ASL). The plants maintained in the Botanical garden were used for the experimental purpose like to study the reproductive behaviours, seed biology etc.

Floral phenology and morphology

The study was conducted in the Botanical garden of Department of Botany, Lumami, Nagaland. The reproductive phenology viz, time of rhizome sprouting, time of budding, flowering of male and female plant, fruit and seed setting were studied. The female and male plants were maintained side by side for the present study. In order to estimate flower production, total number of flower per plant was counted manually in the selected plants. Seeds per pod were counted to quantify production of pods.

Pollination

The pollination of *Dioscorea villosa* was studied by identifying the visitors of the flowering plant. The frequency of visit per flower was observed during the flowering period. Maximum visitation rate of the insects during the day time was also recorded.

Seed biology

Seed collection

The mature fruits were harvested from the Botanical garden. For the present study, the intact matured fruits of about 16 weeks old were harvested randomly from the plant during 2011-2013. The seeds are harvested when the fruits turned yellowish green (**Fig. 8a**). The harvested fruits were brought to the laboratory immediately after harvest. The collection was completely randomized without seeing the size. The fruits were air dried inside the laboratory by spreading on the old newspaper. The collected

fruits were stored in plastic bags at 25ºC till used (**Fig. 8b)**. The seeds removed from the dried fruits and were divided into different groups for different experiments.

Preparation of potting mix

The potting mix for the experimental purpose was prepared by mixing soil, sand and coconut coir at 1:1:1 ratio. The garden soil is crushed into find powder and mix with the coconut coir and sand in the ratio of 1:1:1 and put in a plastic pot and transparent poly bag. The poly bag and plastic pot are made perforated for better aeration. They were kept moist before the transfer of seeds for better germination response. The differentially treated seeds were then sowed on these potting mixed.

Experimental process

A part of the seeds were sowed while others were treated differentially at 4ºC in a refrigerator for 0, 24, 48, 72, 96 hours and sowed as described below:

1. A set of processed seeds were used for filter paper germination. Seeds were sowed in filter paper in a humidity chamber of 90 mm in diameter. The filter paper was moistened with 5ml of water and kept in a laboratory at room temperature (25ºC).

2. Another set of processed seeds were sowed in a poly bag and kept in an incubator at constant temperature of 30ºC.

3. While, another set of processed seeds were sowed on different PGRs containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) fortified with sucrose (3%, w/v) and gelled with agar (0.8%, w/v). The medium was dispensed in a borosilicate test tube (size: 25 x 150 mm) and kept under florescence light (40 µmol m^{-2} s^{-1} illumination) of 12/12 light/dark period.

Table 11: Phenology of the *D. villosa* plant in the garden

Parameter	**Female plant**	**Male plant**
Sprouting of rhizome	March/April	March/April
Budding initiation	September/October	October/November
Flowering period	October	November
Seed maturity	February/March	No seeds
Rhizome maturity	March	March
Rhizome splitting	March/April	March/April

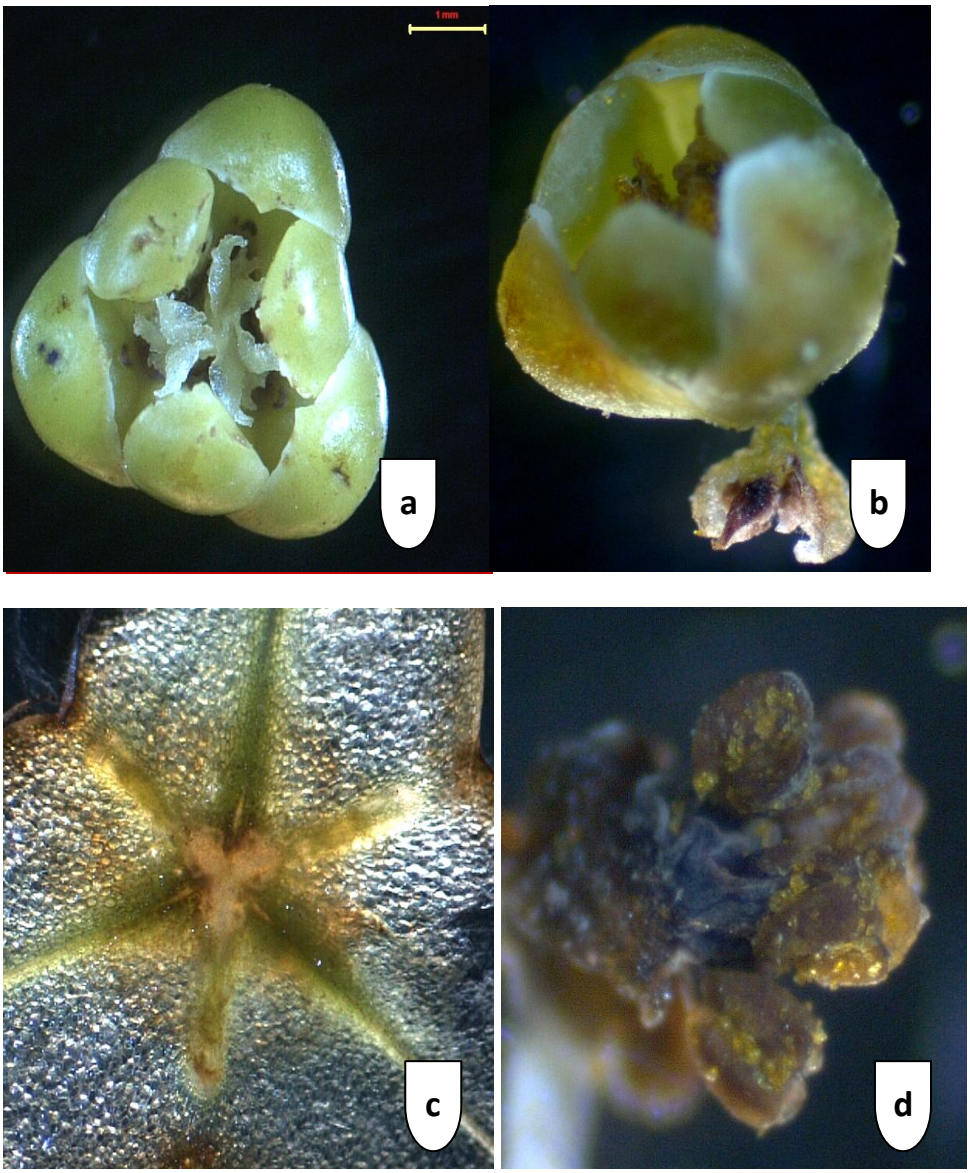


Figure 9: *D. villosa* female flower (enlarged); b. Male flower (enlarged); c. Cross section of the ovary showing empty locule or aborted seeds; d. Cross section of male flower showing bilobed anther.

To study the emergence, survival and growth of seedlings of *Dioscorea villosa* under the above mentioned conditions, 4 replicates of 10 seeds (N=40/test) were used. The potting mix was packed in poly bag and used for seed germination purpose. In each potting mixed 10 seeds were sowed. The seed beds were watered at regular interval. In filter paper germination test, 4 replicate of 10 seeds were sowed in a humidity chamber of 90 mm in diameter. The filter paper was moistened with 5 ml of water on regular basis. The experimental design was completely randomized. The data was collected at regular basis for seed germination and seedling morphology.

The seedlings were maintained in the respective poly bag and watered at regular interval and the seedling morphology was studied. Once the seedlings showed normal functioning like formation of seedling growth, normal leaves etc., the seedling were transferred to the poly house and seedlings mortality were observed.

Results

Plant morphology and floral phenology

Dioscorea villosa is a unisexual or dioecious plant. The rhizome of the species sprouts in the month of March-April and bud initiation occurs in the month of October (**Table 11**) and flowers in the month of October. Seed sets in November and matures in February-March. The rhizome mature during March and ready for harvest. Most of the species of *Dioscorea* produces air potato but *D. villosa* does not produces air potato. Both male and female flowers are pedicellate, perianth fleshy, tepal in two whorled of three, polytepalous, sepaloid. The pistillate flower is yellowish green, tepals in two whorl of three, stigma is wet, bifid, epigynous, ovary trilocular, axial placentation (**Fig. 9a & b)**. In the cross section of the young fruits, in most of the cases the locules were found to be empty/aborted (**Fig**. **9c**). Staminate inflorescence bracteolate. The

Table 12: Fruit setting of *D. villosa* plant

Sl. No.	No. of fruit per inflorescence	Healthy fruit formed per inflorescence	Aborted fruit per inflorescence
1	7	3	4
2	5	3	2
3	10	3	7
4	6	4	2
5	3	2	1
6	7	3	4
7	8	6	2
8	5	2	3
9	11	5	6
10	12	6	6
11	10	5	5
12	5	2	3
13	5	5	0
14	8	4	4
15	6	3	3
16	8	3	5
17	5	3	2
18	6	2	4
19	10	4	6
20	7	4	3
Mean	***7.2***	***3.6 (50%)***	***3.6 (50%)***

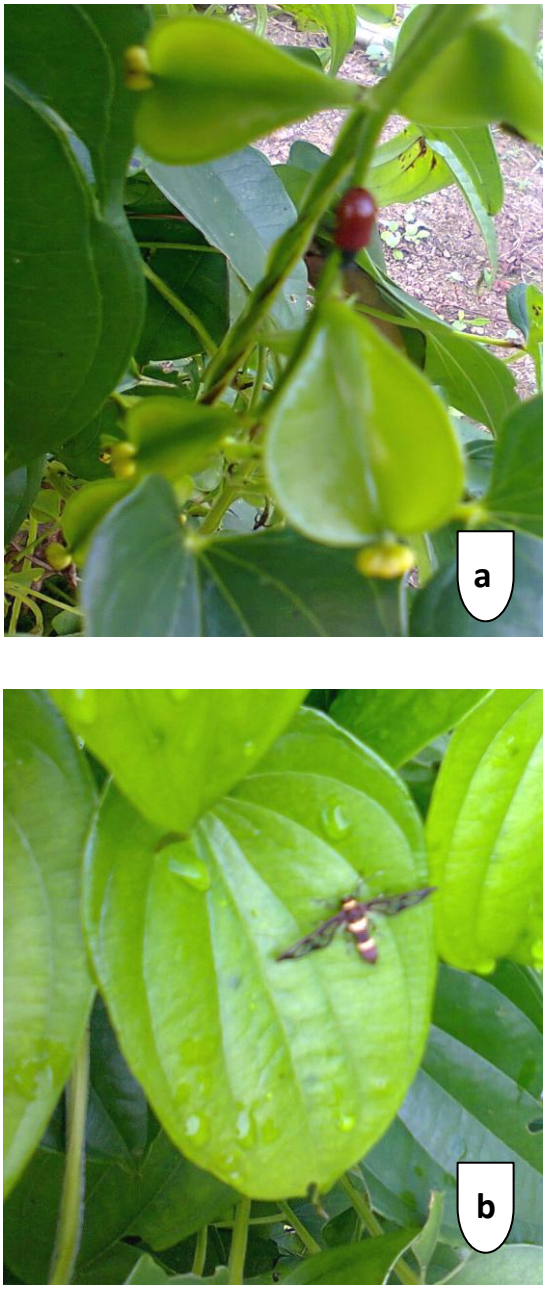


Figure 10: a & b. Some of the visitors (considered to be the pollinators) of *Dioscorea villosa* flowers.

Table 13: Visitation rate of insects on *D. villosa* plant

Time slot	Visitor A	Visitor B	Visitor C	Visitor D	Visitor E	Visitor F
7.30-8. 30 A.M.	2	-	1	**1**	-	1
8.30-9.30 A.M.	1,1,1,1	-	-	**2**	-	1
9.30-10.30 A.M	1	1	1	**-**	-	1,1,1
10.30-11.30 AM	**-**	**2**	**2,3,1**	**2,1**	**-**	**-**
11.30 AM-12.30 P.M	3	-	1	**3**	-	1
12.30-1.30 P.M	-	-	-	**-**	-	-
1.30 -2.30 P.M	-	-	1,1	**2**	-	-
2.30-3.30 P.M	-	-	1	**1,1**	-	-
3.30-4.30 P.M	1	-	-	**1,1**	1	1

Frequency of visit = No. of visits/flower/hour

1**. Visitor a (black and yellow stripe flies)** = 11/83 = 0.13; 2. **Visitor b (blue beetle)** = 3/83 = 0.03; 3. **Visitor c** (**orange beetle**) = 12/83 = 0.14; 4. **Visitor d (red beatle)** = 15/83 = 0.18; 5. **Visitor e (spider)** = 1/83 = 0.01; 6. **Visitor f (black wasp)** = 0.08

Figure 11: *D. villosa* seed germination in incubator.

inflorescence is single and in the axils of upper leaves. The filament is straight, separated, didymous anther lobes and ovate tepals. In male flower, anther bilobed, staminoids six arranged in two whorl of three (**Fig**. **9d**) with short filaments, basally fixed with tepal, the anther bilobe. Both male and female flower are mildly scented of a jasmine like odour. Ovary is trilocular, axial placentation, fruits loculicidal. *Dioscorea villosa* is a highly polymorphic.

During the present investigation during 2010-2013 it was observed that there was an average of 7.2 fruits formation per inflorescence of which 50% of the fruits bearing seeds while remaining 50% of the fruits were seedless (**Table 12**). Seeds produced were very light, winged and brown in colour.

Pollination

The pollination of *Dioscorea villosa* was found to be xenogamous. There was a presence of olfactory in the form of floral scents. The flowers were visited by different insects which include beetles, flies, wasps etc (**Fig. 10**). Maximum visitation rate was observed during morning from 8.30 A.M and in the evening from 1.30 P.M to 4.30 P.M but less visitation of insect was observed during afternoon (**Table 13**). Highest frequency of visit was recorded with beetles.

Seed biology

Freshly harvested seeds (greenish in colour) when sowed on filter paper in humidity chamber failed to show any sign of germination. Seeds sowed in the incubator at a constant temperature of 30ºC showed emergence of radical and shoot formation and germination started within 35 days (**Fig. 11a**) indicating that the species can grow well and adopt to comparatively high temperature for germination. The seed

Table 14: Effects of cold stratification (4ºC) on the seed germination of *D. Villosa* on seed bed

Stratification duration (hour)	% response (±SE)*	Types of response
0	2.5 (0.2)	Seedling formed but not healthy
24	2.5 0.2)	-do-
48	7.5 0.3)	Healthy seedlings
72	3.5 (0.2)	As above
96	10.2 (0.2)	As above

* Standard error from mean.

Seeds sowed in the seed bed; Data represent the mean of 3 replicates.

Each replicate contained 10 seeds.

Table 15: Effect of cold stratification (4^{o}C) on the seeds of *D. Villosa* on filter paper test

Stratification duration (hour)	% response (±SE)*	Types of response
0	-	No response
24	-	-do-
48	-	-do-
72	-	-do-
96	25.5 (1.5)	Germinated seeds formed shoots

* Standard error from mean.

Data represent mean of three replicates.

germination rate and germination time was greatly influence by pre-treatment of the seeds (**Table 14 & 15**). The seeds stratified at 4^{o}C for 96 hrs exhibited optimum germination (10.2%) under the given conditions in the seeds bed compared to non-stratified seeds (2.5%) (**Table 14**). While seeds incubated in the incubator exhibited 25.5% germination from the seeds stratified for 96 hrs. (**Table 15**). There was significant difference in the germination period, germination rate with the pre-treated seeds. The rate of seedling establishment rate was very high though the germination rate was comparatively low. This finding suggests that once the seed germination was achieved, and seedlings were established, the seedling survival rate was high.

Discussion

Floral biology

During the present investigation it was observed that *D. villosa* does not produce air potato which makes the species different from the other species of the genus *Dioscorea*. The male flowers were arranged in 2 whorls of 3, bracted, anther bilobe, didymous. Male flowers served only for the production of pollen and once the anther dehisced, the flowers wither. The female flowers were also arranged in 2 whorls of 3. Though there was high flowering and fruiting, seed production per plant was quite low. This result supports the previous work done on *Crataeva religiosa* by Singh *et. al.* (2003).This could be one region why the plants are rare because seeds are also an important factor for maintaining the plant from generation to generation.

Pollination

The insects visiting the plant include wasps, flies, ants and beetles. Wasps and some beetle are found on the dorsal and ventral surface of the leaves. Beetle stay on the flower throughout the day visiting each flower. Maximum visitation by the insects was

observed during morning and evening. But the insects visits decreases during the afternoon period when the studies were undertaken. The pollen was found to be sticky in nature and orange in colour.

Seed biology

The seed preservation practice is as old as agricultural practices but systematic collection and storage facilities have seen a development in the 20th century. There is an estimate of over 1500 seeds or gene banks around the world containing over 6 million seed accessions. For some plant species, using relatively fresh seeds gives superior germination over stored seeds.

The time for emergence of radicals from the germinated seeds, germination rate, seedling morphology and seedling establishment is influenced by various factors. Plant species differ greatly in their habit preference, temperature requirements, post harvest storage, specific pre-treatment for seed germination, seedling emergence and survival. According to Onan (2006) germination of mugword seed was greatly influenced from temperature, but light condition was almost independent. Dosmann (2002) also reports that seed stratification improves germination of seeds.

In the present study, freshly harvested seeds (green colour) of *Dioscorea villosa* when sowed on seed beds failed to germinate indicating that seeds were dormant at maturity. Effective seed germination was achieved when seed dormancy was broken by cold stratification at 4^{o}C for 96 hrs suggest that *Dioscorea villosa* seeds are morphologically dormant. Findings in the present investigation is in agreement with the previous results that species with underdeveloped embryo require some post harvest period for embryo to grow to some critical threshold before germination occurs, and

require some special treatment before they break morphological dormancy and exhibit germination (Albrecht, 2006).

During the present study, study was carried out on the relationship among rate of germination with pre-treated seeds and non-treated seeds. When seeds were given a constant cold stratification for a period of 96 hr (4ºC) and then moved into warmer temperature (30ºC, incubator), dormancy was broken and seeds germinated to high rate of 40% after 35 days. This suggest that once the critical time period of cold stratification demanded by *Dioscorea villosa* seeds is achieved, seeds are capable of germinating in temperature ≥ 15ºC. This breaking of dormancy by cold stratification without prior warm stratification treatment, indicates that *Dioscorea villosa* seeds has non-deep simple morphologically dormancy seeds (Albercht, 2006). The seeds which were not treated with cold stratification show less percent of germination ~10% only with delayed in the germination period.

The finding in the present study reveals that the seeds of *Dioscorea villosa* are physiologically dormant. Since cold stratification breaks dormancy which suggest that effective dormancy could be broken with 96 hr of cold stratification at 4ºC followed by sowing at warmer temperature of 30ºC. Conversely, this alternating temperature was ineffective at breaking dormancy in *C. canadensis* suggesting that temperature higher than 4ºC is ineffective at breaking physiological dormancy (Albercht, 2006). *Dioscorea villosa* seeds is therefore capable of germinating at a high constant temperature of 30ºC in an incubator, once the critical time period of cold stratification (4ºC) demanded by the *Dioscorea villosa* seeds was achieved.

The dispersal of seed was observed to be mediated through wind in nature. Few seeds which shaded on the forest soil germinated during favourable conditions. Seeds

which fall on the forest leaf failed to germinate due to deficiency of nutrients. In conclusion, this study can guide seedling in the field by following the temperature requirements for dormancy break identified, thereby, facilitating the conservation management strategies which often limit the seed dispersal and scarcity of the plant in nature.

Chapter - 4

Micropropagation of *Aconitum nagarum*

The genus *Aconitum* belongs to the family Ranunculaceae is distributed worldwide with nearly 300 species. In India the genus is represented by about 33 species mainly distributed in sub-alpine and alpine zones of Himalayas (Sharma and Gaur, 2012). Aconitum are highly valuable herb because of the rich source of diterpenoid alkaloid contained. Many reported the presence of diterpenoid alkaloid (Dong *et. al*., 2000; Zhang *et. al*., 2005). The roots/tuber is used as a release of pain to dispel dampness, analgesic activity, cures snake bite, nausea, vomiting, and as antibacterial etc. (Dong *et. al*., 2000; Zhang *et. al*., 2005; Srivastava *et. al*., 2011; Sinam *et. al*., 2012). Besides its high medicinal importance, this species provides excellent ornamental value.

Owing to its high medicinal value, the demand of *Aconitum nagarum* is increasing and the species is being exploited from its natural habitat. The species is also being endangered in its habitat due to various anthropogenic activities, natural

calamities and micro climatic condition. Therefore, there is a need to raise the plant species in large scale to fulfil the increasing demand on the one hand and help the conservation of the species on the other hand. Thus development of micropropagation protocol of *A. nagarum* will help in solving the above problem.

Successful *in vitro* establishment of aseptic cultures of any plant species depends on various factors like developmental stages of the explants sources, type of the explants, quality and quantity of the organic carbon sources, nutrient media, plant growth regulator, microclimatic conditions in the laboratory etc.

Material and methods

Plant material collection, processing and sterilization

The present study was aimed for establishment of culture systems for production of cloning planting materials under *in vitro* condition and propagation of *Aconitum nagarum*. To achieve the overall objectives of the present study and initiation of cultures, the mature fruits were collected during October-November, 2011-2013 from Dzuku valley of Khonoma village, Nagaland and Sirui Hills of Urkhrul district, Manipur, India. The collected mature fruits were transported to the laboratory within 2 days. The seeds were wrapped in old newspapers and kept in a poly bags. On reaching in the laboratory the seeds were removed from the fruits and stored in a seal polythene bags at 4ºC for experimental purpose.

Seed explants

The mature fruits of the plant were harvested randomly from the forest of Dzuku valley, Nagaland and Sirui hills, Manipur and were used for the present study. The processed seeds were surface sterilized with aqueous solution of $HgCl_2$ (0.3%, w/v) for

5 min and subsequently rinsed 4-5 times with sterile distilled water. Finally, the explants were dipped in ethanol (70%, v/v) for ~20 sec and rinsed with sterilized distilled water. After sterilization, the explants were soaked in sterilized water till culture.

Shoot buds

The shoots were collected from the *in vitro* raised plants developed from the germinated seeds. After the plants were taken out in the laminar flow cabinet, the shoot buds were dissected from the harvested shoots and soaked in sterilized distilled water till used to prevent them from desiccation and kept them fresh and was wiped with sterile tissue paper just before inoculation in the culture medium. The shoot buds were harvested in different periodically in different seasons (winter, spring, summer and autumn) to study the seasonal influence on morphogenetic response.

Tissue culture media

For culture of mature seeds and shoots, MS medium was used. The basal media was fortified with sucrose (3%, w/v) and gelled with agar (0.8%, w/v) and supplemented with different quality and quantity of plant growth regulators (PGRs) like α- naphthalene acetic acid (NAA), benzyl adenine (BA) and 2,4-dichlorophenoxy acetic acid (2,4-D) at a different concentrations which were used either singly or in combination.

The *p*H of the medium was adjusted to 5.6 using 0.1 N NaOH and 0.1N HCl and about 12 ml medium was dispensed in each borosilicate test tube (size 25X150 mm) and cotton plugged. The medium was sterilized by autoclaving at 121ºC for 20 min at a pressure of 1.05 Kg Cm^2.

Plant Tissue Culture

Initiation of cultures

Seed: The sterilized seeds was cultured on MS medium containing sucrose (3% w/v) and agar (0.8% w/v) as gelling agent and supplemented with different concentrations of PGRs such as NAA, BA, and 2,4-D at different levels (0-12 µM) either singly or in combination. In each test tube 3 seeds were cultured and for each treatment 30 seeds were maintained. The cultures was maintained at 25±2ºC under cool white fluorescent light provided by white CFL bulbs at an intensity of 40 µmol m^{-2} s^{-1} and 12/12 hr (light/dark) photo period.

Shoot buds: The Shoot buds of ~0.2-0.5 cm size were excised out from the *in vitro* grown plantlets and used for initiation of culture in the present study. The shoot buds were cultured on MS medium containing sucrose (3%) supplemented with different concentrations of PGRs such as NAA and BA (0-15 µM) in combination and congealed with agar (0.8%). All the experiments were repeated at least thrice.

Experimental design

In the present a completely randomized experimental design was performed. In all the experiments each treatment had at least 3 replicates and maintained at 25±2ºC under cool white fluorescent light at 40 µmol m^{-2} s^{-1} and 12/12 each (light/dark) photo period until mentioned otherwise. In each treatment 20 explants were maintained. All the cultures were sub culture at 4-5 wk interval unless mentioned otherwise. The data are recorded at regular interval.

The plantlets/ micro shoots developed from the germinated seeds, cultured shoot buds were maintained for another two-three passages on optimum initiation condition for further differentiation and culture proliferation.

Plantlets regeneration and culture proliferation

The shoot buds/ micro shoots developed in the initiation medium were maintained on initiation medium with optimum supplements for another 2-3 passages. In every sub-culture, the micro shoots/ shoot buds formed were separated and transfer to fresh regeneration medium for further culture proliferation. The micro shoots were maintained for 2-3 passages on regeneration medium before they were transferred to on rooting medium.

Rooting of micro shoots

About 5-6 cm long micro shoots with well expanded leaves from the regeneration medium were selected for inducing roots in the micro shoots before they are subjected to acclimatization and transplantation of the regenerates in the potting mix. The micro shoots were maintained on MS medium containing sucrose (3%) and NAA (0-5 µM) and maintained in normal laboratory conditions.

Hardening of regenerates

The well rooted plantlets were taken out from the rooting medium and transferred on MS medium fortified with sucrose (3% w/v) devoid of any PGRs and maintained in normal laboratory for 4 wk.

Potting mix and transplantation of regenerates

The hardened plantlets were taken out from the culture vials and washed with tap water to remove any traces of agar. The hardened plantlets were then transplanted

Figure 12: a. *A. nagarum* seed germination in culture showing the first sign of germination as emergence of radical; b. Multiple shoot formation rom germinated seed in culture.

Table 16: Effects of PGRs on *in vitro se*ed germination of *A. nagarum* on MS medium

PGRs Conc. (µM) BA	NAA	24D	Time for initial response (wk)	% response (±SE)*	No. of shoot buds formed/explant	Types of response
0	**-**	**-**	**-**	**-**	**-**	**No germination**
3	**-**	**-**	**9**	**5.00 (0.50)**	**-**	**-**
6	***-***	***-***	***8***	***20.0 (1.5)***	***3***	***Plantlets healthy***
9	**-**	**-**	***-***	***-***	***-***	**No germination**
12	***-***	***-***	***7***	***10.0 (1.5)***	***4***	***Plantlets healthy***
-	3	-	-	-	-	No seed germinate
-	6	-	-	-	-	-do-
	9	-	-	-	-	-do-
	12		-	-	-	-do-
-	-	3	-	-	-	-do-
-	-	6	-	-	-	-do-
-	-	9	11	10.00 (1.50)	-	Callus but degenerated
-	-	12	12	20.00 (1.00)	-	-do-

* Standard error from mean; Data represent the mean of 3 replicates on MS Medium with sucrose (3%); Data taken after 12 Wks of culture.

on plastic pots containing a mixture of soil, decayed wood powder, coconut coir at 1:1:1 ratio. The pots were covered with holed transparent poly bags and kept moist through the capillary water for a wk. The acclimatized transplants were finally transferred to the poly house.

Results

Initiation of culture

Seed germination

The mature seeds/embryo was cultured on MS medium containing different supplements. The quality and quantity of PGRs were found to be crucial factors for successful culture initiation. First sign of germination was recorded as the emergence of shoot from the cultured seeds (**Fig. 12a)**.

Effects of quality and quantity of PGRs on germination of seeds

Amongst the different levels of PGRs used for seeds germination, the MS medium containing BA (6 µM) singly supported optimum germination after 8 wks of culture where 20.0% seeds registered germination (**Table 16**). At lower concentration though there was initial sign of germination, failed to convert into seedling while, at higher concentrations reduced the *per cent* germination. Both the auxins (2,4-D and NAA) tested in the present study for seed germination did not support healthy germination. Of the two auxins, NAA across the concentrations did not support any germination while 2,4-D (9 µM) supported callusing but degenerate without shoot-bud formation. The germinated seeds formed plantlets within 12 wk of culture (**Fig. 12b**) followed multiple shoot formation.

Table 17: Effects of seasonal temperature of culture initiation of shoots of *Aconitum nagarum* on *in vitro* morphogenetic response

Month of culture initiation	% response (±SE)*	Types of response
Winter (10-19°C)	**95.50 (1.5)**	Plant healthy with multiple shoot bud formation
Spring (13-21°C)	80.50 (1.50)	Plant healthy with better response
Summer (19-29°C)	60.00 (2.00)	Stunted plant growth with less shoot bud formation, and in most cases plant degenerate
Autumn (15-25°C)	80.00 (2.00)	Stunted plant growth

Data represent the mean of replicates on 2 successive years.

* SE: Standard error from mean.

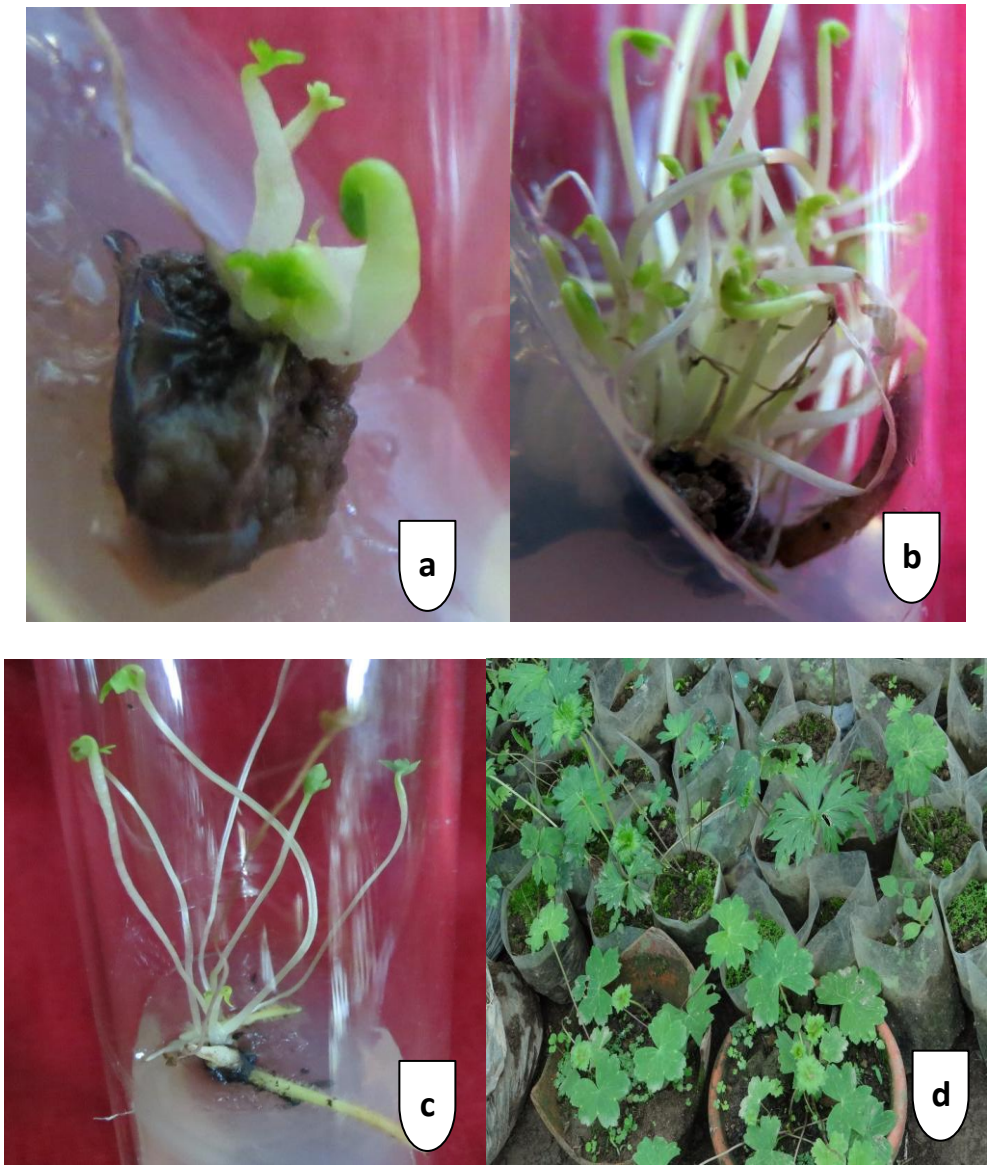


Figure 13: Different stages involves in *in vitro* culture of nodal explants of *A. nagarum*. a. Initial morphogenetic response from the cultured nodal segment; b. Multiple shoot formation on regeneration medium; c. A rooted plantlet; d. Some transplants established in community potting mix in the poly house.

Shoots explants

Cultures were also initiated from the shoot buds harvested from *in vitro* raised axenic cultures developed from the germinated seeds. The shoot buds were harvested in different seasons. The culture was initiated on MS medium fortified with sucrose (3%) and different quality and quantity of PGRs. It was found that establishment of culture and *in vitro* morphogenetic response of shoot buds was greatly influenced by various factors like quality of PGRs and season of explants harvest.

Seasonal effect on *in vitro* culture establishment

Cultures were initiated throughout the year in different seasons to study the effect on *in vitro* culture establishment. The present study was conducted consecutively for three years for culture proliferation and regeneration of the plantlets. In the present study with *Aconitum nagarum*, seasonal temperature of plantlets initiation greatly influenced the plantlets establishment and growth. It was observed that amongst the different seasons investigated for culture initiation on optimum medium and supplements, the shoot bud culture during winter responded optimally where as much as 16 shoot buds formed from single shoot and almost all the cultured explants responded positively (**Table 17**). While, plantlets cultured during summer registered lower morphogenetic response and developed shoots turned pale green and degenerated subsequently. The plantlets culture during autumn were least responding and plant were slightly stunted.

Effects of PGRs

For culture initiation besides seasonal influence, incorporation of the PGRs was a prerequisite. Amongst the different levels of PGRs used for culture initiation and proliferation, Kn was found to be inferior over BA in combination with BA. Under the conditions provided in the present study optimum morphogenetic response was registered on medium supplemented with sucrose (3%) and BA (6 µM). Under this

Table 18: Effect of BA and NAA on the morphogenetic response of shoot buds of *A. nagarum* from *in vitro* source

PGRs Conc. (µM) BA	NAA	Avg. Time for initial response (days)	Mean No. of shoot bud formed /explants (±SE)*	Mean plantlet height (cm)	Types of response
0	0	-	-	-	No response
3	-	8	7.1 (0.2)	3.2	Plant withered
6	**-**	**8**	**16.2 (0.3)**	**2.5**	**Plant healthy with shoot buds**
9	-	8	15.0 (0.5)	1.5	Plant healthy, stunted
12	-	8	10.5 (0.5)	2	Plant healthy
15	-	16	12.0 (0.4)	1	Plant healthy and stunted
3	1	16	13.5 (0.2)	1.5	-do-
6	1	8	4.2 (0.3)	2	Unhealthy plantlets
9	1	8	14.0 (0.4)	2	Plant healthy
12	1	16	13.0 (0.3)	2.5	-do-
15	1	8	8.0 (0.4)	2.3	-do-
3	2	16	11.5 (0.3)	1.2	-do-
6	2	16	6.0 (0.2)	0.5	Callus, plant not healthy
9	2	16	13.3 (0.3)	2	Plant healthy
12	2	16	6.5 (0.3)	2	-do-
15	2	8	10.5 (0.4)	0.8	Plant unhealthy, stunted growth

* Standard error from mean; Data collected after 8 wks of culture on MS medium with 3% (w/v) sucrose; Data represents the mean of 3 replicates

condition cultures established within one week of culture initiation (**Fig. 13a**). At lower concentration number of shoot buds formed was lesser in number and at higher concentrations of BA delayed morphogenetic response. Under optimum condition as many as 16 shoot buds formed per explants (**Fig. 13b & Table 18**). Sprouting of the shoot-lets was initiated within 4 wk of culture and within 6-8 wk multiple shoots/ micro shoots developed. It was found that when BA enriched medium was fortified with NAA, supported lesser micro shoots formation and there was callusing of the shoots at the basal part as well as micro shoots were shunted in growth. Besides this, the cultures developed on medium containing singly BA were healthy whole shoots on Kn enriched medium were slightly etiolated and unhealthy.

Plant regeneration and culture proliferation

The shoot buds/micro shoots developed from explants like seeds, shoot segments on initiation medium were maintained for another two to three passages for culture differentiation and proliferation on the respective initiation medium and with optimum supplements.

Effect of PGRs

The shoot buds from *in vitro* source cultured started proliferation into young plantlets within 4 wk on regeneration media. The different quality and quantity of PGRs incorporated in the regeneration medium exhibited differential response on regeneration and mass multiplication. Amongst the different quality and quantity of PGRs used, BA (6 µM) supported optimum regeneration and mass multiplication of plantlets which produced multiple shoots/buds. As many as 16 shoots/shoot buds developed per explants per subculture (**Fig. 13c**).

Table 19: Effects of different concentrations of NAA on root induction of *A. nagarum*

NAA Conc. (µM)	No. of roots formed/plantlets	Types of response
0	0	No roots
1	2	Plant healthy, etiolated
2	2	Plant healthy, fewer roots
3	2	As above
4	3	Plant healthy with less roots
5	***5***	***Maximum no. of roots with root hair. Plantlets are healthy***

Data collected after 11 Wks of culture on MS Medium containing sucrose (3%, w/v); Data represent the mean of 3 replicates.

Rooting of micro shoots

Regenerated shoots (~3 cm in length, sources directly from regeneration medium) induced roots when transferred on rooting medium. The shoots are treated with NAA (0-5 µm). Of the different concentration of NAA used for inducing roots, a concentration of 5µM supported maximum root growth where as many as 4-5 roots per plant developed after 8 wk of culture (**Table 19 & Fig. 13d**). At lower concentration roots were less with more growth of the shoots. While, at higher concentration roots are healthy.

Hardening of the regenerates and transplantation to potting Mix

The well rooted plants from rooting condition were transferred on MS medium containing sucrose (3% w/v) and agar (0.8% w/v) freed from any PGRs and maintained for 4-6 wk under normal laboratory condition. The hardened plantlets were taken out from the culture vials and washed with running tap water to remove any traces of agar. The hardened plantlets were transplanted onto plastic pots containing a mixture of soil, decayed wood powder and coconut coir at 1:1:1 ratio (**Fig. 13e**). The pots were then made perforated and covered with the holed transparent poly bags and kept moist by keeping in a tub/basin. About 45% of transplants survived after 8 wk of transfer. The hardened plantlets are transferred to the wild after 8 wk.

Discussion

Initiation of culture

Seed germination: Callusing of the mature seeds was observed after 8 wk of culture followed by differentiation into multiple shoot bud/ micro shoots by sub culture in the same medium. Many stimuli are communicated across the plant body by PGRs which

consequently play an important role in diverse aspects of plant growth and development (Mereier *et. al.*, 2003). At a cellular level, auxin affects division, expansion and differentiation. Cytokinins are necessary in concerts with auxin in many cases for cell division at G1-S and G2-M transitions in a variety of cultured plant cells as well as in plants. Progression through the cell cycle is central to cell proliferation and fundamental to growth and development of higher plants (Stals and Inze, 2001; Mereier *et. al.*, 2003; Abhyankar and Reddy, 2007; Dhavala and Rathore, 2010; Mir *et. al.*, 2011). In the present study of the different PGRs incorporated at differential concentrations for culturing seeds explants, NAA, 2, 4-D did not impact impressive morphogenetic response, while BA at a concentration of 6 µM supported early response of shoot buds formation on MS medium containing sucrose (3%).

Shoot buds: In the present investigation, incorporation of at least one of the PGRs was pre-requisite for *in vitro* culture initiation. On PGRs control medium the culture degenerate subsequently without any morphogenetic response. Of the three PGRs incorporated in different concentrations and combinations, optimum response was registered on BA (6 µM) enriched medium where as many as 16 shoots buds developed. Kn in general found to be inferior over BA either singly or in combination with NAA. Earlier Britto *et. al*. (2003) and Karuppusamy *et. al*. (2007) reported the synergistic effect of NAA and BA on nodal explants culture of *Ceropegia bulbosa* and *Hydrocotyle conferta* respectively. Dhavala and Rathore (2010) reported that cytokinin alone could not promote axillary bud breaking in *Embelia ribes* unless one of the auxin especially IAA is incorporated in the medium in conjunction with cytokinin. While in Guava nodal segment culture, incorporation of GA_3 along with BA was prerequisite for axillary bud breaking (Mangal *et. al*., 2008). But in *Adhatoda vasica* Nees. axillary bud proliferation and multiple shoot initiation were optimum on MS medium containing BA

alone (Abhyankar and Reddy, 2007). The findings of the present study are also in agreement with this report. In case of sugarcane leaf culture, NAA and BA in combination promoted shoot bud formation but NAA alone induced somatic embryogenesis (Lakshmanan *et. al*., 2006).

Plant regeneration and culture proliferation

The shoot buds/micro shoots developed on initiation medium were maintained for another two passages. The micro shoots are then maintained on MS medium containing sucrose (3%) and different PGRs. For regeneration of plantlets, incorporation of one of the PGRs was obligatory. In the absence of PGRs all the cultures degenerated. Amongst the three PGRs tested, incorporation of BA singly proved to be superior for shoot proliferation, and plant height. About 16 shoot buds were formed on medium containing BA (6µM) where average plant height was ~3 cm. While BA and NAA in combination exhibited a more or less similar response in the entire range studied and did not support optimum plant regeneration and culture proliferation.

The effectiveness of cytokinin on plant regeneration and culture proliferation is reported by many earlier workers (Selvaraj *et. al*., 2006, Baskaran *et. al*., 2009). In *Acacia confuse*, BA, NAA and Kn in combination (0, 0.05 and 0.05 mg L^{-1} respectively) produced maximum shoot buds where as many as 25 shoot buds developed in culture (Arumugam *et. al*., 2009).

Rooting, hardening and transplantation to potting mix

Regenerated shoots (~3cm in length, sources directly from regeneration medium) induced roots when transferred on rooting medium. The shoots were treated differentially for inducing roots. Under optimum condition, only 4-5 roots were formed

after 8 wk of culture on MS medium fortified with 5 (µM). At lower concentration roots are less with more growth of the shoots. While, at higher concentration roots are healthy. The role of auxin in rooting of the *in vitro* raised micro shoots have been shown in several species, including apple (Moncousin *et. al*., 1992), *Quercus suber* (Manzanera and Pardos, 1990), *Strobilanthes flaccidifolius* (Deb and Arenmongla, 2012). Auxins have been shown to act as a local morphogenetic trigger on the formation of lateral roots in *Arabidopsis*, leading to the specification of founder cells of the new organ from previous differentiated cells (Dubrovsky *et. al*., 2008). It was reported that NAA was better option followed by IAA and IBA. While, in some other reports, IBA was found to be superior over other PGRs for rooting (Abhyankar and Reddy, 2007; Baskaran *et. al*., 2009, Arumugam *et. al*., 2009). The promontory effect of NAA on rooting was also described in rice (Biswas and Mandal, 2007), in *Populus euphratica* (Ferreira *et. al*., 2009).

The rooted plants were hardened on MS medium containing sucrose (3%) and maintained for 4-6 wk under normal laboratory condition. The hardened plants were transfer to potting mixed as mention in material and methods. The plants were successfully transferred to poly house. About 100 plants were tested for survival and about 45% survival was registered after two months of transfer.

During the present investigation, protocols were established for culture initiation from shoot explants from *in vitro* source, seed explants of *in vivo* source. These techniques open new routes for *in vitro* mass multiplication of this economically important species of North-East India in general and Nagaland in particular. The protocols established for culture initiation from seed explants and shoots explants indicates the possibility of using alternative explants. The protocols may be used for

production of clonal planting material in commercial scale and could be used by the pharmaceutical industry. Future research warrants on utility of these plants.

Chapter - 5

Micropropagation of *Dioscorea villosa*

Dioscorea villosa belongs to the family Dioscoreaceae which comprises about 650 species belonging to ten genera. They possess rhizomes and tubers. The *D. villosa* plants sprout out during the month of March-April. The plants are unisexual (dioecious plants). The seeds are winged, show orthodox seed storage characteristics and can exhibit considerable dormancy. The species is use as a source of saponins for preparation of steroids in the pharmaceutical (Dweck, 2002). The rhizome extract is a medicinal herb used to treat symptoms of rheumatoid arthritis and menopause, colic (Kaimal and Kemper, 1999; Wojcikowski, 2008). As skin cream – diosgenin as a source of natural hormones, antifungal, use in urinary tract

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problems in chiness traditional medicine, treatment of asthma and cardiovascular (Kaimal and Kemper, 1999). The species also possess anti carcinogenic potential (Chao-chin *et. al.*, 2007). The species exhibits very poor seed setting and seeds viability. Due to the many fold uses, the plant is facing problems. Therefore development of *in vitro* propagation protocols will help in propagation and conservation of the species.

Material and methods

Plant collection

Dioscorea villosa was collected from Mophunchuket village under Mokokchung District, Nagaland and Lumami village of Zunheboto District, Nagaland, India. The collected species were maintained in Botanical garden of the Department of Botany at a temperature of ~25-29°C in summer and 12-19°C during winter in the experimental garden. The plant population were not in abundant but they are either in a cluster or single wherever they were located.

Selection of explants and sterilization

Seeds: Mature fruits were harvested from the experimental garden and were used for the present study. Once the fruits/capsule was collected, seeds are taken out of the capsule and washed with laboratory detergent. The seeds were then washed with running tap water 2-3 times. The seeds are surface sterilized with a solution of $HgCl_2$ (0.03%, w/v) for 5 min followed by washing 4-5 times with sterile water under the laminar air-flow. The seeds were dipped in 70% (v/v) ethanol for 20 sec just before inoculation in the medium.

Nodal explants from *in vivo* source: The nodal explant for the present study was collected from the plants maintained in the experimental garden. The *in vivo* source nodal explants were washed with running tap water and leaves were removed and then scrubbed thoroughly with laboratory detergent 'Labolene' (1:100, v/v ratio) followed by rinsing 3-4 times with running tap water. The explants were cut into segments with one or two nodes in each segment. The explants were sterilized with $HgCl_2$ (0.03%, w/v) for 3 min followed by ethanol (70%, v/v) for 20 sec inside the laminar air flow. The explants were subsequently rinsed 4-5 times with sterilized distilled water after every treatment. After sterilization the sterilized nodal segments were maintained in the sterile distilled water till used to avoid leaching exudates.

Nodal explants from *in vitro* source: The nodal explants were harvested from the axenic source cultures produced either from the cultured seeds or pre-existing cultures. The plantlets were taken out inside the laminar flow cabinet carefully and dissected out the nodal segments and soaked in sterile distilled water till used.

Leaf explants from *in vitro* grown plants: The leaves were harvested from the *in vitro* grown plants after 2-3 wk of emergence. The leaves were soaked in sterile distilled water after harvest till used.

Tissue culture medium

For initiation of *in vitro* culture as well as for the regeneration purpose from different explants, MS medium was used during the present study. For seed culture the basal medium was fortified with sucrose (3%) and PGRs like BA, kinetin (Kn), GA_3, indole butyric acid (IBA) (0-15 µM) singly. While for *in vitro* culture of nodal segments (both *in vivo* and *in vitro* sourced) and leaf explants, the basal medium was fortified with organic carbon sucrose (3%), different quality and quantity of PGRs like

BA, Kn and NAA at a concentration of 0-15 µM which were used either singly or in combination. The basal medium was also fortified with different quality and quantity of antioxidants like activated charcoal (AC), ascorbic acid and polyvinyl pyrollidone (PVP) at concentrations of 0-400 mg L^{-1}. The medium was gelled with agar (0.8%) before autoclave. The *p*H of the medium was adjusted to 5.7 using 0.1 N NaOH and 0.1N HCL and about 12 ml medium was dispensed in each borosilicate test tube (size: 25×150 mm) and cotton plugged. The medium were sterilized by autoclaving at 121ºC for 20 min at a pressure of 1.05 kg cm^{-2}.

Plant tissue culture

Initiation of cultures

Seed: The mature seeds were cultured on MS medium containing sucrose (3%) and different concentrations of PGRs such as BA, Kn, GA_3 and IBA (0-15 µM). For each treatment 20 test tubes were maintained and in each test tube one seed was cultured. **Nodal explants from *in vivo* source:** *In vivo* grown explants were collected and culture on MS Medium fortified with sucrose (3%), different quality and quantity of PGRs like BA, Kn and NAA (0-15 µM) either singly or in combination, different antioxidants and agar (0.8%) as gelling agent. The sterilized nodal segments of about 0.5-1 cm long were culture on the initiation medium. For each treatment 20 explants were maintained. The cultures were maintained at 25±2ºC under cool white florescent light at 12/12 hr (dark/light) period.

Nodal explants from *in vitro* source

The processed nodal explants harvested from the axenic sourced plantlets were cut into uninodal segment and cultured on MS medium supplemented with sucrose

(3%) and different quality and quantity of PGRs viz. BA, Kn and NAA (0-15 µM) either singly or in combination. For each treatment 20 nodal segments were cultured. The cultures were maintained at 25±2ºC under cool white florescent light at 12/12 hr (dark/light) period.

Leaf explants

Leaves of *Dioscorea villosa.* (~5.0 cm long) were isolated from *in vitro* stock plants (donor plants). The donor plants were raised from nodal segment cultured on MS medium containing sucrose (3%, w/v) and NAA (1µM) and Kn (9µM) in combination. The isolated leaves were soaked in sterilized distilled water till used, to maintain the freshness of the plant. The leaf explants were cut into ~1.0 cm pieces. Besides leaf segments, whole leaf was also cultured for comparative study. The leaf segments were cultured on MS medium containing sucrose (3%), and supplemented with different concentrations of different PGRs such as NAA, BA and Kn (0-15 µM) either singly or in combination.

Experimental design

The experimental design was completely randomized. In all the experiments each treatment had at least 3 replicates. The cultures were maintained at 25±2ºC under cool white fluorescent light at 40 µmol m^{-2} s^{-1} intensities and 12/12 hr each (light/dark) photo period until mentioned otherwise. All the culture was sub-cultured at 3-4 intervals until mentioned otherwise. *In vitro* response was evaluated based on the percentage of explants responded and number of propagules formed in the culture after specific period of time (as stated in the table) and data was expressed as mean of replicates.

The shoot buds/ propagules formed in the initiation medium from germinated seeds, nodal segments, and leaves were maintained for another two passages on the optimum initiation medium for further growth and differentiation. The propagules so developed were harvested and maintained on different culture conditions for plant regeneration and culture proliferation.

Culture proliferation and plantlets regeneration

The shoot buds /propagules of 3-4 wk old were cropped from the initiation medium and maintained on different strengths of MS medium (0, 1/4th, ½, 3/4th and full strength inorganic salts and full strengths of organic nutrients). All the nutrient media were supplemented with different organic carbon sources like dextrose, fructose and sucrose (0-4%, w/v) and optimum PGRs and antioxidant for culture initiation. All the cultures were maintained at full laboratory illumination and for 2-3 passages for plantlet development and culture proliferation.

Hardening of regenerates

The well rooted plantlets (~6-8 cm) were taken out from the regeneration medium and transfer on MS medium containing sucrose (2%, w/v), agar (0.8%, w/v) devoid of any PGRs and maintained in normal laboratory conditions for 4 wk prior to transferring to the potting mix.

Potting mix and transplantation of the regenerates

The hardened plantlets were taken out from the culture vials and washed under running tap water to remove all the traces of agar with the help of fine brush. The hardened plantlets were then transplanted onto community potting mix (CPM) containing a mixture of sand, soil at the ratio of 1:1 with coconut coir topping. The

Table 20: Effects of different PGRs on *in vitro* seed germination of *Dioscorea villosa*

PGRs Conc. (µM) BA	Kn	GA_3	IBA	Time for initial response (wk)	% response (±SE)*	Types of response
0	0	0	0	0	0	No germination
3	-	-	-	5	10.5 (0.3)	Radical emerged
6	-	-	-	5	14.3 (1.0)	As above
9	-	-	-	6	14.3 (1.0)	Radical and hypocotyls emerged
12	-	-	-	6	14.3 (0.5)	Radical and hypocotyls emerged
15	-	-	-	5	14.5 (0.3)	As above
-	3	-	-	6	15.2 (0.4)	As above
-	6	-	-	5	14.2 (0.2)	Seedlings formed
-	9	-	-	4	14.3 (0.4)	As above
-	*12*	*-*	*-*	*4*	*29.50 (1.5)*	***Seedlings with 1st set of leaves formed***
-	15	-	-	-	-	-
-	-	3	-	-	-	-
-	-	6	-	4	14.0 (1.0)	Radical emerged
-	-	9	-	5	14.5 (0.5)	-do-
-	-	12	-	-	-	-
-	-	15	-	-	-	-
-	-	-	3	-	-	-
-	-	-	6	5	14.2 (0.4)	Radical emerged
-	-	-	9	6	14.0 (0.5)	As above
-	-	-	12	6	12.2 (0.5)	As above
-	-	-	15	-	-	

* Standard error from mean; Data represent the mean of 3 replicates, one seed cultured in each test tube.

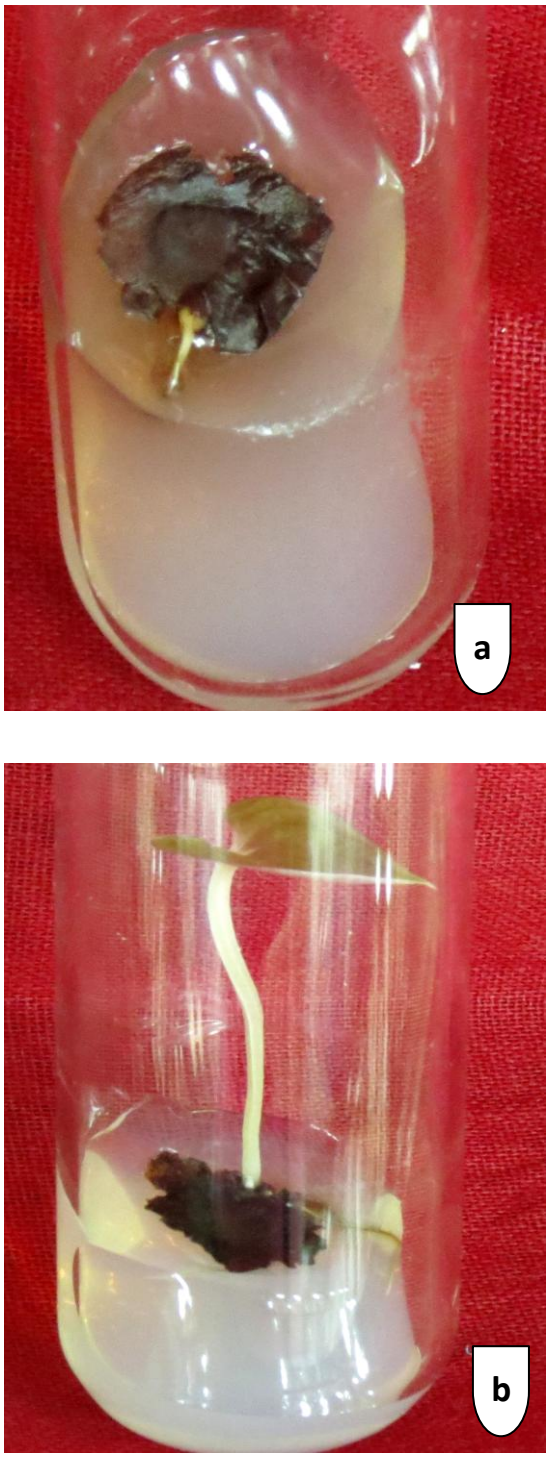

Figure 14: *In vitro* culture of *D. villosa* seed. a. Germinated seed showing the emergence of radical; b. Seedling with secondary leaf developed from the germinated seed in culture.

Figure 15: Different stages of *in vitro* morphogenetic response of nodal segments of *D. villosa* from both *in vivo* and *in vitro* source. a. Cultured nodal segment from *in vivo* source showing initial response; b. Multiple shoot/micro shoots formation from cultured nodal segment from *in vivo* source; c. Cultured nodal segment from *in vitro* source showing initial response; b. Multiple shoot/micro shoots formation from cultured nodal segment from *in vitro* source.

CPM were covered with holed transparent polybag and kept over a tub for continuous supply of capillary water for 8 wk. The plants were transfer to poly shade before transferring to the garden.

Results

Initiation of cultures

Seed/embryo culture: The seeds were cultured on MS medium containing different supplements. Incorporation of carbon source quality and quantity of PGRs were found to be crucial factors for successful culture initiation. Swelling of seeds was the first sign of germination. Of the different PGRs tested, BA across the concentration did not support healthy germination and supported only radical emergence but failed to produce plantlets in most of the cases. Similar response was also registered on medium enriched with GA_3 and IBA. Under the given conditions, MS medium fortified with sucrose (3%) and Kn (12 µM) singly supported optimum germination (20%) after 4 wk of culture (**Table 20 & Fig. 14a**). Plantlets developed within 6-7 wk from the germinated seeds and released the secondary leaves (**Fig. 14b**).

Nodal explants from *in vivo* source: The culture was initiated from the nodal explants of *in vivo* source. Swelling of the nodal region was recorded as the first sign of *in vitro* morphogenetic response (**Fig. 15a**). Sprouting of the shoot bud from the nodal region of the segment was initiated within 3 wk of culture (**Fig. 15b**). Of the three different types of PGRs used for morphogenetic response, in general BA was found to be better PGR for *in vitro* morphogenetic response from the *in vivo* grown nodal explants. Cultures initiated on other two PGR fortified medium did not support multiple shoot buds formation. The *in vivo* grown explants released excessive phenolic exudation in

Table 21: Effects of different antioxidants on plant regeneration from nodal explants of *Dioscorea villosa* from *in vivo* source

Antioxidant type and Conc. (mg L^{-1})	**% Response ±SE***	**Types of response**
0	30.00 (1.50)	Browning of medium and no further response
PVP		
100	70.20 (2.70)	Less Browning of medium
200	72.50 (1.30)	-do-
300	72.00 (2.00)	No browning of the medium
400	65.50 (2.20)	-do-
Activated charcoal		
100	75.20 (1.20)	Less browning of medium
200	80.50 (3.00)	Slight browning of medium
300	**90.60 (2.00)**	**Healthy explants, no browing in the media. Axillary bud sprouted from both the side of the node**
400	88.00 (2.50)	No browning of medium, plant healthy with maximum no. of shoots
Ascorbic acid		
100	80.00 (1.50)	Browning of medium
200	80.00 (2.00)	-do-
300	50.50 (1.50)	Less browning of medium
400	50.00 (1.50)	-do-

* SE: Standard error from mean; Data represent the mean of 3 replicates; Data collected after 8 wk of culture on MS Medium contain (3%, w/v) sucrose with PGRs of BA (6 µM).

Table 22: Effects of PGRs on the initiation of culture from the nodal explants of *in vivo* source of *Dioscorea villosa*

PGRs Conc. (µM) BA	Kn	NAA	No. of shoots/ explants	No. of roots/ explants	% response ±SE*	Types of response
3	-	-	2	1	80.50 (0.15)	Pl healthy with dark green leaf
6	***-***	***-***	***3***	***1***	***90.60 (2.00)***	***-do-***
9	-	-	1	-	80.20 (2.50)	Pl stunted
12	-	-	-	-	70.00 (0.50)	Nodular swelling
15	-	-	1	-	80.50 (2.50)	Pl stunted
3	-	1	-	2	70.00 (1.00)	Nodular swelling
6	-	1	-	-	70.50 (2.00)	-do-
9	-	1	1	1	80.00 (2.50)	Pl healthy with green leaf
6	-	2	1	-	70.00 (1.50)	Pl not healthy
9	-	2	1	1	70.00 (1.50)	-do-
12	-	2	-	2	80.50 (1.50)	Nodular swelling
15	-	2	-	-	80.00 (2.00)	Merismatic loci with nodular swelling
-	3	-	2	4	80.50 (2.00)	Pl healthy with reddish leaf
-	6	-	1	5	80.00 (1.50)	Pl healthy, stunted
-	9	-	1	4	80.40 (0.75)	-do-
-	12	-	1	2	70.00 (3.00)	Pl thin and etiolated
-	15	-	1	1	80.00 (2.00)	Pl healthy with reddish leaf
-	3	2	1	7	80.20 (1.60)	-do-
-	6	2	-	7	80.50 (1.00)	-do-
-	9	2	1	9	90.50 (1.70)	Pl healthy
-	12	2	-	6	80.20 (2.50)	Shoot bud
-	15	2	-	5	80.20 (1.50)	-do-

* SE: Standard error from mean; Data represent the mean of 3 replicates; Data collected after 4 wk of culture; On MS medium with sucrose (3%), AC (300 mg L^{-1}).
Only the significant treatments are computed.

Table 23: Effects of different antioxidants on plant regeneration from nodal explants from *in vitro* source of *Dioscorea villosa*

Antioxidant type and Conc. (mg L^{-1})	**% Response (±SE)***	**Types of response**
0	50 (1.0)	Browning of medium, plant unhealthy
PVP		
100	82.50 (1.5)	Browning of medium, plant unhealthy
200	86.00 (1.0)	Slight brown, stunted growth
300	85.00 (1.5)	No browning, plant etiolated
400	87.00 (2.0)	-do-
Activated charcoal		
100	91.0 (1.0)	Browning of medium
200	94.5 (1.0)	Browning of medium near the cut end of plantlets
300	**95.50 (1.5)**	**No browning of medium, plant healthy with maximum no. of shoots**
400	92.5 (1.5)	No browning, stunted growth
Ascorbic acid		
100	80.0 (1.0)	Browning of medium, Plant unhealthy
200	82.0 (1.5)	-do-
300	50.0 (2.0)	Browning of medium, plant unhealthy, stunted growth
400	50.0 (2.0)	-do-

* Standard error from mean; Data represents the mean of 3 replicates; Data collected after 8 wk of culture on MS Medium containing sucrose (3%) with Kn and NAA (9+1) in combination

the medium resulted in necrosis of the tissue and supported very poor morphogenetic response. Incorporation of antioxidant resulted in reduced darkening of the medium and improved the morphogenetic response. In the present study three different antioxidants (AC, ascorbic acid and PVP) were used in different concentration. All the three antioxidants supported controlling of browning/darkening of medium and improved morphogenesis compared to antioxidant controlled medium. But the optimum morphogenesis was registered on medium supplemented with AC (300 mg L^{-1}) (**Table 21**). Under the given conditions optimum morphogenetic response was registered on MS medium enriched with sucrose (3%) and BA (6 µM) (**Table 22**). About 90% explants responded positively with the sprouting of shoot buds from the nodal regions where as many as 3 shoot buds developed per node. The cultures maintained on Kn enriched medium though did not support shoot bud formation across the concentrations studied, supported large number of roots from the nodal zone.

Nodal explants from *in vitro* source: Cultures were also initiated from the nodal segments from *in vitro* source. Sprouting of the shoot buds from the nodal region of the segments was initiated within 1 wk of culture (**Fig. 15c)**. It was found that for successful morphogenetic response from the cultured nodal segments incorporation of antioxidant was prerequisite as cultured initiated on antioxidant control medium resulted darkening of medium and poor morphogenesis. To control the browning of medium three antioxidants were tested in different concentrations. Amongst the different quality and quantity of antioxidants tested, AC at 300 mg L^{-1} reduced the browning substantially and ~95% explants exhibited morphogenetic response (**Table 23**). Besides antioxidant, incorporation of sucrose and PGRs in the initiation medium was obligatory for initiation of culture. Of the different PGRs tested at different concentrations medium enriched with BA supported fewer shoot buds formation while,

Table 24: Effects of PGRs on morphogenetic response of nodal explants from *in vitro* source of *Dioscorea villosa*

PGRs Conc. (µM)			No. of shoots/explants	No. of leaves/explants	No. of roots/explants
BA	Kn	NAA			
0	0	0	-	-	-
3	-	-	2	3	3
6	-	-	2	2	-
3	-	1	2	1	4
9	-	1	2	2	-
3	-	2	2	3	3
-	3	-	2	3	2
-	6	-	2	3	1
-	9	-	1	2	3
-	12	-	1	2	1
-	3	1	1	3	2
-	6	1	1	2	3
-	9	1	3	4	4
-	12	1	2	2	3
-	15	1	2	3	3
-	3	2	2	2	2
-	6	2	3	3	2
-	9	2	1	1	1
-	12	2	1	3	5
-	15	2	2	3	3

Only the significant treatments are computed; Data represent the mean of 3 replicates

Data collected after 8 wk of culture; On MS medium containing sucrose (3%).

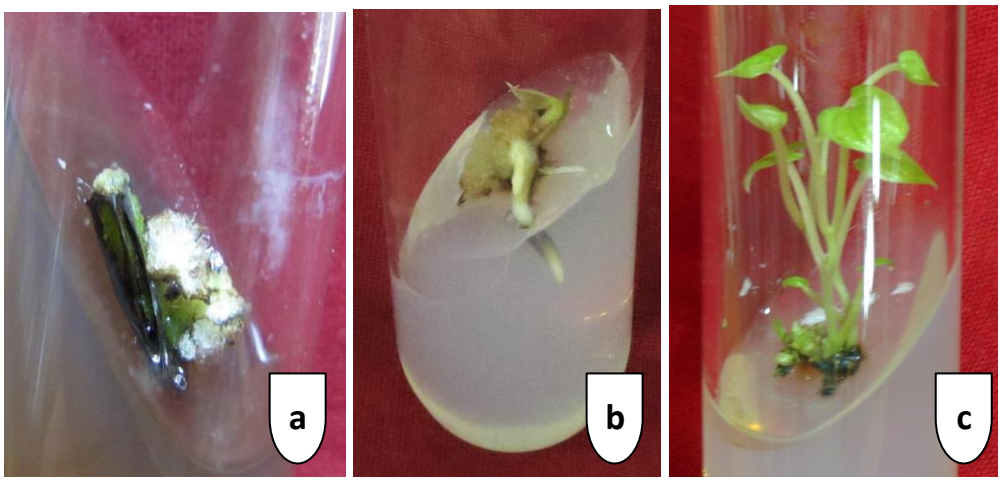

Figure 16: *In vitro* morphogenetic response of foliar explants of *D. villosa*. a. Cultured foliar segments showing swelling and initiation of callusing; b. Formation of shoot buds from the callus developed from cultured foliar segment; c. Multiple shoot buds/plantlets formed from the cultured foliar segment.

Table 25: Effects of PGR on morphogenetic response of foliar explants of *Dioscorea villosa* from *in vitro* source

PGRs Conc. (µM)*			Time for initial response (days)	% response (±SE)**	Types of plant response
BA	**NAA**	**Kn**			
0	**0**	**0**	-	-	No response
3	**-**	**-**	23	40.50 (0.50)	Callus
6	**-**	**-**	28	43.50 (0.75)	Callus at the cut end
9	**-**	**-**	28	45.00 (1.50)	Callus at the petiole
12	**-**	**-**	28	45.00 (1.00)	-do-
15	**-**	**-**	23	34.50 (1.50)	Callus formation
3	**1**	**-**	23	38.00 (0.50)	callus all over the foliar segment
6	**1**	**-**	23	42.50 (0.50)	Callus
9	**1**	**-**	18	45.00 0.50)	Callus
12	**1**	**-**	18	50.00 (1.50)	-do-
15	**1**	**-**	18	50.00 (2.00)	-do-
3	**2**	**-**	28	60.00 (2.00)	-do-
6	**2**	**-**	23	60.00 (1.50)	-do-
9	**2**	**-**	23	70.00 (1.00)	-do-
12	**2**	**-**	22	70.00 (0.75)	-do-
15	**2**	**-**	28	55.0 (2.00)	-do-
-	**-**	**3**	-	-	no response
-	**-**	**12**	20	45.00 (2.00)	Callus
-	**-**	**15**	20	40.00 (2.00)	Callus
-	**2**	**3**	30	35.50 (1.50)	-do-
-	**2**	**6**	26	56.00 (0.50)	-do-
-	**2**	**9**	24	65.50 (1.50)	-do-
-	**2**	**12**	20	80.00 (1.50)	Plant healthy with 6 roots and 1 leaf
-	**2**	**15**	-	-	No response

* Only the responding concentrations are computed.

** SE: Standard error from mean; Data collected after 50 days of culture.

Kn enriched medium supported moderate response. But optimum number of shoot buds formation and per cent response was registered on medium fortified with NAA and Kn in combinations. As many as 3 shoot buds developed at a single node on MS medium supplemented with sucrose (3%) and NAA + Kn (1+9 µM in combinations) (**Table 24 & Fig. 15d)**. About 95% explants responded positively with the sprouting of shoot buds from the nodal regions under optimum conditions. Positive response was recorded with both single and combined treatments of NAA and BA.

Foliar explants

Cultures were also initiated from the foliar explants of ~5-6 wk old from *in vitro* source (**Fig. 16a**). The foliar explants were cultured on MS medium enriched with sucrose (3%) and different PGRs. In most of the treatments callus formation was recorded from the cultured explants. The callus formation was initially initiated from the cut ends followed by expanded throughout the leaf segment (**Fig. 16b**). But explants cultured on NAA and Kn enriched medium formed shoot buds (**Table 25 & Fig. 16c**). After 8 wk of culture initiation meristematic activity was invoked at the basal ends of the explants. Single treatment as well as in combination of PGRs such as NAA, BA and Kn did not support healthy organogenesis and in most of the cases explants callused while, lone treatment with Kn supported moderate organogenesis. But optimum morphogenetic response was recorded on basal medium enriched with NAA (2µM) and Kn (12µM) in combination (**Table 25**). About 80% of cultured explants responded positively after 8 wk of culture initiation on MS medium containing sucrose (3%) and NAA + Kn (2 and 12 µM respectively in combination) where shoot buds invoked and formed shoot buds.

Table 26: Effects of different organic carbon sources on plant regeneration of *Dioscorea villosa* from *in vitro* source

Source & Conc. (%) of organic carbon	Avg. plantlet height (cm)	Avg. no. of leaves formed/ plantlet	Avg no. of shoots formed/plantlet	Avg. no. of roots formed/plantlets
0	**0**	**0**	**0**	**0**
Sucrose				
1	**1.5**	**1**	**1**	**-**
2	**1**	**2**	**2**	**-**
3	***6***	***5***	***3***	***2***
4	**2.5**	**3**	**3**	**2**
Dextrose				
1	**1.5**	**1**	**1**	**-**
2	**2.5**	**3**	**1**	**-**
3	**1.8**	**2**	**1**	**2**
4	**1.5**	**3**	**2**	**1**
Fructose				
1	**1.5**	**1**	**1**	**-**
2	**1.5**	**2**	**2**	**-**
3	**1.5**	**1**	**1**	**-**
4	**0.4**	**2**	**2**	**-**

Data represent the mean of 3 replicates; Data collected after 4wk of culture

MS Medium containing Kn and NAA (9+1 µM) in combination.

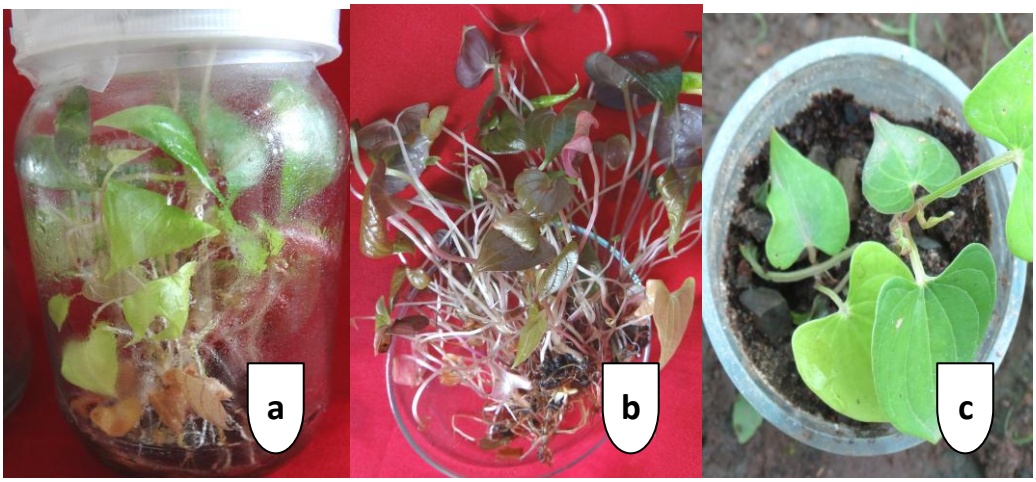

Figure 17: a. *Dioscorea villosa* rooted plantlets under hardening condition; b. Rooted plants harvested for transferring to potting mix; c. Transplant established in the community potting mix in the poly house.

Plantlet regeneration and culture proliferation

The shoot buds develop from seeds, foliar and nodal explants were maintained further for two more passages on optimum initiation condition for further differentiation. The shoot buds were then maintained on different strengths of MS medium (0, 1/4th, 3/4th, 1/2 and full strength inorganic salts and full organic adjuncts) containing different level of organic carbon source (0-4%). Effects of different antioxidant on the regeneration of plantlets were also observed.

Effects of different organic carbon sources

The basal medium was fortified with different quality and quantity of organic sources like dextrose, fructose and sucrose (0-4%). The cultures maintained on dextrose and fructose enriched medium supported poorer regeneration with fewer secondary shoot buds formation and less plant height resulting in few nodal region formations. Among the three different organic carbon sources incorporated in the regeneration medium, in general cultures maintained on sucrose enriched medium outperformed the cultures maintained on medium with other organic carbon sources under otherwise identical conditions. The optimum culture differentiation and plant regeneration was achieved when MS medium was incorporated with sucrose (3%, w/v) (**Table 26**). Under this condition as many as 3 shoot bud formed per plant per subculture where the average height of the plantlets, leaves number, root formation was recorded highest (**Fig 17a**).

Effect of PGRs on the regeneration and growth of plantlets

The different quality and quantity of PGRs incorporated in the regeneration medium exhibited differential response on regeneration and *in vitro* propagation. None of the single concentration of different PGRs on regeneration produced healthy plantlets. Among the different quality and quantity of PGRs used, Kn and NAA (9+1 in

Table 27: Effects of different strengths of MS Medium on plant regeneration of *Dioscorea villosa*

Strength of MS medium	No. of leaves formed per shoot & length (cm)	No. of shoot formed/explants	No. of roots formed/explants
Water agar	-	-	-
MS full	4 (4cm)	3	4
3/4th	3 (2.5 cm)	2	1
1/2	3 (2 cm)	2	-
1/4th	2 (2.5 cm)	1	3

Data represent the mean of 3 replicates; MS Medium containing sucrose (3%, w/v) and Kn and NAA (9+1µM) in combination.
Data collected after 8 wks of culture.

combination) supported optimum regeneration of plantlets which produced 3 multiple shoot buds with maximum plantlets height and roots.

Effect of different strengths of MS medium

The strength of nutrient medium had remarkable influence on the culture proliferation and plant growth. At lower concentration of the basal strength, growth of plant was slower with reduced plant proliferation. With increase in strength of basal medium improved the plant growth and proliferation. Optimum response was observed on full MS medium where maximum plant growth as well as plant proliferation was registered better (**Table 27**).

The regenerated plantlets were maintained on optimum regeneration condition for 2-3 passages for culture proliferation and plantlet growth. The healthy plantlet with about 6 cm long, 4-5 leaves with well rooted of about 6-7 roots from the regeneration medium were taken out from the regeneration medium and subjected to *in vitro* hardening (**Fig. 17b)**.

Hardening and field trail of the regenerates

The well developed plants from the regeneration and rooting medium were transferred to 1/2 strength MS medium containing (2%, w/v) but free from any PGRs and maintained for 4 wk under normal laboratory condition. The hardened plants were transfer to CPM (**Fig. 17c**). The transferred hardened plants were then covered with holed transparent poly bags and maintained for 1 wk. The potted plants were watered on regular basis after which the potted plant were further kept in the shade house for 3-4 wk. The potted plants were watered regularly in the poly house and left in the normal full day light condition in the garden. About ~90% of the transplants survived to form fully developed plants after two months of potting.

Discussion

Initiation of culture

Seed/embryo culture

In each *Dioscorea* capsule contains about 6 winged seeds. The seeds are functionally underdeveloped and capitates shaped embryos (Albretch, 2006). The finding shows that seed germination was greatly influenced by the PGRs used. This result support the finding that successful seed germination and or immature embryo culture was greatly influenced by several factors like developmental age of the embryo/seeds, quality and quantity of nutrient media, different media supplements, quality and quantity of PGRs (Sharma and Tandon, 1990; Deb and Temjensangba, 2006a, b, 2007a, b; Deb and Sungkumlong, 2008, 2009; Sungkumlong and Deb, 2008; Temjensangba and Deb 2005a, 2006, Pongener and Deb, 2009, 2011a).

In the present investigation, in the absence of PGRs, seeds failed to differentiate further and germinate. The PGRs in the germinating medium showed a marked effect on the growth, differentiation and development of the seeds. Kn had stimulated the germination of seeds of *Dioscorea villosa*. KN was more effective than BA, GA_3 and IBA. Seed germination of *Dioscorea villosa* on optimum PGRs was evident after 8 wk of culture and formed root and shoot buds. While, seeds maintained on BA, IBA and GA_3 rich medium delayed germination and germination frequencies were also significantly lower. In *Rhynchostylis gigantea* NAA (0.2 mg L^{-1}) and BA (0.05 mg L^{-1}) supported optimum germination. But Nagaraju *et. al.* (2004) reported that in *Dendrobium* hybrid, the single leaflet in the PLB developed when basal medium was

supplemented by BAP alone while, BA singly supported better germination in *Malaxis khasiana* (Deb and Temjensangba, 2006a).

Initiation of culture from leaf

In the current investigation cultures were also initiated from the foliar explants from *in vitro* source. Wimber (1965) successfully developed PLBs from the leaves of *Cymbidiums*, which opened up an effective alternative to apical shoot meristem culture. Since then the regenerative competence of foliar explants were positively tested for more than 60 orchid species (Temjensangba and Deb, 2005b). However, the success is restricted mostly with epiphytic orchids and only few species from terrestrial orchids suggesting thereby that the ground orchids are less amenable to *in vitro* regeneration (Deb and Sungkumlong, 2010). In the present study, initiation of morphogenetic response from the foliar explants of *Dioscorea villosa* was studied. The morphogenetic potential of leaf base has been reported in *Dioscorea cayenensis* (Shu *et. al*., 2005); *Dioscorea floribunda* (Chaturvedi, 1979); *Dioscorea alata* (Heena and Lele, 2012); *Coelogyne, Dendrobium, Oncidium* and *Phalaenopsis* (Abdul Karim and Hairani, 1990), *Acampe praemorsa* (Nayak *et. al.*, 1997), *A. labrosa* (Deb and Temjensangba, 2007a), *C. racemiferum* (Temjensangba and Deb, 2005b), *C. suaveolens* and *Taenia latifolia* (Deb and Sungkumlong, 2010), *V. coerulea* (Vij and Aggarwal, 2003). Mathews and Rao (1985) considered the leaf base to be the decisive factor for culture initiation from foliar explants. Sinha and Hegde (1999) reported the development of meristematic activity along the entire leaf in *Renades Arunoday* hybrid.

The incorporation of PGRs to the basal medium was obligatory for the initiation of culture. The explants failed to respond, when cultured on PGRs free medium. The role of growth hormones in stimulating meristematic activity and promoting

proliferation in leaf explants is well documented in orchids (Abdul Karim and Hairani, 1990; Vij and Pathak, 1990; Yam and Weatherhead, 1991; Arditti and Ernst, 1993; Vij *et. al.*, 1994; Nayak *et. al.*, 1997; Temjensangba and Deb, 2005b; Deb and Temjensangba, 2007a; Li and Xu, 2009; Deb and Sungkumlong, 2010). Murashige (1974) opined that *in vitro* plant regeneration occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. In *Dioscorea villosa* initiation of shoot bud was observed after 8 wk of culture. Amongst the different PGRs tested, optimum response was registered on MS medium containing sucrose (3%), NAA and Kn (1 and 12 µM respectively in combination).

Initiation of culture from nodal explants

In the present study, cultures were also initiated with nodal segments of *Dioscorea villosa* from *in vitro* as well as from *in vivo* source. Morphogenetic response and sprouting of shoot buds from the nodal region of the segments were initiated within 1 wk of culture from *in vitro* source. While in *in vivo* source, morphogenetic response was observed after 3wk of culture initiation. A combined treatment of NAA and Kn was more effective over single treatment for *in vitro* source explants. About 3 shoot buds developed from each node on MS medium supplemented with sucrose (3%) and Kn + NAA (9+1 µM each) in combination. Present study is in agreement with the reports in *Phalaenopsis* where a combined treatment of BA (2 mg L^{-1}) and NAA (0.5 mg L^{-1}) was found to be optimum for breaking axillary buds and formation of multiple shoot buds (Kosir *et al*, 2004) and observed that incorporation of NAA was promotory. Other workers like Tisserat and Jones (1999), Roy and Banerjee (2003) also observed that an appropriate combination of NAA and BA stimulated multiple shoot buds formation. While from *in vivo* source, amongst the different PGRs tested, optimum

response was registered on MS medium containing sucrose (3%), BA (6 µM) where as many as 2-3 shoot bud developed after 3 wk of culture. While Kn enriched medium either singly or in combination supported moderate response. Present study is in agreement with Arditti and Ernst (1993), Pongener and Deb (2011b) reported that BA promotes morphogenetic response but addition of NAA reduced induction and regeneration. The morphogenetic potential of nodal segment has been reported in *Dioscorea fordii* Prain et Burk (Yan *et. al*., 2011); *Dioscorea alata* (Heena and Lele, 2012); *Dioscorea remotiflora* (Antonia *et. al*., 2012); *Dioscorea nipponica* Makiro (Chen *et. al*., 2007); *Dioscorea composite* Hemsl (Alizadeh *et. al*., 1998); *Dioscorea bulbifera* L. (Chu *et. al*., 2002); *Dioscorea oppositifolia* (Poornima and Ravishankar, 2007, Maheswari *et. al*., 2012); *Dioscorea pentaphylla* (Asha and Nair, 2010, Poornima and Rai, 2007); *Dioscorea floribunda* (2002, Chaturvedi, 1975); *Dioscorea wightii* (Mahesh *et. al*., 2010).

Regeneration of plantlets and culture proliferation

The shoot buds formed from the cultured seeds, foliar explants, nodal segments were maintained for another two passages for further differentiation and proliferation on optimum initiation conditions. The advanced stage shoot buds, tiny plantlets developed on initiation medium were transferred on regeneration medium for plantlets formation and culture proliferation.

Effect of different organic carbon sources

Various organic carbon sources such as fructose, glucose and sucrose (0-4%) were incorporated in the regeneration medium where sucrose rich medium was found to be superior for this plant species. Incorporation of at least one of the organic carbon sources was obligatory for regeneration and mass multiplication of plantlets. There was

no regeneration in the absence of the organic carbon sources. The requirements of the quality and quantity of exogenous supply of the organic carbon sources varies with the species, the media compositions used, the endogenous level of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005a, c; Pongener and Deb, 2011a). Sharma and Tandon (1990) reported that 2-3% of sucrose; D-Fructose and D-Glucose were the suitable organic carbon sources for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata*. In the present investigation, amongst the different organic carbon sources tested, optimum regeneration and shoot bud formation in *Dioscorea villosa* were obtained on basal media containing sucrose (3%) where as many as 3 shoots were formed per subculture. The media enriched with glucose supported fewer shoot bud formation whereas, fructose enriched media did not support healthy regeneration.

Effect of PGRs

Different quality and quantity of PGRs marked a pronounced effect and elicit different responses in the seedling development. Inclusion of PGRs in the regeneration medium was obligatory for successful regeneration of plantlets and mass multiplication. In the absence of PGRs, there was no regeneration and cultures degenerated subsequently. Amongst the different concentrations of NAA, BA and Kn tested, a combined treatment of NAA and Kn at 1+9 µM supported healthy regeneration and multiple shoot formation. Cultures with combined treatments of Kn with NAA were found to be better as compared with single treatment of Kn. Bhadra and Hossain (2004) reported highest number of multiple shoot buds formation from nodal segment of *Micropera pallida* when medium was supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} BA. While the PGRs like BAP singly or in combination with IAA

were best used for initiation of cultures and development of healthy plantlets from leaf explants of *Saccolabium papillosum* (Kaur and Vij, 2000).

Effect of strength of basal medium

Amongst the different strengths of MS medium tested full strength MS medium supported highest numbers of plantlets formation and better culture proliferation followed by 3/4th strength and 1/2 strength. At lower strength of MS medium (1/4th and 1/2 strengths) most of the plantlets were either stunted in growth with fewer new shoot buds formation or cultures degenerated. At 3/4th strength of MS medium the plantlet height was comparatively better than 1/4th and 1/2 strength media. In this condition, though regenerated plants were healthy but supported fewer shoot buds, roots and leaf formation. This perhaps could be due to difference of chemical constituents with MS medium or deficient as to the requirement of the developing plantlets. Chen *et. al.* (2004), George and Sherrington (1984), Temjensangba and Deb (2005a), Pongener and Deb (2009, 2011a) argued that the change in culture conditions and media could alter the pattern of organogenesis in orchids and such behaviour can be judiciously exploited to achieve desirable response in many orchid taxa by altering the nutrient regime.

Effects of antioxidants

In some species exudation from of the explants and browning of medium is a common problem and is generally considered due to oxidation of phenolic compounds released from the cut ends of the explants (Bhat and Chandel, 1991). In the present study, browning of medium was one of the bottlenecks for successful establishment of culture from the explants sources in general and nodal explants in particular. The browning of medium could be prevented by incorporating different antioxidant like

PVP, A.C and ascorbic acid (100-400 mg L^{-1}). Deb and Tandon (2004) could successfully control the browning of medium by incorporating 200 mg L^{-1} PVP and 100 mg L^{-1} citric acid in combination but, in *Embelia ribes* incorporation of PVP did not reduce the browning of the medium (Dhavala and Rathore, 2010). Gupta (1980) reported that in case of shoot tip culture of apple and teak, agitating the plant materials for an hour or incorporation of PVP and β-mercaptoethanol (0.5-2%) helped in preventing oxidation of phenolic compounds. While, Narayanaswamy (1994) discussed that culture medium fortified with antioxidant like ascorbic acid and citric acid (500-2000 mg L^{-1}) could curtailed the effects of phenolic exudates. In the present study, phenolic exudation could be controlled by using different antioxidants viz. AC, PVP, ascorbic acid where AC (300 mg L^{-1}) exhibited better control over others.

During the present investigation, protocols were established for culture initiation from seeds foliar explants, nodal explants from both *in vivo* and *in vitro* sources of *Dioscorea villosa*, regeneration of plantlets and mass multiplication. These techniques open new routes for *in vitro* mass multiplication of this economically important medicinal plant. The protocols established for culture initiation from foliar explants, nodal explants segments indicates the possibility of using alternative explants. The protocols may be used by the commercial pharmaceutical industries for multiplication and extraction of the diosgenin for preparation of the steroid progesterone.

Chapter - 6

Summary

Northeastern region of India is blessed with a wide range of physiography and eco-climatic conditions, lying between 21º34'N to 29º50'N latitude and 87º32'E to 97º52'E longitude and covers an area of ca 2, 62,060 sq km. Northeast India represents the transition zone between the Indian, Indo-Malayan and Indo-Chinese biogeographic regions and a meeting place of the Himalayan Mountains and Peninsular India. Northeast India is thus the geographical gateway for much of India's flora and fauna, and as a consequence, the region is one of the richest in biological values. The vegetation of the Northeast regions accounts for approximately 7500 species of angiosperms. Out of 315 families of angiosperms in India, more than 200 are represented in Northeast India and this region accounts for nearly 50% of the total number of plant species in India as a whole. It is of interest to note that about one third of the flora of Northeast India is endemic to this region.

The primary forest of Northeast India is losing day by day due to *'Slash and Burn'/* Jhum Cultivation practice by the local people, fragmentation of forest, indiscriminate collection of the plant species, unplanned developmental activities, natural calamities like land slide, drought, forest fire etc. As a result many important plant species are facing threat or on the verge of extinction in their natural habitat.

Therefore, it is necessary to develop protocols for reproductive behaviour and mass multiplication through *in vitro* conservation of these threatened plant species. During the present study, I have worked with two economically important plant species viz., ***Aconitum nagarum*** Stapf. (Ranunculaceae) and ***Dioscorea villosa*** L. (Dioscoreaceae) for their certain reproductive behaviour and *in vitro* propagation and establishment in the natural habitats. The cultures were initiated from different explants sources like embryos/seeds, nodal explants, foliar explants of the two selected plant species and certain reproductive behaviour like floral biology, seed biology and pollinators were studied.

1. *Aconitum nagarum* Stapf. (Ranuculaceae)

Floral biology

During the present investigation it was observed that *Aconitum nagarum* start budding from September with peak flowering from October first week. The flowers are blue, in slender raceme, petals and filaments glabrous, carpel 5, hermaphrodite. The flowers bloom acropetally i.e., flower starts blooming from the base of the inflorescence to the tip of the inflorescence thus the fruits also mature acropetally. The anthesis was observed between 6-6.30A.M. Anther dehisced longitudinally from 7A.M till 9.30A.M. The number of anther was 49 per flower. It was observed that flowers colour changes as the plant fully dehisced. The flowering duration per flower varies from 4-6 days followed by fruit formations and matures within 10-15 days. The

average flowers per plant varies from 8-28 and common pollinator was found to be bee. Mean seeds per plant was 270-540 and pollen per anther was 1000-2000.

Seed biology

For the study the seeds were stratified at 4ºC and used for germination testing under different conditions. In the filter paper test, maximum germination was achieved from the seed stratified for 48 hrs at 4ºC followed by 96 hrs when transfer to laboratory (25ºC) with minimum days taken to germinate. Under this condition 38% and 15.38% seed germination recorded after 13 days and 10 days of sowing respectively while maximum germination was recorded with the seeds treated with 96 hrs at 4ºC with the seed that were sown in the seed bed whereas no seeds germination was recorded from the seed maintained in the incubator at 30ºC across the stratification period. The seed germination rate, germination time was greatly influence by pre-treatment of the seeds. There was significant difference in the germination period, germination rate with the stratified and non-stratified seeds.

Besides temperature requirement for seed germination, seeds were also tested for their post harvest storage tolerance. The present study shows that germination rate and germination period decreases as the period of seed storage increased. The first sign of seed germination was the emergence of radical from the seed coat followed by a pseudo cotyledonary leaf formation and cotyledonary leaf was replaced by a true leaf within 58-60 days after germination as the pseudo leaf turn yellowish in appearance and slowly wither giving way for the true leaf to succeed.

***In vitro* propagation**

Cultures were initiated from seeds. The cultured seed exhibited germination of ~20%. Callusing of the seeds was observed after 7-8 wk of initiation of culture.

Germination rate of ~10% was achieved on MS medium containing sucrose (3%, w/v). Amongst the different levels of PGRs used for seed germination, MS medium containing BA (12 µM) singly supported optimum germination (~10%) after 7 wk of culture. Treatment of NAA (0-12 µM) did not show any germination. Where treatment with 2, 4-D (0-12µM) shows callusing but failed to initiate shoot. The cultures were maintained under full light condition (40 µmol $m^{-2}s^{-1}$ light intensity) at 12/12 hr (D/L) photoperiod.

Cultures were also initiated from the nodal explants from in vitro source. After 8 days of culture initiation, meristematic activity was invoked by producing small shoot buds. About 80% of cultured explants responded positively after 8 days of culture initiation on MS medium containing sucrose (3%) and BA (6 µM) where as many as 16 meristemoids/shoot buds invoked and formed shoot buds. The shoot buds formed from seed germination, shoot explants cultured on MS media with different adjuncts were maintained for 2-3 passages for regeneration of plantlets and culture proliferation.

The healthy plantlets on the regeneration medium of about 4-6 cm long plantlets/shoots with well expanded leaves from the regeneration medium were selected for inducing rooting. The micro-shoots were maintained on MS medium containing sucrose (3% w/v) and NAA (0-5µM) and maintained in normal laboratory conditions. The well rooted plantlets were taken out from the rooting medium and transferred on MS medium fortified with sucrose (3% w/v) devoid of any PGRs and maintained in normal laboratory for 4 wk for hardening. The hardened plants were transferred to community potting mix containing a mixture of soil, coconut husk, (at 1:1 ratio). The transplants were covered with holed transparent poly bags and maintained in the laboratory (ca 70% filtered light) for 2-3 wk. The potted plants were left in the poly. About 60% of the transplants survived to form fully developed plants after two months

of potting.

2. *Dioscorea villosa* L. (Dioscoreaceae)

Floral biology

The male flowers were arranged in 2 whorls of 3, bracted, anther bilobe, didymous. The female flowers were also arranged in 2 whorls of 3. Though there was high fruiting, mean seed production per plant was quite low. This could be one region why the plants are rare because seeds are also an important factor for maintaining the plant from generation to generation.

Pollination

The insects visiting the plant include wasps, flies, ants and beetles. Wasps and some beetle are found on the dorsal and ventral surface of the leaves. Beetle stay on the flower throughout the day visiting each flower. Maximum visitation by the insects was observed during morning and evening. But the insects visits decreases during the afternoon period when the studies were undertaken. The pollen was found to be sticky in nature and orange in colour.

Seed biology

The time for emergence of radicals from the germinated seeds, germination rate, seedling morphology and seedling establishment is influenced by various many factors. Plant species differ greatly in their habit preference, temperature requirements, post harvest storage, specific pre-treatment for seed germination, seedling emergence and survival. Effective dormancy break was occurred in *Dioscorea villosa* following cold-stratification in a temperature of 4ºC for 96 hr; suggest that *D. villosa* seeds are morphologically dormant. During the present study, investigation was carried out on

the relationship among rate of germination with pre-treated seeds and non-treated seeds. When seeds were given a constant cold stratification for a period of 96 hr (4ºC) and then moved into warmer temperature (30ºC, incubator), dormancy was broken and seeds germinated to high rate of 40% after 35 days. The seeds which were not treated with cold stratification show less percent of germination ~ 10% only with delayed in the germination period.

The dispersal of seed was observed to be through wind in nature. Few seeds which fall on the soil germinated during favourable conditions. Seeds which fall on the litter fail to germinate due to deficiency of nutrients. In conclusion, this study can guide seedling in the field by following the temperature requirements for dormancy.

***In vitro* propagation**

Cultures were initiated from the foliar explants of *in vitro* source. Callusing of foliar segments was observed within 20 days of culture from the cut ends followed by either shoot buds formation or callus formation within 8 wk. Of the different PGRs tested for the purpose medium supplemented with NAA and Kn (2+12 µM) in combination supported ~80% response either in the form of shoot bud formation or callusing of foliar explants.

Cultures were also initiated from the nodal explants from *in vitro* as well as from the *in vivo* sourced nodal explants. Swelling of the nodal region was the first sign of response both from *in vitro* and *in vivo* sourced explants. Sprouting of the shoot bud from the nodal region was achieved within 3 wk of culture from the *in vivo* source nodal explants whereas from *in vitro* source nodal explants it was observed after 1 wk of culture. Of the different quality and quantity of PGRs tested, BA (6 µM) alone supported optimum response from *in vivo* source nodal segments, whereas optimum response from nodal segments of *in vitro* sourced was achieved on medium conjunct

with Kn and NAA (9+1 µM) in combination. Maximum numbers of shoot bud (3) develop on MS medium supplemented with sucrose (3%, w/v) in both the *in vivo* and *in vitro* explants source. About 95% explants responded positively with the sprouting of shoot buds from the nodal explants.

The shoot buds developed from the nodal segments and foliar explants were maintained on regeneration medium for culture proliferation and plantlets regeneration. The basal medium was supplemented with different quality and quantity of different organic carbon sources (dextrose, glucose and sucrose at a concentration of 0-4%) and it was found that sucrose enriched medium supported optimum regeneration and culture proliferation. Optimum regeneration of plantlets and culture proliferation was achieved on MS medium fortified with sucrose (3%) and Kn and NAA (9+1 µM in combination).

Different antioxidant like PVP, Ascorbic acid and Activated charcoal were also incorporated in the regeneration medium to study their effect on plant regeneration and plant morphology. It was observed that incorporation of AC had prominent effect on culture proliferation and plant growth. The cultures with AC as antioxidant responded better and faster as compared to AC controlled medium.

The well rooted plants from rooting condition were transferred on ½MS salt solution containing sucrose (2%) and devoid of any PGRs and maintained for 6-7 wk under normal laboratory condition. The hardened plantlets were taken out from the culture vials and washed with tap water to remove any traces of agar before transplanting to plastic pots containing a mixture of soil, sand, at 1:1 ratio with coconut coir topping. The pots were covered with holed transparent polybags and watered at week interval for two months for 3-4 wk and maintained in polyshade with Ca.70% of shading sunlight and finally after two months, the plantlets were left in the normal full

day light condition. About 100 plants were tested for survival and about 70% survival was registered after two months of transfer.

Conclusions

The present study was undertaken to understand certain reproductive behaviours of *Aconitum nagarum* and *Dioscorea villosa*, their seed biology. Works were also carried out on development of *in vitro* culture protocols of these two medicinally important threatened species with the objective to produce clonal planting materials for mass propagation. The outcome of the floral biology and seed biology will help in working out the conservation strategies of these two species and seed propagation of the species. The *in vitro* propagation protocols from different explants open up newer route for alternative propagation of these species. The protocols so developed may be used for commercial production of planting materials.

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