POLLINATION BIOLOGY OF THREE *CLERODENDRUM* SPECIES IN A TROPICAL FOREST OF MIZORAM

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CERTIFICATE

This is to certify that the thesis entitled **"Pollination biology of three *Clerodendrum* species in a tropical forest of Mizoram"** submitted by **Puyam Devanda Singh** in fulfillment of Doctor of Philosophy in Forestry is an original work and has not been submitted elsewhere for other degree. It is recommended that this thesis be placed before examiners for the award of the degree of Doctor of Philosophy.

Dated: Supervisor

Place: Aizawl

(Dr. Kewat Sanjay Kumar)

DECLARATION

MIZORAM UNIVERSITY

NOVEMBER, 2022

I, **Puyam Devanda Singh,** hereby declare that the subject matter of this thesis entitled **"Pollination biology of three *Clerodendrum* species in a tropical forest of Mizoram"** is the record of the work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Forestry

(Puyam Devanda Singh)

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Chapter 1

Introduction

The forests accomplish the needs of the rural communities for their livelihood and subsistence. Indigenous peoples are very familiar with their surroundings' floral and faunal diversity and have developed a deep relationship with their nature and natural resources. Non-timber forest products (NTFPs) are any forest items obtained from forests, aside from timbers. Thus, Non-Timber Forest Products (NTFPs) are all biological materials that are obtained from forests for human use except timber (Soe & Yeo-Chang, 2019). NTFPs play a vital role in the rural economy, creating employment generation, revenue earning potential and socio-cultural and life support opportunities. Rural people in developing countries earn 20-25% of their income from NTFPs (Uprety et al., 2016). NTFPs are regarded as secondary forest products and have been historically and culturally used for hundreds of thousands of years by indigenous societies for foods, medicines, fuel woods, fodders, fibres, building materials, and other uses (Shackleton, 2015). The traditional health care system depends mainly on NTFPs in the form of medicinal plants with ethnic and cultural significance. To treat common ailments and other diseases, local people used to prepare traditional medicines from the plant species found in their natural landscape.

Among the different categories of NTFPs, medicinal plants are more important than other categories. Many species of NTFPs have medicinal benefits for treating various ailments such as stomach aches, cuts and wounds, diarrhoea, ulcers, infertility, malaria, fever, and blood purification (Popoola & Obembe, 2013). Both domestically and globally, demand for medicinal plants is increasing. Ayurveda, Chinese, Unani, Siddha, Tibetan, and other traditional medical systems worldwide rely on medicinal and aromatic plants (Gewali & Awale, 2008). Numerous drugs or medicines are extracted/synthesized from wild plants or their extracts. According to the World Health Organization (WHO), 80% of the population traditionally uses plant-based medicine for their basic healthcare since it is accessible to the general public and easy to assess (Uritu et al., 2018).

The International Union for Conservation of Nature and the World Wildlife Fund (IUCN) estimates that between 50,000 and 80,000 plant species are utilized in both modern and traditional therapeutic systems (Romanciuc, 2019). About 20,000 species of higher plants are contributed by India, with 500 plant species classified as having medicinal potential and one-third of them being endemic (Krishnan et al., 2011). Almost all human societies have used medicinal plants as a source of healing purposes. In tropical region medicinal plants are considered as the most important Non-timber forest products (NTFPs). $2/3^{rd}$ of the rural population in the tropics still depends on traditional medicinal plants for their primary healthcare (Muthu et al., 2006).

Medicinal plants contain physiologically active biochemicals that have therapeutic effect, such as saponins, tannins, essential oil, terpenoids, flavonoids and alkaloids (Okigbo et al., 2009). This various chemical composition is present in the form of secondary plant metabolites. Many diseases, such as malaria, diabetes, sickle cell anaemia, mental health issues, and microbial infection are treated with plant-based medications, medicines, and extracts (Okigbo et al., 2009). Examples of plant-based medications, health supplements and aromatic products include pharmaceuticals, multivitamins, natural health products, beauty aids, cosmetics, personal care products, aromatic and essential oils, and diverse goods.

North East region of India is rich in biodiversity and ethnicity. The diverse topography of North East India is a huge source of medicinal plants that the indigenous population uses to manage their primary health care. Medicinal plants, which are relatively common in their natural habitat in the region are now quickly diminishing due to forest fragmentation, forest fire, jhum cultivation, logging, and landslide, thus threatening to the valuable plant biodiversity of the forest (Deb et al., 2015). Other factors include climate change, urban development, industrialization, pollution, destructive harvesting practices, and indiscriminate use have decreased the wild population of medicinal plants. Consequently, the ICUN has listed medicinal plants as "vulnerable" and "threatened" depending on their population status (Smitha & Thondaiman, 2016). Thus, there is a need to educate the local population for

conservation and sustainable use of natural recourses particularly the medicinal plants found in the wild (Kennish, 2002).

The genus *Clerodendrum* (Lamiaceae) is one of the important medicinal plants genera. They possess various secondary metabolites and are used in various ethnic and folk medicine, indigenous systems such as Ayurveda, Unani, and Homeopathy, and as a source of drugs in various pharmaceutical industries (Poonam and Singh, 2009). Members of the genus are used in the treatment for hypertension, cardioprotection, diarrhoea, dysentery, anti-colics, abdominal discomfort, dizziness, gastric disorders, cough, and skin problems. Beside used as, anthelmintic, anticonvulsant, analgesic, blood clotting, antioxidant, anticancer, antimalarial, antifungal treatment have also been documented (Kalita et al., 2012). The genus *Clerodendrum* includes 580 species (Shendge et al., 2018) which are found worldwide in tropical and subtropical regions of the world. Majority of the genus' species are found in tropical Africa and southern Asia, but they are rarely found in tropical America and northern Australasia. A few species are distributed in the north and continue into the temperate zone in eastern Asia (Mabberley, 2008). About 23 species out of 580 species (Kar et al., 2014), are found in the north-eastern part of India. The present study was concentrated on three *Clerodendrum* species, namely *Clerodendrum colebrookianum* Walp., *Clerodendrum infortunatum* L. and *Cleroldendrum serratum* (L.) Moon.

C. colebrookianum is an important edible plant for its medicinal value. It is used as a home remedy in the treatment of hypertension by different tribes of Northeast India; the young leaves of the plant are mostly cooked and boiled for eating (Yadav, 2012). In Mizoram local traditional medicinal practitioners use the plant for anticolics pain in infants (Sharma et al., 2001). In Nagaland the plant is used for the treatment of helminthic infection, dizziness, and greenish (Jamir et al., 1999); in Arunachal Pradesh it is used for stomach disorders (Namsa et al., 2011) and in Manipur it is used for skin diseases, cough and dysentery treatment (Singh & Singh, 2009). The ethnic tribe in Dibrugarh district, Assam, has reported *C. colebrookianum* as vulnerable shrub (Gogoi & Nath, 2021). A study by Choi et al., (2004) reported that

C. colebrookianum has antidiabetic, antihypertensive, and sedative properties. There have been claims that leaves contain fatty acids, octacosanol, sitosterol, colebrin, and clerosterol (Yang et al., 2000)

C. infortunatum is a shrub with medicinal benefits for treating blood disorders, mood swings, and thirsty and burning sensations (Rej et al., 2014). Bark juice relieves stomach pain and indigestion, while leaf extracts serve to treat scorpion stings, ease pain, and have expectorant and vermifuge properties (Nandi & Lyndem, 2016). It is used in homoeopathy as a treatment for diarrhoea and fresh wounds (Helen et al., 2017). Several pharmacological effects of *C. infortunatum* such as anthelmintic, anticonvulsant, analgesic, blood clotting, antioxidant, anticancer, antimalaria and antifungal activity, are documented (Bhattacharjee et al., 2011 and Saha et al., 2018). Additionally, *C. infortunatum* contains flavonoids, glycerides of stearic acid, linoleic acid, and lignoceric acid, as well as saponin, diterpene (Clerodin), triterpene (lupeol), and steroid (-sitosterol) (Bhattacharjee et al., 2011).

C. serratum has been one of the most important medicinal plants since ancient times. The plant species are used to treat a variety of human ailments. Different parts of the plant are used for various medicinal treatments; the root and leaves are more commonly used. The plant's roots are bitter and are used to treat a variety of ailments including ulcers, wounds, rheumatism, snakebites, bronchitis, cholera, drops, eye problems, fever, inflammations, and malaria (Patel et al., 2014). External applications of the plant's leaves are used to cure ophthalmia and cephalalgia.

In Assam, the tender leaf extract and leaf juice of *C. serratum* are used for the treatment of helminthic diseases, dysentery, seasonal fever, and fruit for dietary purposes (Yadav et al., 2018). The Jaintia tribes of the North Cachar hill district of Assam used *C. serratum* for curing different ailments (Sajem & Gosai, 2006). The leaves and roots of *C. serratum* are used as medicine for treating malaria, febrile and catarhitic infections, fever, cephalalgia, and snake bites in Western Ghats of India (Raviraja, 2005). The root of *C. serratum* is used to treat asthma in Andhra Pradesh (Savithramma et al., 2007). The ethnomedicinal uses of *C. serratum* have been reported from different region of India and also from many countries of the world.

China, Japan, Korea and Thailand also reported the use of *C. serratum* for various medicinal treatments such as syphilis, typhoid, cancer, jaundice, and hypertension (Yadav et al., 2018). The roots of *C. serratum* contain sapogenins, D-mannitol, and stigmasterol, and the leaves contain flavonoids and phenolic acids (Apana et al., 2021). Deforestation, high exploitation and low seed germination of *C. serratum* are some of the result for causing reduction in the natural plant population (Sharma et al., 2009). The Chhattisgarh Medicinal Plant Board has reported *C. serratum* as a threatened species (Upadhyay & Koche, 2015). *C. serratum* is also enlisted as "vulnerable" and "endangered" species in India (Apana et al., 2021).

Understanding of the pollination biology of wild and domesticated plant genetic resources is essential for management of the rapidly diminishing tropical biodiversity and increasing plant species diversity, evenness, and productivity (Krishnan et al., 2020). Medicinal plants have received much less attention in reproductive and genetic studies and species improvement programs than agricultural and forestry crops. There is an urgent need to develop a database that is essential for the conservation and management of medicinal plant resources since there is a severe lack of baseline data on pollination biology for tropical medicinal plant species, especially from North East India. The current study was undretaken to discern detail information about the anthesis, anther dehiscence, female receptivity, pollen production interactions, pollen production, *in vitro* pollen germination and pollen storage of three important *Clerodendrum* species in the tropical forest of Mizoram.

1.1. Floral and pollination biology

Despite of enormous importance for medicinal purposes and other uses, the raw material for *Clerodendrum* species is still primarily taken from the wild; hence proper cultivation methods need to be established. The cultivation of this medicinal plant is a challenging task since the floral and pollination biology of *Clerodendrum* species is less known. Understanding floral biology enables us to understand the various phenophases of a species as well as the processes that take place from the creation of gametes to the germination of seeds as well as the limitations on the natural reproduction of the plant species. Studies on the floral and

pollination biology of vulnerable medicinal plants species will offer useful knowledge information, such as the timing of flowering, fruiting and plant-pollinator interactions. This will help in the hybridization, cultivation, sustainable use and conservation of that particular plant species in their habitat.

The length of time and frequency play a key role in defining flowering and fruiting patterns. There is a need to identify the limiting factors influencing the floral and pollination biology of medicinal plants, such as time of onset, synchronization, duration of flowering, amplitude and variation in flowering quantity. The onset of flowering may be regulated by a variety of weather conditions, such as temperature, humidity, rainfall, and solar radiation (Requile et al., 2021). The stability of the fruit and seed production of various commercially significant crops is severely hampered by ineffectual pollination. Limitation of pollen on flowers may occur when the flower depends on pollinators for pollination. A reduced pollinator activity might affect reproductive success in producing fruit and seed sets. Major biotic elements influencing pollination success in animal pollinated plant species include the population density of pollinators, their diversity, frequency of visits, and the quantity and quality of pollen that reaches the stigma (Kremen et al., 2007).

Pollination and fertilization of a flower occur in different stages (a) time of opening flower- anthesis, (b) stomium split at base and apex of the theca – anther dehiscence, (c) released of pollen from the dehisced thecae, (d) released pollen from anther are deposited and germinated on stigma and (f) pollen tube growth. Reproductive success in the plant is related to floral arrangement, the number of flowers that bloom per day, and the frequency of legitimate pollinator visits for pollination and fertilization (Thakur et al., 2018). The degree of outcrossing and selfing within a plant population depends on synchronous and asynchronous flowering, which affects plant reproduction (Fuchs et al., 2003). The majority of the angiosperms produced hermaphroditic flowers and developed a range of strategies to encourage outbreeding (Lora et al., 2011). Dichogamy, i.e., time separation in the maturity of male and female reproductive organ, is observed in angiosperm species (Endress & Lorence, 2004). Timing and duration of stigma receptivity are critical factors in regulating the

separation of the male and female reproductive phase, which controls the pollen's adherence, hydration, and germination. Stigma receptivity directly influences the duration of adequate pollination time and, subsequently on fruit yield. Different species have different lengths of stigma receptivity which can be influenced by the environmental factor (Hedhly et al., 2003).

Scarce information about the mating behaviour and incompatibility in medicinal plants was available in spite of their medicinal uses. The improvement, conservation and management of plant species are impossible without the proper knowledge of their type of mating system. Scientific studies on floral features and pollinators responsible for the mating system will help to formulate conservation measures for rare, vulnerable and endangered plants of ecological and economic importance.

1.2. Pollen production in mating success of plants

Pollen is a male gametophyte of seed plants that creates male gametes. Compared to other plant organs, pollens are anatomically simple (Crang et al., 2018). The germination of pollen and pollen tube growth is essential for fertilization and seed development. The biotic and abiotic factors help in the success of sexual reproduction, which play an important part in the successful mating and fruit set (Kremen et al., 2007). The study of pollen grain production is essential because it determines the pollen availability in and across the populations by gene flow which impacts mating success (Allison, 1990). Flowering plant species produce and release enormous amount of pollen into the atmosphere every year. The number of the stamen and flower in the individual plant is reported to be positively correlated with the number of pollen grains produced (Mazzeo et al., 2014). Pollen production is influenced by the number of flowers, the inflorescence number of a plant and the environment in which it develops. In addition, the size of the pollen grains and the length of the anthers are the main determinants of pollen production (Pletsers et al., 2015). The flower density in the plant is influenced by climate factors (Khanduri et al., 2013). Anemophilous plants produce enormous volumes of pollen to fulfil their fundamental role (Piotrowska, 2008). Information about the pollen production of a particular species will help to aero biologists to predict the pollen season and amount

of pollen emission to the air (the amount of pollen in the air is influenced by plant density, flowering season length, and pollen production), tree breeders for making timely crosses, foresters and silviculturists to improve the quality of the wood through provenance testing and mating design and ecologists to study natural selection and evolution of plant species.

Between species and populations, the production of pollen per anther and the quality of pollen vary considerably for the same plant species. *Dactylis glomerata* provides an excellent example of pollen production estimation under various climatic and geographic situations (Severova et al., 2022). Pollen production varies greatly depending on the location and altitude of the same species. The differences in pollen production among the population define the species' reproductive costs, which significantly impact an individual's fitness (Kyogoku, 2015). Within a species and across sites and years, the amount of pollen production in woody species varies greatly and is regulated by environmental conditions (Barwise & Kumar, 2020). In animal pollinated plants, the quality of pollen supplied during pollination offers reproductive assurance when pollinator numbers are limited (Timerman, & Barrett, 2021). The production of seeds and fruits in various plant species have been linked to pollen limitation which results because the pistil gets insufficient pollen grain to fertilise all the ovules (Harder, & Johnson, 2008). Although the quantity of pollen grain that land on the stigma is significantly higher than the number of accessible ovules but fertilization and fruit set does not occur. A minimum number of pollen grains on the stigma are required to enable pollen germination and pollen tube growth which is associated with pollen production effect and is linked to a phenomenon i.e. pollen density dependent. Pollen production has an important role in natural regeneration of the plant communities in tropical forests and had a substantial impact on a species reproduction (Schwartz et al., 2017). Production of a high number of pollens enables stigma to receive sufficient pollen, which helps in high fruit/seed set, ultimately affecting plant abundance and population viability and raising the possibility of a successful plant mating system (Khanduri et al., 2015b).

1.3. Pollen viability

Pollen sterility is a crucial obstacle to sexual recombination. The viability and vigour of the pollen determine the rate of pollen quality. To understand sterility issues, hybridization programmes, fruiting, breeding programmes, and evolutionary ecology, pollen viability, artificial pollination and inbreeding experiments are crucial.

The pollen grains develope inside the anther and they start to dehisce in a dehydrated and metabolically inactive state at the time of anther maturity. Once the pollen is released from the anther they act as autonomous functional unit and are exposed to ambient environment and the part of pollen gets transferred on to the receptive stigmas through different pollinating vectors at a limited period of time, within the limit of pollen grain viability, which led to pollination. After deposition on the stigma, pollen germinates within a few hours to several days and pollen tube grows within the style and reaches to the embryo sac. It has been reported that in most angiosperms, pollen grains germinated by the time when the ovule structure and embryo sac have fully developed. Hence, fertilization normally occurs with days and month after pollination (Chen & Fang, 2016).

The time period between the anthesis and receptivity of stigma is influenced by numerous distinctive environmental factors which might reduce the viability rate of pollen and there are chances for dryness of stigma when exposed to high temperature. During reproduction process plant might face many environmental factors. To achieve successful reproduction, plant modifies their reproductive system to accommodate pollination success and fertilization. Encountering the above view in mind, artificial pollination and breeding experiments are done for estimation of pollen viability to understand the sterility problems and hybridisation programs, fruiting breeding programs and evolutionary ecology (Bandeira et al., 2021).

The presence of pistillate tissue in nature makes it difficult to examine pollen germination and pollen tube growth *in vivo*. *In vitro* pollen germination has therefore been extensively applied on a variety of pollen architecture. The study of *in vitro* pollen germination is essential because it can provide a wealth of information about the physiology and biochemistry of pollen germination and pollen tube growth.

Pollen viability and *in vitro* pollen germination have a linear connection, and in many species, it has been found to be directly correlated with fruit and seed production.

It is critical to know an appropriate method for measuring pollen viability while breeding of plant species (Dafni & Firmage, 2000). A variety of dyes have been used for testing of the viability of pollen. The dye which is used for testing the viability of pollen might also have the chances of staining the death pollen. TTC, MTT, X-Gal, aniline blue with lactophenol, acetocarmine and Alexander's produce are few of the common dyes for testing the viability of pollen, but all the above dye has recently been criticised strongly that they also stain the death pollen (Rathod et al., 2018). Therefore, the viability test conducted through the above dyes give us a confused idea that weather the dyes are able to differentiate between live/viable pollen and death pollen and which pollen can germinate and which cannot germinate. So, using above mentioned dyes for pollen viability test cannot tell whether the pollen is alive or not. To support the pollen viability test further *in vitro* pollen germination test is necessary.

1.4. Influence of growth hormones on pollen germination in medicinal plants

Comparatively little study has been done on its propagation in comparison to the importance of *C. colebrookianum*, *C. infortunatum*, and *C. serratum* in medicine and reproductive biology (Lalramnghinglova, 2016). Pollen viability, germination, and storage are essential parts of the reproductive biology and breeding of a plant species because effective sexual plant reproduction depends on viable and fertile pollen. Understanding sterility and hybridization through artificial pollination and inbreeding experiments depends on the viability and vigour of the pollen, which affect the pollen quality rate (Shivanna, 2019). Proper germination and growth of pollen grains are essential for fertilization, fruit, and seed development (Shivanna & Rangswamy, 2012). Numerous pollen viability studies rely primarily on *in vitro* pollen germination (Hao et al., 2022). In plant developmental biology, study on pollen germination is essential. It can provide a wealth of information regarding the

physiological and nutritional needs for pollen germination and growth (Shivanna & Rangswamy, 2012).

Plant hormones influence the growth of pollen tube, in addition to pollen germination. Water, amino acids, sugars, boron, calcium, growth stimulating substance such as gibberellins, auxins, kinetin, Indolebutyric acid (IBA) and Indole-3-acetic acid (IAA) are some of the main components for pollen germination culture media. Sucrose, boric acid, calcium nitrate, potassium nitrate and magnesium sulphate are also some of the organic and inorganic substances which had an effect on *in vitro* pollen germination. According to reports, gibberellins are a group of endogenous plant growth hormone that play a key role in a variety of characteristics of plant growth, including seed germination, trichome development, stem and leaf elongation, flower induction, anther development, and fruit and seed development. Study from different authors have shownthat the gibberellins help in the enhancement of pollen tube growth *in vitro* (Binenbaum et al., 2018). Auxins help in promoting pollen tube growth via an effect on Ca^{2+} channel activity in apical pollen tubes, with the influenced of ATPase activity regulation (Gao et al., 2019). The application of kinetin at low concentrations in *in vitro* culture improves pollen germination and at high concentration of kinetin it can inhibit pollen tube growth (Soni & Bohra, 2021). Exogenous indole-3-acetic acid (IAA) facilitate *in vitro* pollen tube growth (Zhang et al., 2018) and they involved in the pollen-pistil interaction directly or indirectly. During plant sexual reproduction, IAA plays an important role for controlling the development of stamens and ovaries, promoting the maturation of egg cells and inducing the axial polarity and polar development of embryo (Aloni, 2021).

1.5. Pollen storage and its practical applications in medicinal plants

For plant breeding, particularly in asynchronous flowering species, and for germplasm transmission, long-term pollen storage is crucial. Pollen's storage time varies from one plant species to another and from minutes to months. Thus, it is possible to determine and standardise pollen grain storage conditions to preserve pollen grains' viability over an extended period for establishing crosses between two

types or species that flower at different periods. In order to extend pollen viability and enable species hybridization to create new species of novel importance, the optimal temperature must be determined through further experimentation.

For breeding programmes, hereditary preservation, artificial fertilisation and self-incompatibility, pollen storage is beneficial. With respect to plant species and storage circumstances, pollen has a significantly variable life lifetime (Mesnoua et al., 2018). Numerous techniques are taken after these days to preserve the viability of pollen beneath storage conditions. Further, storing pollen is essential for the artificial hybridization of fruits cultivated under controlled conditions. Pollen is used as an explant source in tissue culture to create haploid plants (Lone et al., 2020). There are several different methods for preserving pollen such as natural solvents, refrigeration, freeze drying and cryopreservation (Sidhu, 2019). By adjusting the temperature, relative humidity and storage environment the amount of time pollen stored can be increased (Yang et al., 2010; Mesnoua et al., 2018; Jaskani & Naqvi, 2017).

Considering the economic and medical implications of *C. colebrookianum*, *C. infortunatum* and *C. serratum* and to increase its natural populations understanding pollen biology, *in vitro* pollen germination, pollen tube growth rate and pollination mechanism is essential. The knowledge about the functional quality of pollen will help in establishing a relative method to monitor pollen vigour during storage, genetic and pollen-stigma interaction studies, crop improvement and breeding, incompatibility and fertility studies. Pollen grains can be temporarily stored and used in hybridization, breeding, supplementing and pollination research in the future. Keeping in view the all above facts in consideration, this study was conducted in three valuable medicinal plants with the following broad objectives:

Objectives:

1. Anthesis, anther dehiscence, female receptivity and pollen- pollinator interaction in relation to the time of the day and associated weather conditions.
2. Assessment of pollen production, pollen/ovule ratio and its impact on reproductive success.
3. Effect of growth regulators on *in vitro* pollen germination with pollen longevity tests.

Chapter 2

Review of Literature

2.1. Medicinal utilities of *Clerodendrum* species:

Research interest in medicinal plants and their traditional uses during the past few decades has raised worldwide (Rossato et al., 1999). According to the World Health Organization (WHO) survey, between 70-80 percent of the world's population uses herbal plant medicine for their primary healthcare (Al-Snafi, 2016). Medicinal plants are being used to extract important medicines and biologically active substances (Al-Snafi, 2015). The commercial demand for natural health products (nutraceuticals), herbal medicines, and secondary metabolites of medicinal plants has increased throughout the world (Nalawade et al., 2003). According to a conservative estimate, the earth is losing at least one key drug candidate plant species every two years, and the rate of plant extinction is currently between 100-1000 times higher than what is expected from natural extinction (Pimm et al., 1995). Many medicinal plants are lost due to overharvesting and habitat damage (Sharma et al., 2010). According to the IUCN Red List, 91% of plant species are threatened due to habitat loss and degradation (Hoffmann et al., 2008). Understanding the ecological requirements of endemic and threatened plants is required for using plant species wisely (Kala et al., 2006). The perennial shrub *Clerodendrum,* family Lamiaceae, is common in the tropical parts of Asia, such as India, Myanmar, Bangladesh, Malaysia, Indonesia, Thailand, Bhutan, and Nepal, as well as in temperate Tibet (Chathuranga et al., 2019). The *Clerodendrum* genus has a number of species that have been reported to have significant ethnomedical use in several indigenous medical systems and as folk remedies (Chakraborthy & Verma, 2013). For the treatment of numerous life-threatening disorders like syphilis, typhoid, cancer, jaundice, and hypertension, the genus is specifically utilized as medicines in the Indian, Chinese, Thai, Korean, and Japanese systems of medicine (Chakraborthy & Verma, 2013). *Clerodendrum* spp. is frequently used to treat respiratory conditions broadly (Chakraborthy & Verma, 2013). *Clerodendrum* genus reported for its anti-inflammatory, anti-nociceptive, antioxidant, anti-hypertensive, anti-cancer, hepatoprotective, memory-enhancing, and neuroprotective properties (Wang et al., 2018). Twelve species of *Clerodendrum*

were reported to have natural chemicals that could be used as SARS-CoV-2 treatment options, given that these species are frequently used for respiratory conditions (Kar et al., 2021). The root of *C. colebrookianum* is recorded to possess pharmacological properties such as anthelmintic, anti-bacterial, and anti-fungal, used in the treatment of bronchial asthma, gastrointestinal tract diseases, syphilis, gonorrhea, and hematological disorders. Local peoples in the North-Eastern region of India utilize this plant's leaves and leaf twigs as a home treatment for high blood pressure (Rajbongshi, 2014). The Mizo inhabitants of this region claim that the prevalence of hypertension in their society is extremely low, and this is because they regularly consume the tender leaves and shoots of *C. colebrookianum*, locally known as "Phuihnam" (Devi & Sharma, 2004). It is grown by rural and urban residents in kitchen gardens, and private nurseries in urban areas sell the saplings for Rs. 10.00 each (Bordoloi & Borthakur, 1997). Traditional healers have used *C. colebrookianum* to treat intestinal tapeworm infections because of its potent anthelminthic qualities (Yadav, 2012). Leaves and roots were reported to possess sterol glycoside clerosterol, colebrin A-E, clerodolone, octacosanol, daucosterol, triacontane, (24s) ethylcholesta–5,22,25–triene–3β–ol, α–amyrin, β–sitosterol and fatty acids (Yang et al., 2000 and Rajbongshi, 2014).

C. colebrookianum leaves are being consumed for better health in the North East region of India since they have definite cardioprotective potential (Devi & Sharma, 2004). Rajlakshmi et al., (2003) reported that the leaf extract of *C. colebrookianum* increased the blood's antioxidant capacity and had an inhibitory effect on the liver's and kidney's basal levels of lipid peroxidation. Phytoconstituents with antioxidants potential, prevent the formation of uric acid, anti-tumour, enhancing zinc's bioavailability, against high blood pressure were identified in the *C. colebrookianum* through GCMS analysis (Payum, 2020). *C. infortunatum* extracts were reported to be used in Ayurveda to treat leprosy, worm dyspepsia, itching, cough, and colds; its leaf was used in treating scorpion stings, and bark juice was effective at treating indigestion and abdominal pain (Nandi & Mawkhlieng, 2016). It is reported to use in Indian homeopathy for treating fresh wounds, post-natal problems, and diarrhoea (Nadkarni & Nadkarni, 2002). Various parts of plants such

as roots, stems, and leaves are reported to be utilized as a medicine in India by the tribal people of the Chotanagpur plateau in eastern India to treat conditions like asthma, cataracts, malaria, and blood, skin, and lung problems (Singh, 2007). Similar uses have been documented in Thailand; the leaves and roots were used to treat kidney failure and intestinal infections (Islam et al., 2013). The leaf juice is applied externally to treat tumours, skin conditions, snake bites, and scorpion stings in Bangladesh, and it is also used as an anthelmintic, emetic, moderate laxative and cholagogue (Ghani, 2003). The plant extracts of *C. infortunatum* were found to contain high antioxidant and pharmacological properties that support the traditional therapeutic claim for wound healing activity (Gouthamchandra et al., 2010). Traditional medicine uses extracts from the roots, leaves and stem of *C. infortunatum* to treat a variety of diseases, including diarrhoea, respiratory issues, tuberculosis, and intestinal disorders (Waliullah et al., 2014). The essential oils (fatty acids and their esters, aromatic monoterpenes like limonene, a-pinene, ß-pinene, p-cymene, and myrcene and sesquiterpenes), are extracted from the leaves and root bark of the plant *C. infortunatum* which is highly used in Ayurveda (Jirovetz et al., 1999).

C. serratum plant has been reported to have significant ethnomedical value in several traditional medicinal systems including Ayurveda, Siddha, and Unani, for the treatment of fatal illnesses, including syphilis, typhoid, cancer, jaundice, and hypertension (Singh et al., 2012). Additionally, it is said to have been traditionally used as an anti-rheumatic, anti-asthmatic, febrifuge, cephalalgic, and ophthalmic remedy. Roots are recorded for their anti-oxidant, anti-bacterial, anti-fungal, anti-tumours and anti-inflammatory (Singh et al., 2012 and Dongare et al., 2020). Icosahydropicenic acid (IHPA), a new pentacyclic triterpenoid saponin isolated from the roots of *C. Serratum* can be used for the treatment of asthma (Bhujbal et al., 2010). The aerial portions and roots of *C. serratum* have been traditionally used to treat a wide range of inflammatory illnesses because they have anti-rheumatic effects (Shareef et al., 2013). It is reported that the plant drug Sirutekku is extracted from the root of *C. serratum*, which is used in Siddha medicine (Narayanan et al., 2014).

2.2. Plant-pollinator interactions in plants

Pollination biology is a mutualistic relationship between plants and their animal pollen carriers, with energy rewards as the foundation for co-evolution (McCallum et al., 2013). Animal pollinators and pollination have long been thought to have been crucial to the diversity of the angiosperms (Coyne & Orr, 2004). There has been a surge in research on the pollination ecology of agricultural crops, forest trees, and ornamental plants in recent years, but comparatively less research on the reproductive biology of plants with medicinal importance from natural stands (Kulloli & Sreekala, 2009).

Insects play a vital role in transporting pollen, enabling a plant species to outcross with other conspecific members, thus helping in gene flow at a population level (van Ginkel & Flipphi, 2020). The length of the flower, colour, odour, nectar, pollen, and other flower rewards have a significant impact on pollinators (Faheem et al., 2004); thus, floral features influence pollination efficiency. Flowers that are pollinated by bees are bright in colour, reflect light in the blue to violet spectrum, and have nectar guides for nectar during the day (Faheem et al., 2004). Bright or light-coloured flowers with suitable odour and nectar guides that produce nectar at night are pollinated by moths and bats (Zariman et al., 2022). Pinkish and red flowers are well-known to attract butterflies (Abrol, 2012). The odour or fragrance of the flower attracts specific pollinators on them; flowers pollinated by insects mostly release order or fragrance, while flowers pollinated by birds are odourless (Johnson & Govender, 2022). For long-distance advertising, nocturnal flowering plants with a distinctively strong and pervasive floral odour are required.

Over 2, 50,000 species of angiosperms occur in the world, of which 70% of plants rely on insect pollinators (Pannure, 2016). Nectar is predominantly a sugar solution; it has been regarded as the most crucial floral food reward for animal pollinators (Nepi et al., 2012). Nectar is a vital source of nutrition and energy for pollinators, it consists of a complex mixture of carbohydrates, amino acids, proteins, lipids, vitamins, antioxidants, alkaloids, organic acids, and inorganic substances like minerals (Burkett,1998), and they play a significant role in pollination. Generally, a

small amount of concentrated nectar is available in the insect-pollinated flowers, thus encouraging insect pollinators to visit more flowers to quench their thirst, which increases the floral visit and the level of outcrossing (McCallum et al., 2013). Bats and hawk moths pollinated flowers secrete a copious amount of nectar at night, while flowers that bees, butterflies, and birds pollinated one do so during the day (Lemaitre et al., 2014). Many insects, especially apidae, beetles, flies, thrips, and butterflies, depend on pollen as a food source and nutrition (Cane, 2016). According to flowers, pollinators' foraging range varies from 3 to 12 km, and their foraging rates also change (Anonymous, 2003). Bumble bee foraging rates are twice as fast as honeybee foraging rates, although solitary bee foraging rates vary greatly depending on size (Goulson et al., 2002). Honeybees, flies, bees, butterflies, and hawk moths have all been observed moving swiftly between flowers during foraging (Kulloli et al., 2011).

Attitude, temperature, light, wind, and rainfall influence the foraging behaviours, flower visits, and pollination efficiency (Akhtar et al., 2018). The foraging behaviour of pollinators is influenced by the altitudinal gradient, for example, hummingbirds are more effective pollinators at higher elevations than bees, and moths are at intermediate and lower altitudes (Klomberg et al., 2022).

Pollinators are significantly impacted by temperature (Conrad et al., 2017). Since no foraging occurs below 8°C, some activity occurs between 8°C and 16°C, optimum activity occurs between 16°C and 32°C and foraging is reduced above 32°C though in some situations foraging may continue up to a temperature of 42–48°C, temperature fluctuations have a substantial impact on bee foraging (Abrol, 2011). Habitat fragmentation hinders the interaction between plants and their pollinators; for example, two plant species, *Acacia brachybotrya* and *Eremophila glabra*, planted in linear vegetation, received less pollen than conspecifics in adjacent reserves (Jabeen & Bhat, 2013).

The buzz-pollination syndrome is the term used for the removal of pollens from the anther by vibrations (De Luca et al., 2022). Bees and other insects harvest pollen from the anthers utilizing vibrations known as sonication or buzzes (De Luca et al.,

2022). The vibrations of bees help to add pollen collection from various plant species with different morphologies, such as *Cistus*, *Papaver*, *Pedicularis*, *Myrtaceae*, and *Solanum* (Gottsberger, 2012).

2.3. Plant-pollinator interactions in *Clerodendrum* species

Rohitash (2018) reported that anthesis occurs between 0600-0630 h, anther dehiscence between 0730-0900 h and stigma receptive between 1100-1400 h in *Clerodendrum splendens*. Furthermore, he observed only black ants as floral visitors in *C. splendens*, and they are highly self-incompatible. Further, he also mentions that the reason for self-incompatible might be the absence of nectar reward and effective pollinator (Rohitash, 2018). Jai (2010) reported *Xylocopa*, *Eumenes* sp. and *Componotus campestris* (black ant) are the most effective pollinator visitors of *C. splendens*.

The anthesis of *Clerodendrum inerme* was recorded between 1500-1600 h in afternoon, and the anther dehiscence after an hour form anthesis with the longitudinal split of the anther (Aluri et al., 2016). Nectar was released during the post-anthesis phase, and the stigma with forked lobes becomes receptive between 2-3 days after anthesis (Aluri et al., 2016). *C. inerme* is reported to be pollinated by hawk moths (Primack et al., 1981), and pollination of flowers is mostly adapted towards entomophily (Aluri et al., 2016). Raju & Kumar (2016) observed bird and hawk moth pollination and nectar robbery in *C. inerme*. Bees and butterflies pollinate *C. laevifolium*, according to Keng, (1990). Rohitash, (2017) reported *Macroglossum* sp., *Apis cerana*, *Apis dorsata*, *Bumbus lapidaries*, *Danaus genutia*, *Neptishylas papaja*, and *Eurema hecabe* as floral foragers of *C. inerme* and he also noted that honey bees and bumble bees were more active than butterflies.

The anthesis of *C. infortunatum* was observed in the morning hours with the physical separation of male and female (distinct herkogamy), and stigma was receptive mostly during the female phase (Mukhopadhyay & Quader, 2022). *C. infortunatum* flowers exhibit temporal dioecy and are extremely protandrous, herkogamous, and dichogamous (Kumar et al., 2017). The papilinoid butterflies (*Papilio polytes*, *P. polymnestor*, and *Atrophaneura hector*) are observed to pollinate on *C. infortunatum*,

through pterogotribic pollination by striking the anthers and stigma with their wings, according to Byragi & Subba (1995). *Tapinoma melanocephalum* and *Trichomyrmex destructor* harvest nectar for *C. infortunatum* without pollinating the flower (Mukhopadhyay & Quader, 2018).

Sakamoto et al., (2012a) observed the influence of three pollinators on the fruit and seed development of *Clerodendrum trichotomum*: *Papilio spp*., *Macroglossum pyrrhosticta* and *Xylocopa appendiculata,* and they found out that pollinator *Macroglossum pyrrhosticta* promote self-pollination among the flowers.

Clerodendrum molle is visited by nocturnal pollinators, which includes ants, spiders, hawk moths, and roaches, and they are also visited by diurnal pollinator such as carpenter bees and ants (McMullen, 2011). Pollinators play a significant role in a *Clerodendrum* species that reproduces primarily by xenogamy and geitonogamy (Mukhopadhyay, & Quader, 2020).

Clerodendrum trichotomum and *Clerodendrum izuinsulare* were reported to be visited by insects such as diurnal hawkmoths, bees, swallowtails, and nocturnal hawkmoths. They were pollinated nocturnally and diurnally, and shared pollinators are common to both species (Miyake & Inoue, 2003).

Numerous plants may be suited to various pollinator types based on differences in floral appearance. Lepidoptera, nectar-feeding bees, Hymenoptera, Coleoptera, Diptera, Japanese black swallowtail butterflies, nocturnal hawk moths, diurnal hawk moths are among the pollinators of *C. trichotomum* or *C. izuinsulare* (Mizusawa et al., 2014).

Long-tongued hawkmoth is pollinator for the tubular flower *Clerodendrum viscosum*; many black ant and butterflies were observed moving from flower to flower. Though the flower appears hermaphrodite during anthesis, *C. viscosum* stigma cannot accept self-pollens due to their arrangement (Liza et al., 2010).

Three floral visitors behaviours i.e., *Macroglossum pyrrhosticta*, *Xylocopa appendiculata* and *Papilio dehaanii* was studied in *Clerodendrum trichotomum* and

it was concluded that pollination dynamics differ among pollinator species, rapid visitation behaviours of pollinators and flower visits and pollination rate are not equal (Sakamoto et al., 2012a).

2.4. Pollen production and reproductive success in plants

Pollen production varies widely among anemophilous and entomophilous plant species (Mondal & Mandal, 1998). Numerous investigations have focused on pollen production per plant across diverse species (Khanduri et al., 2015b). Microsporogenesis the process by which pollen grains form inside the anther can be influenced by both genetic and environmental stress (Garcia Mozo et al., 2005). During the dispersal phase, pollen is subjected to harsh environmental variables that may reduce pollen viability and germination capacity (Bots & Mariani, 2005). Relative air humidity at pollen movement and shedding are the key factors affecting viability (Fonseca & Westgate, 2005).

Pollen limitation, inadequate compatible pollen receipt by stigma and ovules, is common among animal-pollinated plants (Knight et al., 2006). Pollen limitation is often considered a negative consequence of small population sizes or fragmentation (Jump & Penuelas, 2006). Low reproductive success due to pollen limitation is widely reported in angiosperms (Larson & Barrett, 2000) which is predicted to be the most prominent in an environment where pollinator services are unreliable or low in abundance (Burd et al., 2009). Scientific studies have reported the effect of pollen production and yield of flower on seed settings in entomophilous (Khanduri & Sharma, 2001) and anemophilous plant species (Damialis et al., 2011).

Pollen production is crucial to plants' reproductive fitness and success in forest ecosystems. Anemophilous plants emit enormous volumes of pollen into the atmosphere each year. Most temperate tree species are pollinated by the wind, and successful fertilization requires a large amount of airborne pollen (LaDeau & Clark, 2006). Numerous temperate trees, including conifers, experienced interannual variation in pollen production that led to varied fruit sets and seed production (Shibata et al., 2002). Pollen is a rich reservoir of food resources since they contain

essential amino acids, trace elements, enzymes, B-complex, and vitamins C and E naturally (Godswill et al., 2020). The length of the anthers and the size of the pollen grains have a significant impact on pollen production (Piotrowska, 2008). Environmental factors such as temperature and precipitation influence pollen production; increase in atmospheric carbon dioxide has an impact on pollen production in *Ambrosia artemisiifolia* (Ziska & Caulfield, 2000).

Among anemophilous plant species, pollen production varies widely (Quamar & Bera, 2014). Increased temperatures can hurt plant sexual reproduction, which can lower fertility. Male gametophytes are more susceptible to heat stress during all stages of development; it impacts pollen production and shape, cell wall structure, and most significantly, pollen metabolism (Hedhly, 2011). Different species have different sensitivity to heat stress levels when the high-temperature modes are applied (Parrotta et al., 2016). More than 12000 species in the Poaceae family are pollinated by wind. Pollen from the Poaceae family is now recognized as the world's most significant source of airborne biological pollution and the main factor contributing to pollen allergies (Garcia, 2017). Piotrowska, (2008) study the structural characteristics and pollen production of five allergenic anemophilous plants species such as *Betula verrucosa, Secale cereale, Rumex acetosella, Plantago major* and *Artemisia vulgaris* and observed pollen production per anther was highest in *Secale cereale* 22360, followed by *Betula verrucosa* 11160, *Rumex acetosella* 10850, *Artemisia vulgaris* 9580 and lowest in *Plantago major* 5870.

According to Jai, (2010) *Clerodendrum splendens* has 2160±380 pollens per flower, with 4 ovules per flower and a pollen ovule ratio of 540:1. He concludes that just 2% of the flowers in *Clerodendrum splendens* exhibit open pollination with significantly less seed set percentage. In *Clerodendrum inerme*, the pollen productivity per anther is 796±51.2, while the overall pollen productivity in individual flowers with four and five stamens is 3184 and 3980, respectively (Aluri et al., 2016). Gupta et al. (1982) observed pollen production in *C. viscosum* 1799 to 2646 grains/ stamen and 7979 to 9595 grains/flower. A total of 58 angiospermous plant species have been determined for pollen production, increase in pollen production from herbs<shrubs<trees was

observed and analyzed (Mondal & Mandal, 1998). Pollen production varies among the anemophilous, entomophilous and amphiphilous plants. The average number of pollen production per anther in *C. japonicum* was observed as 9097, and pollen production per flower as 36388 with four anthers per flower. The average pollen production per anther for *C. indicium* was 11137, and pollen production per flower was 44548 with four anthers per flower (Mondal & Mandal, 1998).

Pollen/ovule (P/O) ratio and breeding system correlations have typically been explained by the sex allocation hypothesis or the idea that P/O represents pollination efficiency (Gallardo et al., 1994). Plant breeding systems have been roughly estimated using the pollen: ovule ratio (P/O). Plant breeding techniques have been proven to be connected to specific floral features. Correlation between P/O ratios and breeding methods within some plant groupings has been demonstrated (Alarcon et al., 2011). Autogamy is connected with low P/O levels, while outcrossing is correlated with higher P/O values (Cruden, 1977). Species with specific pollen packaging strategies (pollinia, polyads, or viscin threads) have much lower P/O ratios than species without such strategies because pollen is transferred more effectively, and species offering pollen as a reward have much higher P/O ratios than species offering nectar because large amounts of pollen are consumed (Cruden, 2000). Other characteristics of plants, such as life form and life history, have an impact on P/O ratios as well (Alarcon et al., 2011).

According to Cruden, (1977) cross-pollinated plants generate more pollen grains than self-pollinated plants because they are less efficient at pollination and have larger P/O ratios. Higher P/O ratios in wild species compared to domestic species were explained by Shanker & Ganeshaiah, (1984) as a result of the higher pollination hazards that the wild species had to deal with. In a different study Shanker & Ganeshaiah, (1984) found a correlation between the decline in P/O ratio with increasing Croton age and the likelihood that pollen grains will successfully reach the stigma. Etcheverry et al., (2012) determined P/O in 21 Leguminosae species; he classified 15 species as obligate xenogamous, despite some of them having been

recorded as facultative xenogamous, and have concluded that P/O variability is influenced by the taxonomic position and pollination strategy.

The angiosperms contain a wide range of pollen and ovule for each flower and considering how important these spores are to reproduction (Burd, 2011). One of the most common patterns is that the ratio of pollen to ovules per flower (P:O ratio) is frequently an approximative indicator of a breeding system (Cruden, 1977); local mate competition influences sex allocation. Self-pollination through geitonogamy or autogamy creates local rivalry between them, which favours selection favouring higher female investment in sex and hence a lower P:O ratio.

The reproductive capabilities of 32 species of legumes from the Mediterranean region by Galloni et al., (2007), noted that the species with high P/O values are suggestive of poor pollen transfer efficiency, which is typical of xenogamous species. While species with the highest P/O had primary pollen presentation, those with the lowest P/O exhibited brush or explosive tripping mechanisms. Their study got to the conclusion that reducing autogamy rates requires the stigmatic cuticle. The importance of P/O ratio is a close indication of species breeding strategy was confirmed by productivity studies. The pollen/ovule ratio of *Clerodendrum inerme* is low, indicating highly effective hawkmoth pollination of this specie (Primack et al., 1981).

In hermaphroditic plants, the ratio of pollen to ovule determines the plant mating system higher the P: O ratio, the greater the possibility of outcrossing (Khanduri et al., 2015b). Flowering plants are hermaphrodites; the amount of seeds fertilized by self and outcross pollen varies greatly among species, from predominate self-fertilization to exclusively outcrossing (Goodwillie et. al., 2005). The degree of outcrossing or selfing (the mating system) among individuals and populations can affect the genetic makeup of populations, the rate of gene flow, the size of an effective population, and the manifestation of inbreeding depression (Barrett & Harder, 2017). Research on the evolution of mating systems has concentrated on factors that encourage self-fertilization within populations, such as reproductive assurance when pollen transfer possibilities are scarce and the genetic transmission

benefit of selfing in populations with minimal inbreeding depression (Butcher et al., 2011). The majority of facultative xenogamous species are found in the climax or other stable ecosystems; delayed autogamy, self-compatible, evolved cross-pollination, self-compatible, and flower pollinator activity may be limited or unreliable (Cruden & Lyon, 2019).

2.5. Pollen viability, *in vitro* pollen germination and pollen longevity

The rate of pollen tube growth and the speed at which pollen grains germinate are significantly correlated to pollen vigour (Sulusoglu & Cavusoglu, 2014). *In vitro* pollen germination assays have been used to calculate the pollen germination percentage and can also be used to evaluate pollen vigour by monitoring the rate of germination over time or the length of pollen tubes (Shivanna & Ram, 1993). The study of pollen vitality is essential in the investigation of pollen biology. Incompatibility and fertility research, genetics and pollen-stigma interactions, crop development and breeding programmes, maintaining gene banks, testing pollen germinability after exposure to specific conditions and analysing dispersal and gene flow are some of the subjects discussed (Dafni & Firmage, 2000). Pollen quality is determined by pollen viability and vigor. In the hybridization process, pollen viability and fertility are of the utmost importance. Most plants need to be successfully pollinated in order to fertilize and produce seeds, and any sensible strategy to boost pollen production must take into account in pollen biology, including pollen viability, pollen germination, and pollen tube expansion (Rathod et al., 2018). High temperatures reduce pollen viability, resulting in fruit production decline (Paupiere et al., 2014). Numerous methods are used to evaluate pollen viability. Such as counting the number of seeds that are produced after pollination, tracking pollen germination and pollen tube growth *in vivo*, tetrazolium salts to detect dehydrogenase activity, aniline blue to identify callose in pollen walls and pollen tubes, acetocarmine or Alexander stain to identify cytoplasmic contents, and determining the plasma membrane after *in vivo* test and the choice of approach depends from species to species and on establishing a link between the test and fertility (Dafni & Firmage, 2000). The ability of pollen to accomplish fertilization

and seed set is the most precise means of determining pollen viability (Impe et al., 2020). The staining approach for determining pollen viability is unreliable for many plant species; hence *in vitro* pollen germination must be done (Sulusoglu & Cavusoglu, 2014). The spread of species fitness and the survival of plant generation depend on viable pollen. It is also necessary for plant breeding and crop improvement. Pollen performance, such as fertilization potential, germinability, and stainability, are crucial aspects of pollen viability (Dafni & Firmage, 2000). Rodriguez and Dafni, (2000) tested the viability of pollen using four different dyes to distinguish between fresh pollen and dead pollen. They discovered that the peroxidase test and 2,5-diphenyl monotetrazolium bromide (MTT) did not stain dead pollen; thus, techniques effectively differentiate pollen quality. High humidity (>95% RH) and temperature (38°C) or storage stress of *Nicotiana tabacum*, *Agave sp*., *Tradescantia virginiana* and *Iris sp*. affected pollen vigour before affecting pollen viability (Shivanna et al., 1991). 83% viability and 58% *in vitro* pollen germination with 763.65±34mm long pollen tube were observed for *Clerodendrum splendens* (Rohitash, 2018) with 5% and 8% pollen germination on the stigmatic surface.

Different biochemicals such as sugar, starch, lipids, phytic acid, and mRNA are found in pollen grains; this stored product plays an important role in pollen germination and pollen tube growth (Patel et al., 2014). The osmotic pressure is maintained by sucrose, which also serves as a substrate for pollen metabolism (Linskens & Kroh, 1970). 7.5-20% sucrose solution is needed for optimal pollen germination (Kumari et al., 2009). The optimal germination rate was found to be 10% sucrose in *Bambusa vulgaris* (Koshy & Jee, 2001), *Datura metel* and *Najas marina* (Patel & Mankad, 2014), 15% sucrose in *Bassia latifolia* (Singh & Singh, 2001), 11–15% sucrose in *Asclepias syrica* (Kevan et al., 1989), 20% sucrose in *Abelmoshus esculentus* (Dabgar & Jain, 2001), and 30% sucrose in *Catharanthus roseus* (Patel et al., 1997). It was found that 5% of the *Selix* species and 15% of the water chestnut species had the best germination rates (Hoque & Arima, 2000). With increasing concentrations of boric acid, gibberellic acid, and IAA in basic sucrose and agar media (0.5-1.0 ppm), pollen germination and tube growth were enhanced

(Bigdeli et al., 2016). At 15% sucrose concentration *Cunninghamia lanceolata* showed pollen germination and tube growth, and at 0.01% boric acid promoted pollen germination and tube growth (Fragallah et al., 2019).

External factors such as temperature, boric acid, fungicides, and the presence of heavy metals affect pollen germination (Radovic et al., 2016). Plant hormones affect pollen germination (Tosun & Koyuncu, 2007); they enhance the growth of pollen tubes (Sotomayor et al., 2012). After pollination, the IAA levels in the pistil increases, it was observed that IAA enhances pollen tube growth (Wu et al., (2008a). The effect of auxin (IAA) and gibberellin (GA3) on pollen germination and *in vitro* pollen tube growth was observed for five almond cultivars (Radovic et al., 2016). Pollen tube length increased from 23 to 86% when treated with auxin, and from 6 to 22%, pollen tube increased when treated with gibberellin. The germination of seeds, the growth of trichomes, lengthening of stems and leaves, induction of flowers, development of anthers, development of fruits and seeds, and many other aspects of plant development are impacted by GA3 (Hedden & Phillips, 2000). According to Acar et al., (2010) *Pistacia vera* pollen germinates at 20 percent sucrose with 10, 25, 50, 75, and 100 ppm of boric acid (H3BO3) and gibberellic acid (GA3). He concluded that GA3 inhibit pollen germination and boron promotes pollen germination slightly. The effect of IAA and IBA on two species of *Bauhinia* and GA3 and kinetin on *Spathodea campanulata* was found suitable for pollen germination, and during the first 24 hours, maximum germination was recorded (Sanjay et al., 2016). Ascorbic acid and indole butyric acid (IBA) promote distinct pollen germination and pollen tube growth in *Nuomici litchi*. Application of 5.0-10.0 mg/L ascorbic acid or 2.5-5.0 mg/L IBA to *Nuomici litchi* flowers can encourage pollination and fertilization (Zeng et al., 2018). Indole-3-acetic acid (IAA), regulates the growth of stamens and ovaries, maturation of egg cells, and induces axial polarity and polar development of the embryo, and it is vital for plant sexual reproduction (Wu et al., 2008b). IAA may be directly or indirectly involved in the pollen–pistil interactions during pollen germination and pollen tube growth of *Nicotiana tabacum* (Chen & Zhao, 2008). Growth regulators such as gibberellic acid, indole-3-acetic acid, indole-3-butyric acid, indole-3-propionic acid, 1-

naphthalene acetic acid and kinetin promote pollen germination in tomato (Karapanos et al., 2006). IBA concentration increases the number of female flowers in the cucumber cultivar Wisconsin MR28 (Diola et al., 2008). The addition of indole-3-acetic acid (IAA) to media containing buthionine sulfoximine (BSO) restored pollen germination and early elongation of the pollen tube to *Arabidopsis thaliana* (Zechmann et al., 2011). IAA plays an important role in pollen tube growth of *Torenia fournieri* (Wu et al., 2008b). Gibberellin and ethylene appear to act at the early and late pollen tube stages, respectively, while auxin appears to act at the germination stage. Abscisic acid (ABA), ethrel (ETH) and indole-3-acetic acid (IAA) increased pollen germination while ABA and ETH enhanced pollen tube elongation in *Arachis hypogea*. Thus, pollen treated with IAA, GA3 and ETH caused maximum elongation of pollen tubes (Malik et al., 1976).

The asynchronous flowering among genotype cultivars frequently requires pollen storage for the genetic improvement of plant species. Short-term pollen storage is necessary for late and early flowering genotype hybridization because pollen quality quickly degrades at moderate temperatures and humidity (Towil, 2010). Cryopreserved pollen can be found in pollen banks, where it is simple and quick to obtain pollen for any purpose (Towill & Walters, 2000). For seed orchards and improvement programs, pollen banks have been established in germplasm systems (Walters & Pence, 2021). For studies on the basic physiology, biochemistry, and fertility, as well as for biotechnology projects involving gene expression, transformation, and *in vitro* fertilization, to facilitate crossings in breeding programmes, transport and exchange germplasm among locations, and protect nuclear DNA of germplasm pollen is kept in storage (Mondo et al., 2020). For germplasm banks, pollen preservation is a supplement to seed or clone preservation (Towill & Walters, 2000). Four storage temperatures (4°C, -4°C, -20°C, and -76°C) were used to keep herbaceous peony pollen for more than a year. It was observed that pollen stored at 4 °C could be used for hand-pollination during the asynchronized flowering season (Du et al., 2019). Before storage, a certain amount of drying must be done since pollen with high initial water content cannot be successfully preserved at extremely low temperatures (Barnabas & Rajki, 1976).

According to Sedgley & Harbard, (1993), pollen of *Acacia auriculiformis, Acacia iteaphylla, Acacia karroo* and *Acacia mangium* was stored at 25, 5, -18, and -196°C for up to 3 years, and concluded that pollen should be vacuum dry before storing and pollen should begin storing from -18°C.

Chapter 3

Material and Methods

3.1. Geography and the forest

Mizoram has global significance because it is located in northeast India, an important portion of the Indo-Myanmar biodiversity hotspot, and home to a variety of flora and fauna, as well as some of the world's biologically richest regions. However, it simultaneously faces various threats, mainly anthropogenic-induced. Hence the study site is of prime importance for its rich natural heritage with conservation value. Mizoram, the 23rd state of India, with 11 districts, is situated between bordering countries like Myanmar in the east and Bangladesh in the west and touches it boundaries with neighboring states Assam and Manipur in the north and Tripura in the north-west. It is located at latitudes of 21°58′ and 23°35′N and 92°15′ and 93°29′E (Lalrinchhana et al., 2015). An altitude ranges between 21 and 2157 meters above mean sea level and receives rainfall between 2000 and 3200 millimeters yearly. The Tropic of Cancer passes across the center of Mizoram. Mizoram's topography consists of high hills and narrow gorges with six parallel hill ranges surrounded by deep river valleys, with a total geographical area of about 21081 km2. The climate of Mizoram is pleasant throughout the year. Temperature is mild during the summer, from 14.0°C to 30°C while in winter not too cold temperature varies from 6°C to 21°C. Soil is acidic in nature with rich in organic matter; soil texture is mainly sandy loam to loam. According to the 2015 India State of Forest Report, Mizoram has a total forest area covering about 18,748 km^2 or 88.93% of the state's total geographical land area, i.e., 21,081 km^2. Jhum cultivation or Shifting cultivation and home gardening are the major traditional agricultural practices for generating livelihood in Mizoram. The state is covered with 21 significant hill ranges or peaks. Phawngpui is the highest mountain rising 2,065 meters above sea level. Furthermore, there is a number of rivers flowing toward the north and south portion of the state. The rivers which flow towards the north portion of Mizoram fall into Barak river Assam which includes Tuirial (Sonai), Tlawng (Dhaleswari), and Tuivawl. And the rivers which drain into the south portion of Mizoram are Chhimtuipui (Kolodyne),

Mat, Tuichang, Tiau and Tuipui. Based on height, rainfall, and composition of the dominant species, Gogoi et al., (2022) has reported 6 forest type of Mizoram, i.e. (a)Tropical Wet Evergreen Forest (below 900 a.m.s.l.) (b) Montane sub-tropical Forest (between 900-1500 a.m.s.l.) (c)Temperate Forests (above 1600 a.m.s.l.) (d) Bamboo Forests (above and below 1600 a.m.s.l.) (e) Quercus Forests (f) Jhum land.

3.2. Study Sites

The study was conducted during the flowering seasons of *C. coolebrookianum, C. infortunatum,* and *C. serratum* in two successive years from 2018 to 2021 in a tropical moist natural forest of district Aizawl, Mizoram. The following study sites were selected for the study. Sairang: The study site is located at 23°48′18.53″N latitude, 92°37′56″E longitude, with an altitude of 179.832 m above sea level. Tanhril is situated in around forest area at 23°44′15″N and 23°43′37″ N latitude and 92°39′44″ E and 92°40′ 23″ E longitude and altitude range from 330 to 880 m above mean sea level. Hlimen: The study site is located at 23°40′29″N latitude, 92°43′17″ E longitude, with an altitude of 1140 m above sea level. Durtlang is situated at 23°46′27″ N latitude, and 92°43′47″ E longitude at an altitude of 1161 m above mean sea level. Aizawl, Mizoram, experiences a humid and tropical climate with long, hot summers and mild, dry winters with little to no precipitation. The yearly rainfall averages 2162 mm, and the average annual temperature is around 24.9° C. The year-round average temperature is mild, ranging from 10° C to 36° C, and does not vary considerably. Low-elevation site at Sairang forest are rich in bamboo species and following major tree species *Schima wallichii*, *Mallotus* sp. *Albizzia chinensis*, *Alstonia scholaris*, *Callicarpa arborea*, *Toona ciliata*, *Cassia* sp., *Oroxylum indicum, Duabanga grandiflora*, *Dalbergia pinnata*, *Sterculia villosa*, *Terminalia bellerica*. In mid-elevation site, Tanhril, the major forest trees found are *Schima wallichii*, *Callicarpa arborea*, *Albizzia chinensis*, *Anogeissus acuminata*, *Albizzia procera*, *Castonopsis tribuloides*, *Sterculia villosa, Rhus semilata*, *Mallotus* sp., *Macaranga* sp., *Sepium* sp., *Dillenia* sp., *Duabanga grandiflora*, *Neolamarckia cadamba* , *Ficus* sp. Common tree species found at high elevation site of Hlimen are

Castonopsis tribuloides, *Schima wallichii*, *Sterculia villosa*, *Syzygium* sp. *Litsea* sp. *Lannea coromandelica*, *Phyllanthus emblica*.

Table 3.1 Study site description of three *Clerodendrum* species:

Geographic variables /Plant Species	Study Sites			
	Sairang	Tanhril	Durtlang	Hlimen
Latitude	23°48′18.53″N	23° 44′15″N	23°46 ′ 27″N	23°40′29″N
Longitude	92°37′56″E	92°39′44″ E	92°43′47″E	92°43′17″E
Altitude	179.832 m	748 m	1161 m	1140 m
C. colebrookianum	+	+	+	_
C. infortunatum	+	+	_	_
C. serratum	_	_	_	+

+ indicates presence of plant species in the site

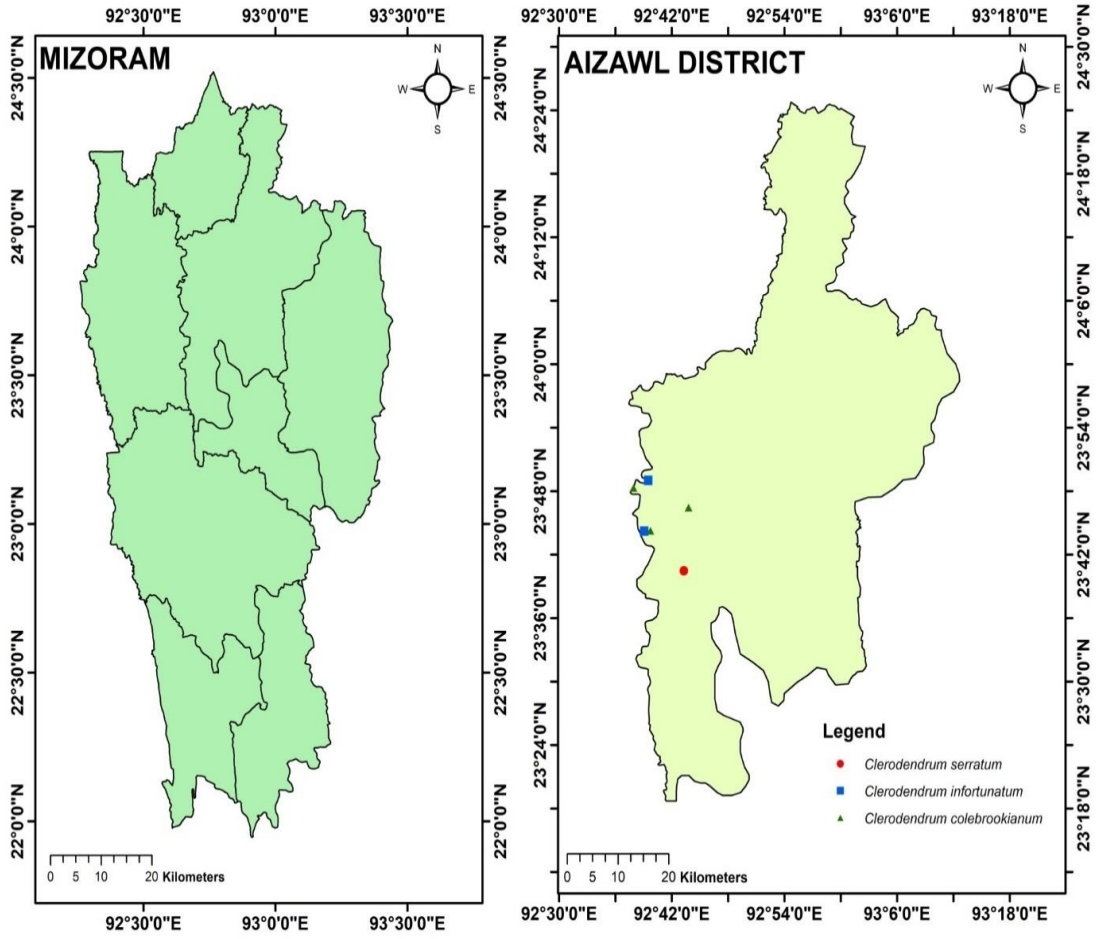


Figure 3.1 Map of Mizoram and Study sites

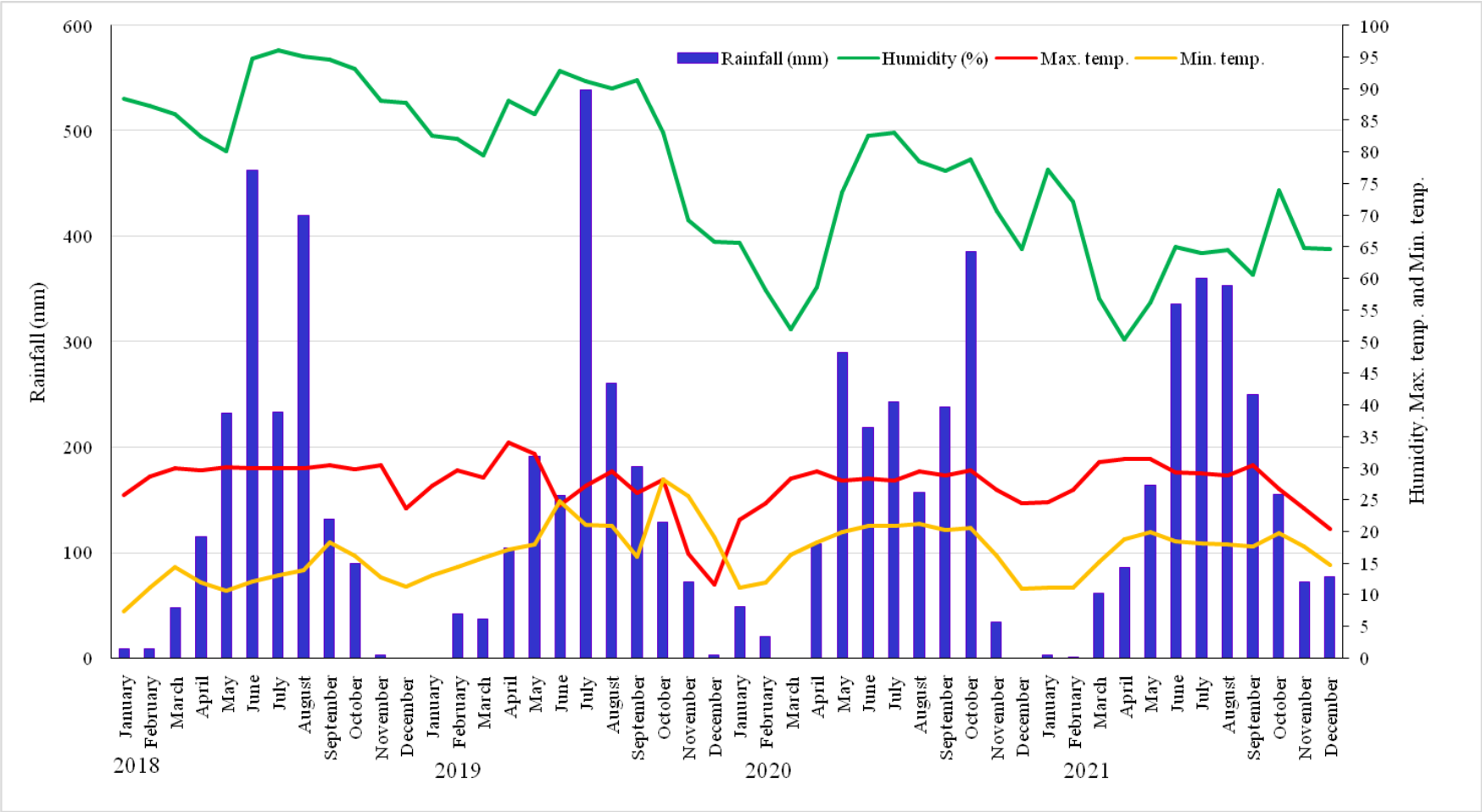


Figure 3.2 Distribution of mean monthly rainfall, maximum temperature, minumum temperature and humidity of study area (Source: DST, Aizawl, Mizoram)

3.3. Study Species

3.3.1. *Clerodendrum glandulosum* Lindl.

Synonym: *Clerodendrum colebrookianum* Walp.

Order: Lamiales

Family: Lamiaceae

Genus: *Clerodendrum*

Habit: Perennial Shrub

Common Name: East Indian glory bower

Local Name: "Phuihnam"

C. colebrookianum with white tubular flower. The flowering and fruiting phase is observed during the month between July – and November; the species is reported to be a vulnerable shrub (Gogoi& Nath, 2021), and the plant species are used as medicine by many local people of northeast India (Yadav, 2012).

In Mizoram *C. colebrookianum*, locally known as 'Phuihnam,' is traditionally used to control hypertension, blood pressure, and intestinal tapeworm infections (Temjenmongla & Yadav, 2005). Leaves and roots are used by tribes of Manipur for skin diseases, cough, and dysentery (Singh& Singh, 2006). Experimentally *C. colebrookianum* has been evaluated for antihypertensive activity (Lokesh & Amitsankar, 2012), antioxidant and hypolipidemic effect (Devi& Sharma, 2004), and anthelmintic activity (Yadav, 2012). The compound of pharmacological importance isolated is colebrosideA (1), colebrin A and B, C-29 sterols, and clerosterol (Yang et al., 2000).

3.3.2. *Rotheca serrata* (L.)

Synonym: ***Clerodendrum serratum* L.**

Order: Lamiales

Family: Lamiaceae

Genus: *Rotheca*

Habit: Perennial Shrub

Common Name: Blue Fountain Bush "Bharangi"

Local Name: "Phuihnamsuak"

Rotheca serrata (L.) (Synonym: ***Clerodendrum serratum* (L.)** flowered and fruited during March-July, and the plant species are found to be found in tropical regions of the world. The Chhattisgarh Medicinal Plant Board has recorded *C. serratum* L. as threatened species (Upadhyay &Koche, 2015). The plant's ethnomedical significance has been documented in numerous traditional medical systems, and Its parts, such as roots and leaves, are used to treat various human ailments across geographical regions. Possess antioxidant, antibacterial, and antifungal mainly found in the roots parts of *C. serratum* (Singh et al., 2012). The root of *C. serratum* contains sapogenins, D-mannitol, and stigmasterol, while the leaves contain high amount of flavonoids and phenolic acids (Apana *et al.* 2021).

3.3.3. *Clerodendrum infortunatum* (L.)

Synonyms: *Clerodendrum viscosum* Vent.

Order: Lamiales

Family: Lamiaceae

Genus: *Clerodendrum*

Habit: Perennial Shrub

Common Name: Hill glory bower

Local Name: "Phui-hnam-chia"

Clerodendrum infortunatum is a gregarious shrub that flowered from March to April in the present study site. Locally leaves are tonic and used in Malaria, scorpion stings, and snake bites. The roots are boiled, and water is used for a bath in case of scabies and other skin diseases. The plant is useful in relieving thirst and burning sensation, foul orders, and blood diseases (Khatry et al., 2005). The plant's root is recommended for treating tumours, several skin conditions, and scorpion bites. (Bhattacharjee et al., 2011). Preliminary chemical research revealed that the leaves of *C. infortunatum* contain saponin, clerodin (a bitter diterpene) 4, 6, and a few enzymes. In addition, leaves have a fixed oil that is made up of glycerides of linoleic, oleic, stearic, and lignoceric acids. From the root sources, luperol and beta-sitosterol.

3.4. Methodology

3.4.1. Floral Development, anthesis pattern and duration of flowering

Floral morphology and structure were studied on twenty inflorescence units, each from five different individuals in each selected plant species. The complete developmental behavior of the flowers, from floral bud initiation up to the mature stage, was judged on randomly selected flower samples (n=50) in each plant species. The observations on the anthesis and time of anther dehiscence were recorded in the field conditions during the flowering seasons. For this purpose, flowers of different individuals (n=10) were labeled, and their anthers were examined at half-hour intervals, each time scoring the dehisced anthers and recording the prevailing weather conditions naturally. For determining the start of the flowering season, 20 fresh flowers per plant on a particular day were kept as standard. To track the length of the flowering season, the initial and last flowering dates were noted. When half of the plant's flowers were opened, the peak flowering time was observed.

3.4.2. Flower and Pollen Production

Ten chosen individuals in each studied plant species were used to estimate flowers and pollen production at their respective study locations. The Three *Clerodendrum* species' height ranges from 215 to 265 cm; thus, the total number of flowers in each branch and sub-branch is counted manually in selected

randomly chosen plants. From each selected ten plants, 25 flowers were collected from each individual for stamen number counting. The floral characteristics, such as the number of sepals, petals, flower diameter (the length and width of the flower were measured using a digital caliper), style length, and ovule counts, were also counted from the collected flower. The ovule number was directly obtained from the dissection of ovaries under a stereoscopic microscope.

The pollen productivity was computed as per Molina et al. (1996). The total quantity of pollen grains per anther was counted from ten randomly chosen fresh flower buds in each individual to estimate the production of pollen grains per flower and per plant. The number of pollen grains was estimated using four anthers from each flower, a total of 40 anthers per plant (10 x 4 = 40). Pollen grains per anther were calculated using the average number of pollen grains from 40 anthers.

From each flower, one anther was taken in a glass slide and mixed with safranin stain; the pinkish colour of the safranin stain is absorbed by the pollen and is easily distinguished and visible under a stereoscopic microscope. The anther is a little crushed using a needle, and the outer cover of the anther is removed using pointed forceps then, it is mixed thoroughly and spread uniformly in the glass slide. Later the glass slide is left for drying; after the glass slide is fully dry, then is mounted with DPX and left for drying. Equal size of the small square box was drawn on the back of the glass slide, then using a stereoscopic microscope, each small square box was observed, and the numbers of pollens in each box were counted visually with a microscope. The average number of pollen per anther multiplied by the average number of stamens per flower, further multiplied by the total number of flowers produced per plant, allowed determining the pollen grain production per plant. Fruit production was calculated by counting every single fruit set in each branch and sub-branch in a random selection of trees. The pollen-ovule ratio was calculated by dividing the expected amount of pollen grains per flower by the projected number of ovules per flower Cruden, (1977).

3.4.3. Diurnal rhythms of pollen concentrations

The diurnal rhythms of pollen concentrations in the ambient air or the occurrence of pollens in the atmosphere were observed by taking pollen air samples at different time intervals between 0500 to 1700 hours of the day for several days on jelly-coated microscopic slides (artificial trappers) as well as on the stigmatic surface of female flowers in relation to prevailing weather conditions. The emasculated flower with stigma was covered with very mesh nylon cloth, and the cover was opened for a particular interval of time; further, the stigma was cut from the flower and stained with safranin, and observed under a microscope for pollen deposition. The observation on the population was carried out from morning 0500 h to evening 1700 h; observation on each block was carried out at a time interval of 2 h, i.e., between 0500 – 0700, 0700 – 0900, 0900 – 1100, 1100 – 1300, 1300 – 1500, 1500 – 1700. The sample glass slide was then observed on the microscope to identify the pollen collected on the slide and count the number of pollen grains present on the 1 cm^2 area of a glass slide. Prevailing weather conditions such as temperature and humidity were recorded.

3.4.4. Pollen Viability

The flower samples were taken in the morning (6-9 am) during anthesis from five individuals growing 100 meters apart from one another for experimentation. To test the pollen viability, freshly opened flowers and unopened flowers (just before anthesis) were selected. 0.5 percent 2, 3, 5-triphenyl tetrazolium chloride (TTC) was prepared in the sucrose solution to determine the pollen's vitality. A few pollen grains were dispersed in the TTC (0.5%) solution and protected from light with cover galas. For 60 minutes, the prepared slides were incubated in dark rooms. The preparation was examined under a light microscope (5 X and 10 X) after incubation; pollen grains dyed red were counted as viable. (Shivanna & Rangaswamy, 2012).

3.4.5. Assessment of pollen longevity

Fresh pollen grains were kept at three different temperatures, 6°C, - 4°C and -20°C, in an airtight vial. The vitality of the stored pollen grains was routinely assessed with 0.5% TTC every 24 hours for another seven days; viability was then examined with a

light microscope every week, at intervals of 14, 21 and 28 days, until the pollen grains were determined to be viable.

3.4.6. Pollen Germination

Studies on *in vitro* pollen germination used the basal media developed by Brewbaker & Kwack (1963). The impact of sucrose compared to the control (distilled water) was examined using 5% and 10% sucrose concentrations. Growth regulators such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), gibberellic acid (GA 3) and kinetin were added to the basal medium at concentrations of 100, 200, and 300 ppm were used to test their effects on *in vitro* pollen germination.

Five replications were blocked in time using a randomized complete block design for the experiment (Tuinstra & Wedel, 2000). A needle was used to transfer pollen grains from fresh anthers to germination material on cavity slides. The cavity slides were set up in a room environment and using a thermo-hygrometer, the average temperature (26.35°F/0.98°C) and average humidity (79.58°F/3.07°C) were recorded. After 24, 48, and 72 hours of incubation, cavity slides were examined under a light microscope. When the length of the pollen tube was more than or equal to the diameter of the pollen grain, the pollen grain was regarded to have germinated (Tuinstra & Wedel, 2000). Ten microscopic views were taken to evaluate the pollen grains in each germination cavity slide. The total number of germinated and non-germinated pollen grains was counted in each view and expressed as a percentage of *in vitro* pollen germination. The statistical method, with the help of Excel 2016, the impact of hormones and their concentrations, sucrose concentrations, time, and plant species were evaluated for *in vitro* pollen germination.

3.4.7. Stigma receptivity, mating system and fruit set

Using stigmatic surfaces to trap the pollen concentration was made to decipher the duration of pollination, time of the day best suited for pollination, and stigma receptivity. This was done by bagging hundreds of flowers (please specify exact number) at the initial stage of development (before anther dehiscence) and was exposed to wind in the batches for desired intervals, which then was removed from

the source plant, and a number of pollen grains deposited on them were counted as per Ornduff, (1975).

The following treatments were used for evaluating the mating system (Modified from Dafni, 1992 and Boulter et al., 2006): (i) natural pollination, in which flowers were not modified; (ii) Spontaneous selfing, in which buds were bagged throughout their flowering period; (iii) Induced selfing, in which flowers anther was emasculated, bagged and hand pollinated with their own pollen; (iv) Geitonogamy, the pollen from the same tree is manually applied after the flower anthers have been emasculated and bagged, (v) Cross – artificial, in which flower buds which were bagged and pollinated with pollen from another plant; and (vi) Cross – natural, in which flower buds are removed and stigma are left open on air. Additionally, fruit setting between spontaneous selfing, induced selfing, geitonogamy, cross-artificial, and cross-natural was compared. The estimated selfing rate was determined in accordance with Charlesworth & Charlesworth, (1987).

3.4.8. Pollinators availability

During the peak flowering period, the frequency of pollinators and their visits were determined on ten randomly selected plant species. The study was conducted for 15 days during the peak flowering season after every alternate day in each site after the first bloom of the flower on the ten selected plants. A data sheet was prepared to collect the data according to its requirement. In the data set, the number of visits and the flowers that were visited, the frequency of visits, contact with the reproductive sections, and interactions with other visitors was all recorded.

The observation on the population was carried out from morning 0500 h to evening 1700 h; observation on each block was carried out at a time interval of 2 h, i.e., between 0500 – 0700, 0700 – 0900, 0900 – 1100, 1100 – 1300, 1300 – 1500, 1500 – 1700. Pollinator frequency was measured in terms of visits/inflorescence/hour. Butterflies, moths, bees, and bugs are the main pollinator recorded during the field observation. Photographs of pollinators were taken during the field observation using a high-resolution camera, and the image where identified using Butterfly of India (Antram, 1924); The book of Indian butterflies (Kehimkar, 2008); The dictionary of

butterflies and moths in colour (Laithwaite et al., 1975), Butterflies and Moths of Pakke Tiger Reserve (Sondhi & Kunte, 2014); Butterflies and Moths of Pakke Tiger Reserve. Second Edition (Sondhi & Kunte, 2018) and Butterflies of the Garo Hills (Sondhiet al., 2013).

3.4.9. Statistical Analysis

MS Excel 2019 and SPSS statistics 25 were used for statistical analysis to estimate mean, standard deviation, and standard error from the data collected from various observations made during field surveys and laboratory observations. Pearson correlation was used to determine the association between floral characteristics, temperature and humidity, as well as pollinators and temperature and humidity. ANOVA for the effect of year and population on the number of flowers, anthers, and pollen grains per plant and fruit set; plant species on the number of flowers, anthers, and pollen grains per plant and fruit set; distill water and sucrose 5% and 10%, concentration and time; and hormones, concentrations, time and species.

Chapter 4

Results

4.1.1. Floral biology: anthesis, anther dehiscence, stigma receptivity in *C. colebrookianum*

C. colebrookianum is a perennial deciduous gregarious erect shrub. The average height of the plant is 265.8±18.42 cm, and the average girth of the plant is up to 17±1.49 cm. The stem and branches are tender. The leaves are simple and opposite to each other. The leaves are large, soft, heart shape, and shiny in appearance with an unpleasant odour when crushed. The flowering begins in July and continues till December, while the peak flowering occurs between September and October. Flowers are white and borne in 4-6 branched corymbose cymes. The flowers are medium, 49.36 ± 0.14 mm long and 19.21 ± 0.16 mm wide, and bisexual. The Calyx is green and polysepalous, consisting of 5 sepals, 3.66 ± 0.04 mm long and 1.69 ± 0.04 mm wide. Corolla is white, with a floral tube (27.94 ± 0.11 mm), 5 lobed at the tip; each lobe is 8.11 ± 0.14 mm long and 2.88 ± 0.03 mm wide. The floral tube has deep-seated nectar (0.5-1 µl). The stamens are 4, epipetalous, 24.88 ± 0.17 mm long, and protrude from the flower. The anthers are purple-coloured, oblong, and 2.31 ± 0.01 mm long. The ovary is globose; the ovule is bi-locular due to the formation of a false septum; it is characteristically a 4-ovule. The ovules are erect, anatropous, and arranged on axile placentation. The style is white, 29.99 ± 0.03 mm, and ends with a simple bifid (two stigmatic lobes) slightly yellowish coloured stigma.

During the study period, it was observed that the time of the flowering phenological phase showed annual variability from one year to the following year, i.e., 2018 and 2019, and also from one population to another in the present study sites. The date of floral bud initiation of the selected study individual plants in *C. colebrookianum* starts in the year 2018 was 9th July, 11th July, and 5th July but in the year 2019, it began on 26th July, 29th July, and 25th July at study sites Tanhril, Durtlang, and Sairang respectively. Whereas in the population, the date of floral bud initiation starts in the year 2018 was 5th July, 6th July, and 2nd July and in the year 2019, it began on 23rd July, 25th July, and 22nd July at Tanhril, Durtlang, and Sairang respectively. The flowering duration of the sample plant during 2018 was

156 d, 154 d, and 160, and in the year 2019, the duration of flowering was 139 d, 136 d, and 138 d at study sites, Tanhril, Durtlang, and Sairang respectively. Whereas at the population, the duration of the flowering in the year 2018 was 167 d, 164 d, and 166 d; in the year 2019, the duration of a flower was 154 d, 144 d, and 147 d at Tanhril, Durtlang, and Sairang respectively. After the floral bud initiation, the anthesis starts on an average of 10-12 days.

The opening of the flower i.e. anthesis started in the morning at 0500 h and between 0700 and 0900 of the day, the anthesis of the flower reached its maximum level (Fig. 4.1 & 4.2). During the beginning of anthesis, the stamen remains coiled, and when the flower is fully opened, the stamen stands erect (Fig. 4.3 & 4.4). A distinct physical separation between stamen and pistil was observed during anthesis (Fig. 4.4). The pistil length is very short and confined to the base of the flower on the first day of anthesis. At noon time, the relative humidity decreases due to an increase in temperature between 1100 and 1300; there is also a decrease in the anthesis of the flower, and it reaches its minimum level. Again, as the day proceeded, when it reached the afternoon, i.e., the time between 1500 and 1700, the slight increase in relative humidity and the temperature decreased. To complete one full flower opening took 44 minutes. There was no opening of flowers during evening and night-time. The correlation analysis also verified this trend as there is a negative (r = -0.538; p = 0.271) and positive (r = 0.537; p = 0.271) relationship of anthesis with temperature and humidity, respectively (Table 4.1).

Anther dehiscence started in the morning hours and coincided with anthesis, and peak pollen release was observed during 0700-0900 hours and continued till 1300-1500; a distinct powdery white appearance of anthers during peak (Fig. 4.2 & 4.5). Pollen was released from anthers through a longitudinal slit and after complete anther, dehiscence stamen bends downwards (Fig. 4.5). There is very weak positive (r = 0.003; p = 0.995) and negative (r = -0.079; p = 0.882) relationship of anther dehiscence with temperature and humidity, respectively (Table 4.1).

The receptivity of the stigma was recorded after 3-4 days from anthesis, it was observed that the length of the stigma becomes longer when it is receptive and the

bifid pistil splits into acute V shape stigmatic lobes (Fig. 4.4 & 4.6). Maximum receptivity of the stigma was recorded between 0900-1100 hours of the day (Fig. 4.2). It was observed that the receptivity lasted only for one day the tip of the stigma colour turns yellowish to brown colour (Fig. 4.6). There is positive (r = 0.487; p = 0.328) and negative (r = -0.533; p = 0.277) relationship of receptivity with temperature and humidity, respectively (Table 4.1).

Figure 4.1 Flowering and fruiting stages of *C. colebrookianum*

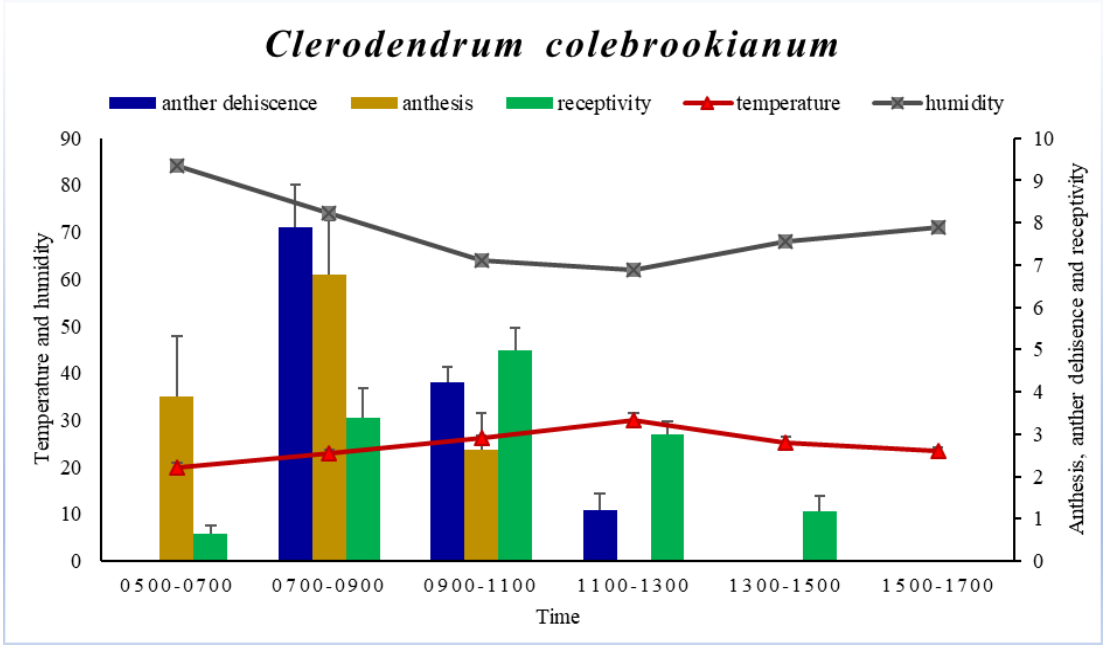


Figure 4.2 Anthesis, anther dehiscence and stigma receptivity in *C. colebrookianum* with respect to site weather conditions

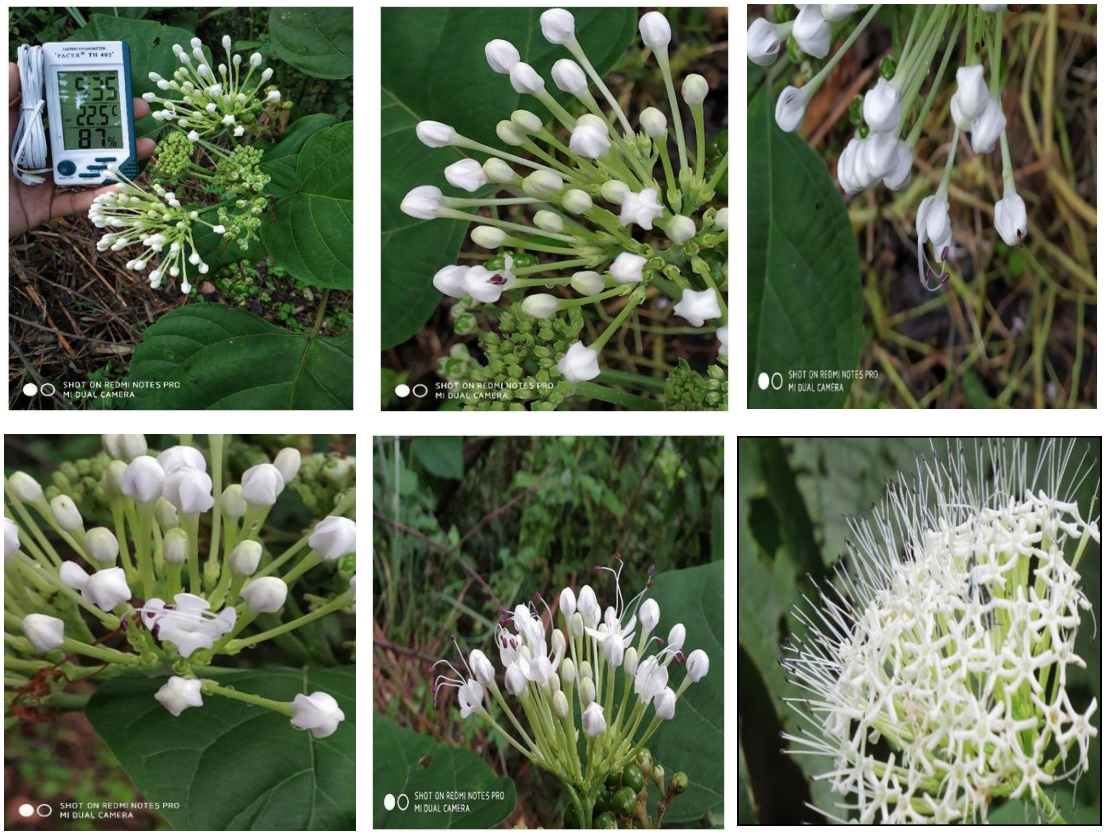


Figure 4.3 Pattern of anthesis in *C. colebrookianum*

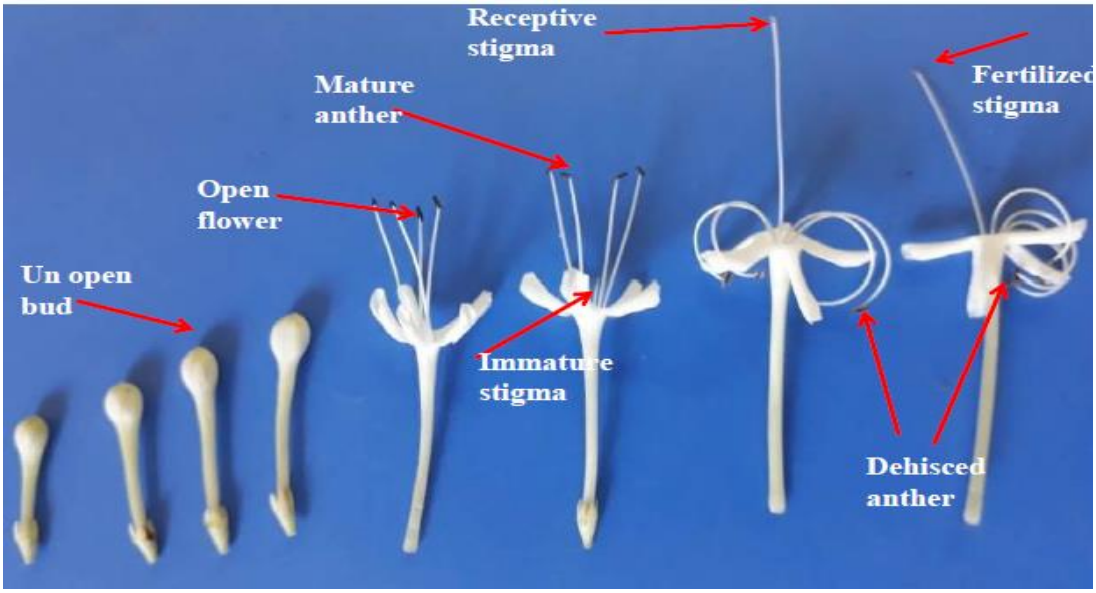


Figure 4.4 Flowering stages of *C. colebrookianum* exhibiting a distinct dichogamy (protandry)

Figure 4.5 Pattern of anther dehiscence (longitudinal slit) in *C. colebrookianum*

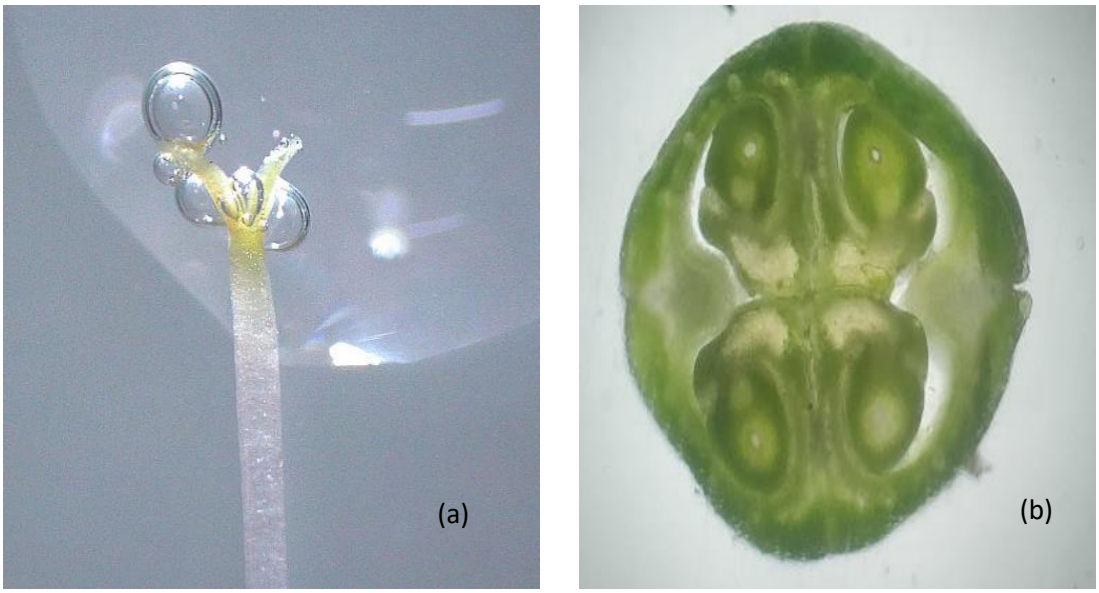


Figure 4.6 (a) Receptive stigma and (b) Transverse section of ovary with four ovules

4.1.2. Floral biology: anthesis, anther dehiscence, stigma receptivity in *C. infortunatum*

C. infortunatum is a perennial gregarious deciduous erect shrub that can reach a height of up to 259.9±9.86 cm and girth of up to 7.88±0.62 cm on average. Leaves are simple and opposite to each other. The leaves are large, elliptical ovate, pubescent, soft, and release an unpleasant odour when crushed. Flowering starts from mid of February and continues till April, and the peak flowering occurs during March. The flower possesses sweet fragrant white with nectar guide pink in colour at the center with peduncle panicle cymes. The flower starts to mature from the base with the shape of a pyramid consisting of 32 to 138 pedicellate flowers.

The flowers are medium 57.96 ± 0.49 mm long and 31.43 ± 0.66 mm wide bisexual and distinct herkogamy and dichogamy (protandry) with deep-seated nectar 3-4 µl at the base of the ovary. The calyx is yellowish green, polysepalous consisting of 5 sepals, 13.83 ± 0.36 mm long, 5.96 ± 0.14 mm wide. Corolla is white, with a floral tube (17.49 ± 0.40 mm), 5 lobed at the tip; each lobe 14.90 ± 0.42 mm long and 6.91 ± 0.17 mm wide. The stamens are 4, epipetalous, 37.28 ± 0.97 mm long, and protrude from the flower. The anthers have purple-coloured, oblong, 2.78 ± 0.04 mm long. The ovules are erect, anatropous, and arranged on axile placentation with 4 ovules. The style is white 43.48 ± 0.36 mm with a bifid stigmatic lobe yellowish coloured at the tip of the stigma (Fig. 10. 11 & 12).

Between 2019 and 2020, the timing of the flowering phenological phase varied from one year to the following year. And also, from one population to another. The date of floral bud initiation of the selected study plants starts in 2019 was on the 25th of February and the 2nd of March, while in the year 2020, it was started on the 3rd of March and the 7th of March at the study sites Tanhril and Sairang, respectively. In contrast, in the population, the date of floral bud initiation began in the year 2019 on the 26th of February and the 4th of March, and in the year 2020, it was started on the 25th of February and the 6th of March at Tanhril and Sairang respectively.

After the floral bud initiation, on an average of 7-9 days, the anthesis starts. The flowering duration of the sample plant in 2019 was 46 days and 39 days, and in the

year 2020, the duration of flowering was 43 days and 35 days at Tanhril, and Sairang, respectively. Whereas in the population, the duration of the flowering in the year 2019 was 48 days and 34 days, and in the year 2020, the duration of the flower were 41 days and 37 days at Tanhril and Sairang, respectively.

Anthesis of the flower begins from morning 0500 h, and between 0700 and 0900 h of the day, the anthesis of the flower reaches its maximum level (Fig. 4.8 & 4.9). Before the anthesis, the stamen and pistil are coiled inside the flower, but when the anthesis starts, the coiled stamen and pistil start to uncoil (Fig. 4.9 & 4.10). The stamen and pistil are physically separated when the flowers are fully opened. Stamens are positioned at the front with a little curve, and the pistil at a back position (Fig. 4.10). At noon time, the relative humidity decreases with an increase in temperature between 1100 and 1300 h. There is also a decrease in the anthesis of the flower, and it reaches its minimum level. Again, as the day proceeds, the relative humidity increases, and the temperature decreases when it reaches the afternoon, i.e., the time between 1500 and 1700. To complete one full flower opening, it took 30-40 minutes. There was no opening of flowers during evening and night time. The correlation analysis also verified this trend as there was a negative (r = -0.658; p = 0.155) and positive (r = 0.350; p = 0.496) relationship of anthesis with temperature and humidity, respectively (Table 4.1).

Anther dehiscence starts in the morning hours and coincides with anthesis, and peak pollen release was observed during 0700-0900 hours and continued till 1300-1500 (Fig. 4.8). A distinct powdery white appearance of anthers during peak dehiscence. Pollen was released from anthers through a longitudinal slit. After complete anther dehiscence stamen bent downwards (Fig. 4.11). There was a negative (r = -0.435; p = 0.389) and negative (r = -0.201; p = 0.703) relationship of anther dehiscence with temperature and humidity, respectively (Table 4.1).

The receptivity of the stigma was recorded on the following day from anthesis. When the bifid pistil split with an acute V shape stigmatic lobe, the stigma became receptive (Fig. 4.12). Maximum receptivity of the stigma was recorded between 0700-1100 hours of the day (Fig. 4.8). It was observed that the receptivity lasted only

for one day the tip of the stigma colour turns yellowish to brown colour (Fig. 4.12). There was weak negative (r = -0.090; p = 0.865) and negative (r = -0.557; p = 0.251) relationship of receptivity with temperature and humidity, respectively (Table 4.1).

Figure 4.7 Flowering and fruiting stages of *C. infortunatum*

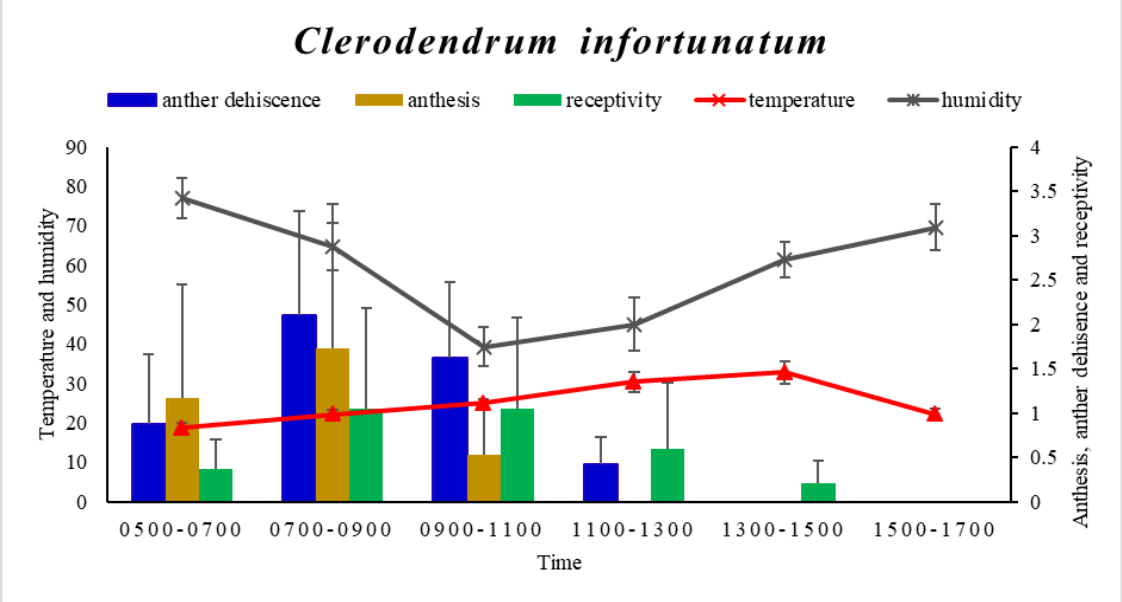


Figure 4.8 Anthesis, anther dehiscence and stigma receptivity in *C. infornatum* with respect to site weather conditions

Figure 4.9 Pattern of anthesis in *C. infortunatum*

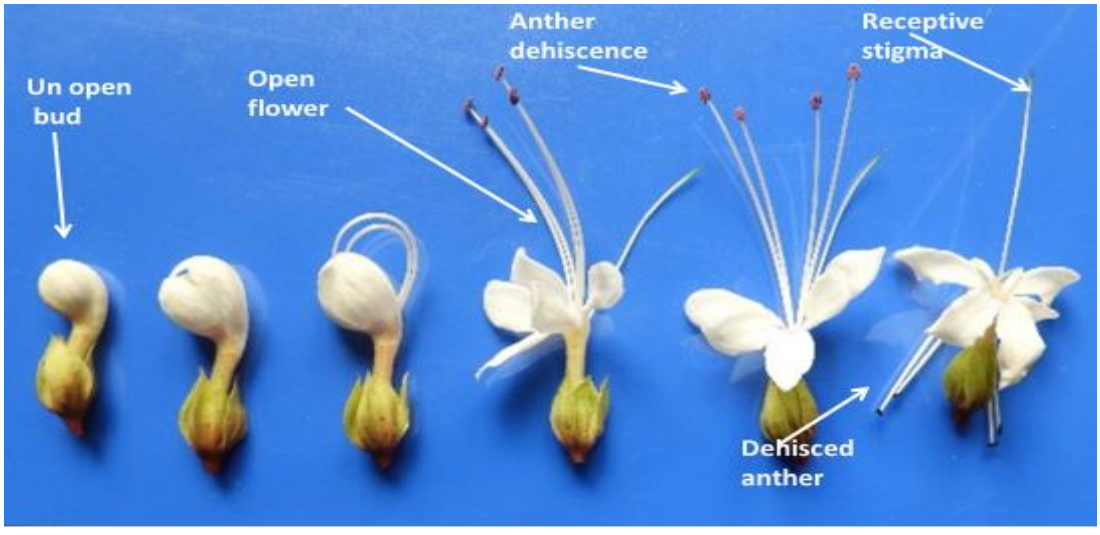


Figure 4.10 Flowering stages of *C. infornatum*, exhibiting a distinct herkogamy & dichogamy (protandry)

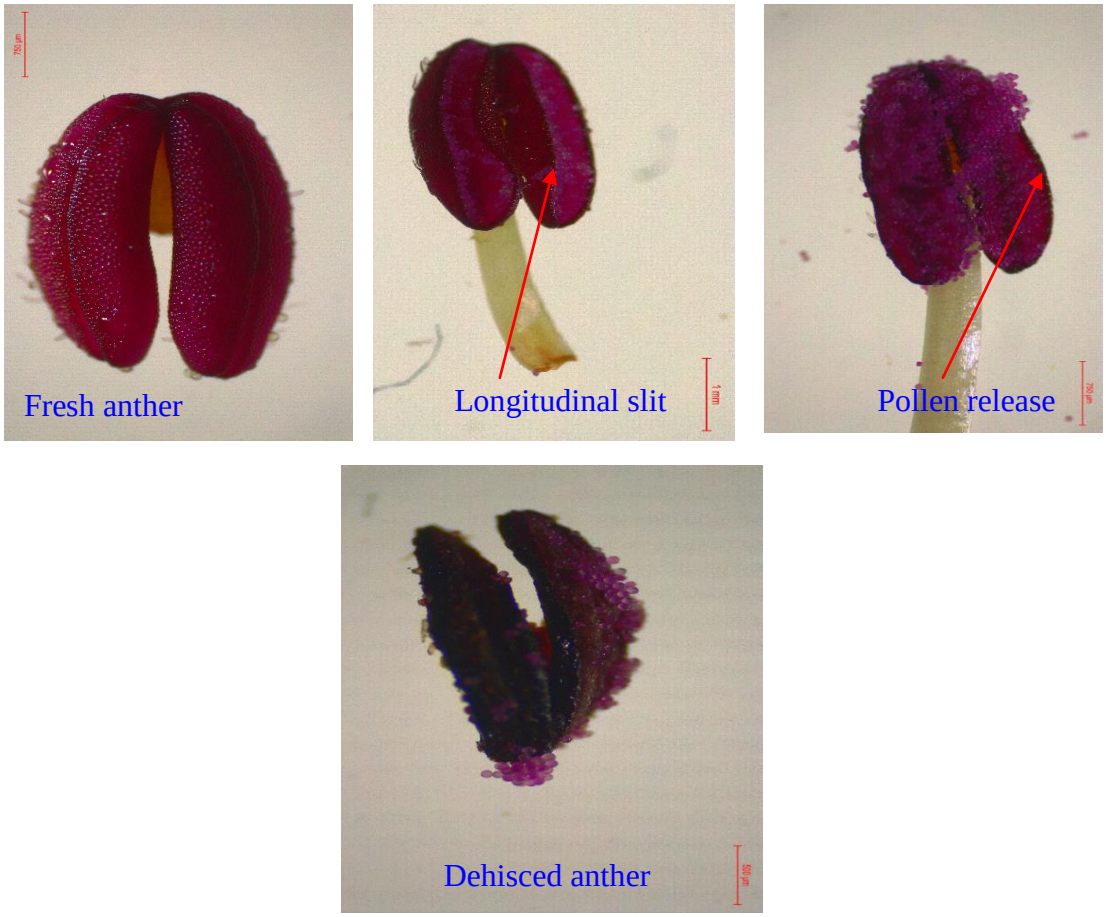


Figure 4.11 Pattern of anther dehiscence (longitudinal slit) in *C. infortunatum*

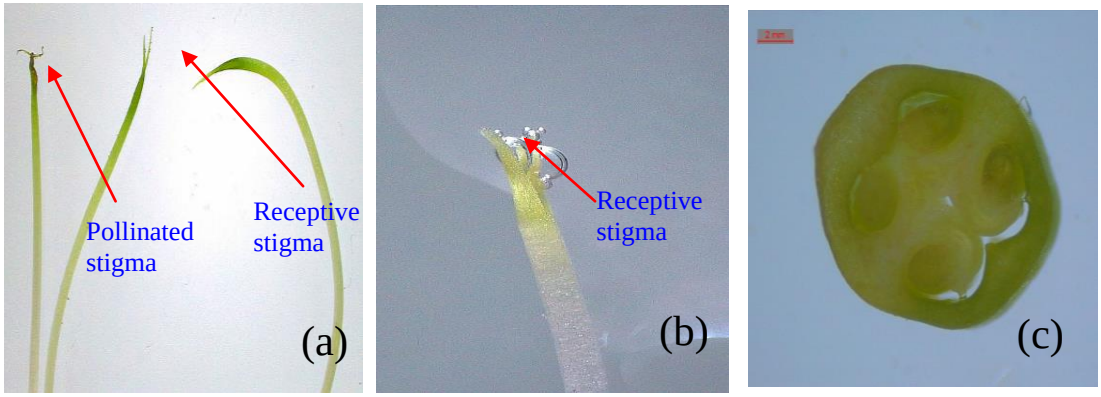


Figure 4.12 (a) Different stages of stigma (b) Receptive stigma and (c) Transverse section of ovary with four ovules

4.1.3. Floral biology: anthesis, anther dehiscence, stigma receptivity in *C. serratum*

Clerodendrum serratum is a perennial deciduous woody shrub with an average height of the plant is up to 215.8±13.76 to 226.7±13.80 cm and an average girth of the plant is up to 1.88±0.12 to 2.29±0.12 cm. The stem and branches are so tendered that they can easily break while pulling. The leaves are simple and opposite to each other. The leaves are oblong and thorny, with an unpleasant odour when crushed. The flowering starts in March and continues till July, while the peak flowering occurs between April and May. Flowers are light purple in colour in compound cymes inflorescence consisting of 27 to 60 flowers.

The flowers are medium, 44.29 ± 0.40 mm long, and 22.43 ± 0.16 mm wide. Bisexual with distinct herkogamy and dichogamy (protandry) with hair around the base of the ovary. Nectar is present in the form of the droplet in very minute quantities on the tip of that hairy structure. The calyx is green, polysepalous consisting of 5 sepals, 5.43 ± 0.05 mm long, 2.89 ± 0.08 mm wide. Corolla is creamy white light purple, with 4 lobed at the tip, each lobe 16.28 ± 0.09 mm long and 6.87 ± 0.10 mm wide, and one lip (nectar guide) with distinct purple color 23.46±0.06 mm long and 3.51± 0.05 mm width. The presence of a lip on the flower is used as a landing platform by insect floral visitors. The stamens 4 epipetalous, purple colour 32.25 ± 0.17 mm long and protrude from the flower. The anthers have purple-coloured, oblong, 2.43 ± 0.05 mm long. The ovules are erect, anatropous, and arranged on axile placentation with 4 ovules. The style is purple colour 37.08± 0.09 mm bifid at the tip of the stigma.

It was noted that between 2019 and 2021, the timing of the flowering phenological phase varied from one year to the next year. The date of floral bud initiation of the sample plant starts in the year 2019 was 15th March, but in the year 2021, it starts on 22nd March, whereas in the population, the date of floral bud initiation begins in the year 2019 was 12th March and in the year 2021 it was started from 19th March. The flowering duration of the sample plant in 2019 was 87 days, and 94 days in the year 2021. Whereas in the population, the duration of the flowering in the year 2019 was

83 days and 89 days in the year 2021. After the floral bud initiation, on an average of 15-18 days, the anthesis starts.

Anthesis of the flower starts from morning 0500 h, and at the time between 0700 and 0900 of the day, the anthesis of the flower reaches its maximum level (Fig. 4.14 & 4.15). Before the anthesis, the stamen and pistil are coiled inside the flower, but when the anthesis start, the coiled stamen and pistil start to be uncoiled (Fig. 4.15 & 4.16). When the flower is open, the stamen and pistil are physically separated; the stamen they are positioned at the front with a little curve, while the pistil is positioned back straight and erect with a little curve at the tip of the stigma (Fig. 4.16). At the noon time, the relative humidity decreases due to increase in temperature between 1100 and 1300 h there is also decreased in the anthesis of the flower, and it reaches its minimum level. Again, as the day proceeds, when it reaches the afternoon, i.e., the time between 1500 and 1700, the relative humidity increases, and the temperature decreases. To complete one full flower opening, it took 50-60 minutes. There was no opening of flowers during nighttime. The correlation analysis also verified this trend as there was a significant negative (r = -0.258; p = 0.621) and positive (r = 0.621; p = 0.188) relationship of anthesis with temperature and humidity, respectively (Table 4.1).

Anther dehiscence started in the morning hours and coincided with anthesis, and peak pollen release was observed during 0900-1100 hours and continued till 1500-1700; a distinct powdery white appearance of anthers during peak (Fig. 4.14). Pollen was released from anthers through a longitudinal slit and after complete anther dehiscence stamen bends downwards (Fig. 4.17). There was a significant positive (r = 0.481; p = 0.334) and negative (r = -0.114; p = 0.830) relationship of anther dehiscence with temperature and humidity, respectively (Table 4.1).

The receptivity of the stigma was recorded on the following day from anthesis; by visual eye receptivity of the stigma can be understood when the bifid pistil curved at U/hook shape the stigma become receptive (Fig. 4.18). Maximum receptivity of the stigma recorded between 0900-1100 hours of the day (Fig. 4.14). It was observed that the receptivity lasts only for one day the tip of the stigma colour turns purple to

brown colour (Fig. 4.18). There was significant positive (r = 0.378; p = 0.460) and positive (r = 0.055; p = 0.918) relationship of receptivity with temperature and humidity, respectively (Table 4.1).

Figure 4.13 Flowering and fruiting stages of *C. serratum*

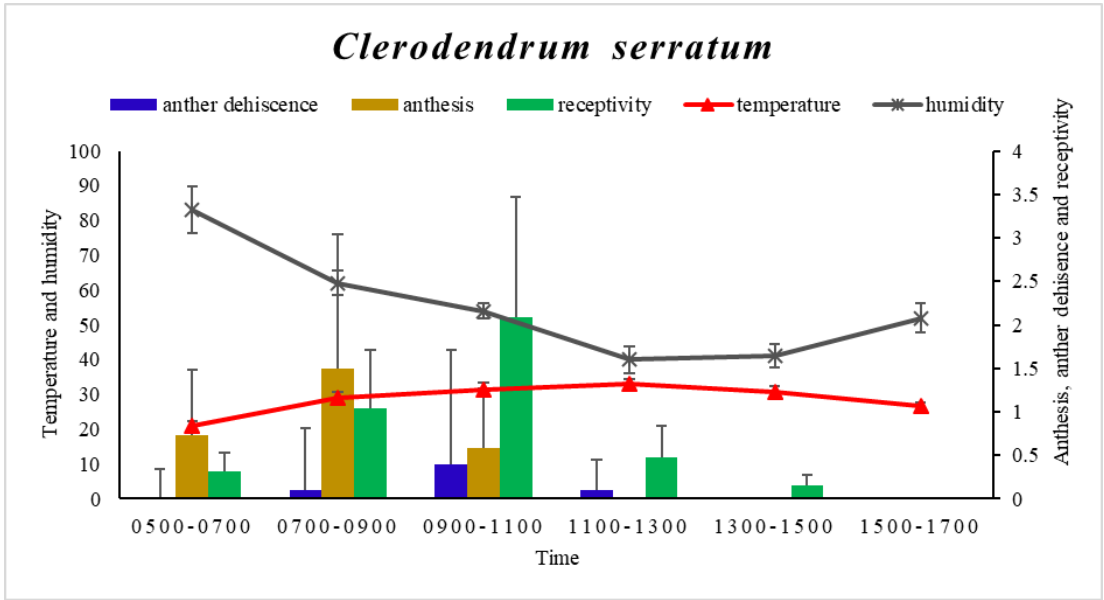


Figure 4.14 Anthesis, anther dehiscence and stigma receptivity in *C. serratum* with respect to site weather conditions

Figure 4.15 Maturation of anther and stigma in *C. serratum*

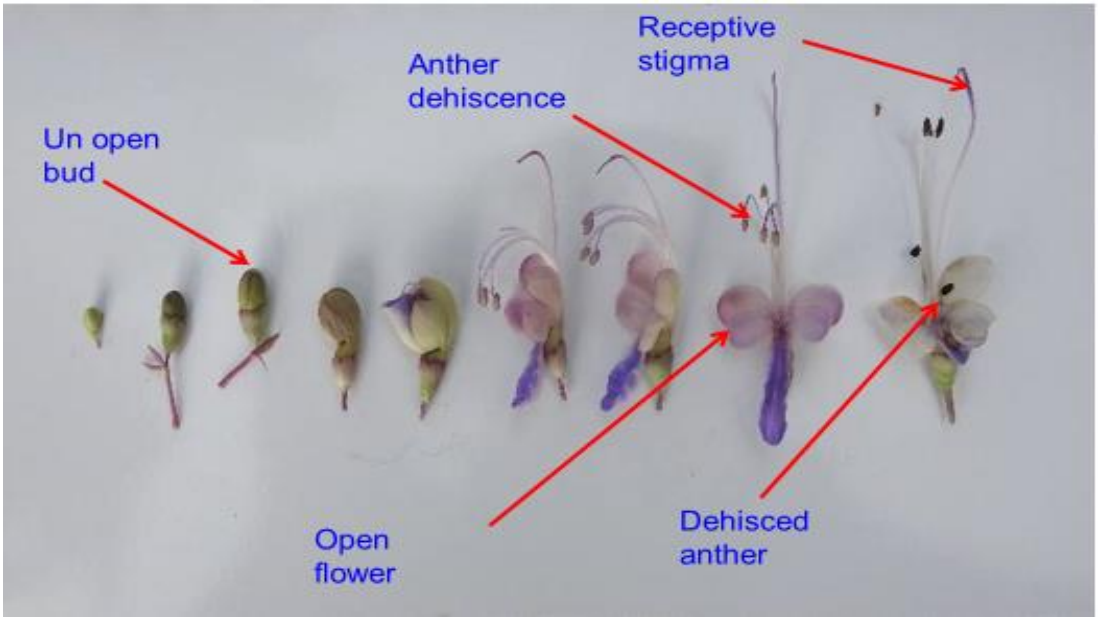


Figure 4.16 Flowering stages of *C. serratum*, exhibiting a distinct herkogamy & dichogamy (protandry)

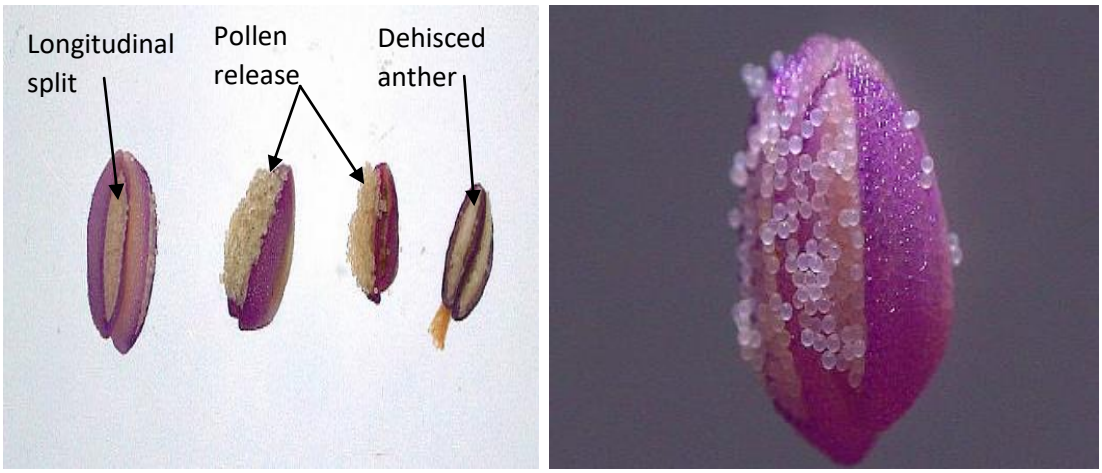


Figure 4.17 Pattern of anther dehiscence (longitudinal slit) in *C. serratum*

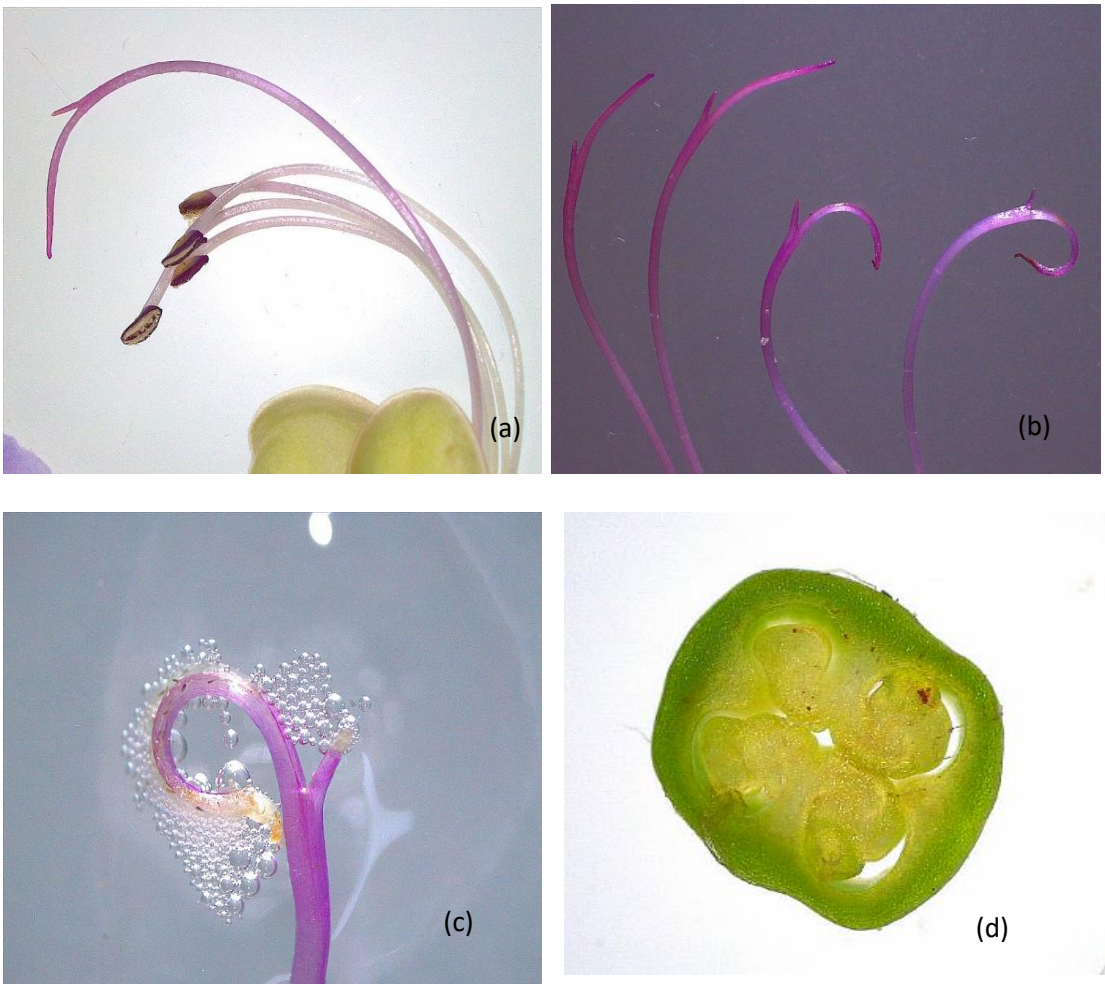


Figure 4.18 (a) A distinct herkogamy and dichogamy (protandry) (b) Different stages of stigma (c) Receptive stigma and (d) Transverse section of ovary with four ovules.

Table 4.1 Correlation coefficient between floral features, temperature and humidity for the three *Clerodendrum* species

Species	Floral feature	Temperature	Humidity
C. colebrookianum	Anthesis	r=-0.538, p=0.271	r=0.537, p= 0.271
C. infortunatum	Anthesis	r=-0.658, p=0.155	r=0.350, p=0.496
C. serratum	Anthesis	r=-0.258, p=0.621	r=0.621, p=0.188
C. colebrookianum	Anther dehiscence	r=0.003, p=0.995	r=-0.078, p=0.882
C. infortunatum	Anther dehiscence	r=-0.434, p=0.389	r=-0.201, p=0.703
C. serratum	Anther dehiscence	r=0.481, p=0.334	r=-0.113, p=0.830
C. colebrookianum	Receptivity	r=0.486, p=0.328	r=-0.532, p=0.277
C. infortunatum	Receptivity	r=-0.090, p=0.869	r=-0.556, p=0.251
C. serratum	Receptivity	r=0.377, p=0.460	r=0.054, p=0.918

4.1.4. Pollinators availability in *C. colebrookianum*.:

Lepidoptera, Hymenoptera, Coleoptera and Hemiptera are the insect orders observed in the *C. colebrookianum* as floral visitors. Twenty-six insect species of the order Lepidoptera have been identified as a floral visitor and has the maximum number of floral pollinators, 4 species of the order Hymenoptera and 1species each from the order Coleoptera and Hemiptera. Butterflies were observed as the most frequently visiting pollinator of *C. colebrookianum* followed by moths and bee species. A total of 16 butterflies (Fig. 4.24) and 10 moths (Fig.4.25) belonging to the order Lepidoptera were observed as floral visitors in the flower of *C. colebrookianum* during the flowering period. Maximum visitation of butterflies was observed between 0800-1500 of the day (Fig. 4.19), and they follow a diurnal pattern, which coincides with anther dehiscence and nectar production. *Papilio polytes, Papilio helenus*, *Delias descombesi*, and *Eurema andersonii* were observed with the highest visitation frequency among the butterflies (Fig 4.19). Moths follow a uniform visitation frequency, and it was observed that they follow a bimodal pattern. Maximum visitation of moths was observed between 0700-1700 of the day (Fig 4.23). No pollinator was observed during the morning hours between 0500-0700 for both the insect groups (butterflies and moths), and fewer butterflies were observed in the evening, i.e. between 1500-1700 but moth pollinators were still observed in evening hours between 1500-1700. *Macroglossum stellatarum*, *Koruthaialos butleri*, and *Matapa aria* were observed as the maximum visitor during this hour (Fig. 4.20). Nectar present in minute quantities is taken as a floral reward by these pollinators.

Three bee pollinators *Amegilla zonata*, *Bombus albopleuralis* and *Trigona species*, were observed as a floral visitor in the flower of *C. colebrookianum* during the flowering period (Fig. 4.21). They follow proclaimed forenoon patterns; maximum visitation was observed during the morning hours between 0700-1100 of the day (Fig. 4.21), coinciding with anthesis, anther dehiscence, and nectar production of the flower. *Amegilla zonata* and *Trigona species* received pollen as a floral reward, and *Bombus albopleuralis* try harvest nectar as a floral reward (Fig. 4.26). Fewer visitations were observed during the afternoon and evening time. Each species of the

order Hemiptera i.e. *Halyomorpha halys* and Order Coleoptera *Charidotella sexpunctata* were observed as a floral visitor in the *C. colebrookianum* (Fig. 4.22).

Temperature and humidity enhance the pollinator activity on flowers. The visitation frequency and activity of butterflies and moths increase with the rise in temperature between 0900-1500 (Fig. 4.23). With the increase in temperature, the visitation frequency of the bee's pollinator increased, and maximum visitation frequency was observed between 0900-1100 but at very high temperatures between 1100-1300, visitation frequencies of the bee's pollinator decreased (Fig. 4.23). In addition, bugs pollinator was observed to increase with an increase in temperature between 1100-1300 of the day (Fig. 4.23). In all the five different groups of pollinators (butterflies, moths, bees, beetle, and bugs) positive (+ve) correlation at (0.05*) was observed (r = 0.879*, 0.903*, 0.297 and 0.877*; p = 0.021, 0.014, 0.567 and 0.022) with temperature and negative (-ve) correlation at (0.05*) was observed (r = -0.794, -0.884*, -0.150 and -0.732; p = 0.061, 0.019, 0.776 and 0.098) with humidity (Table 4.2).

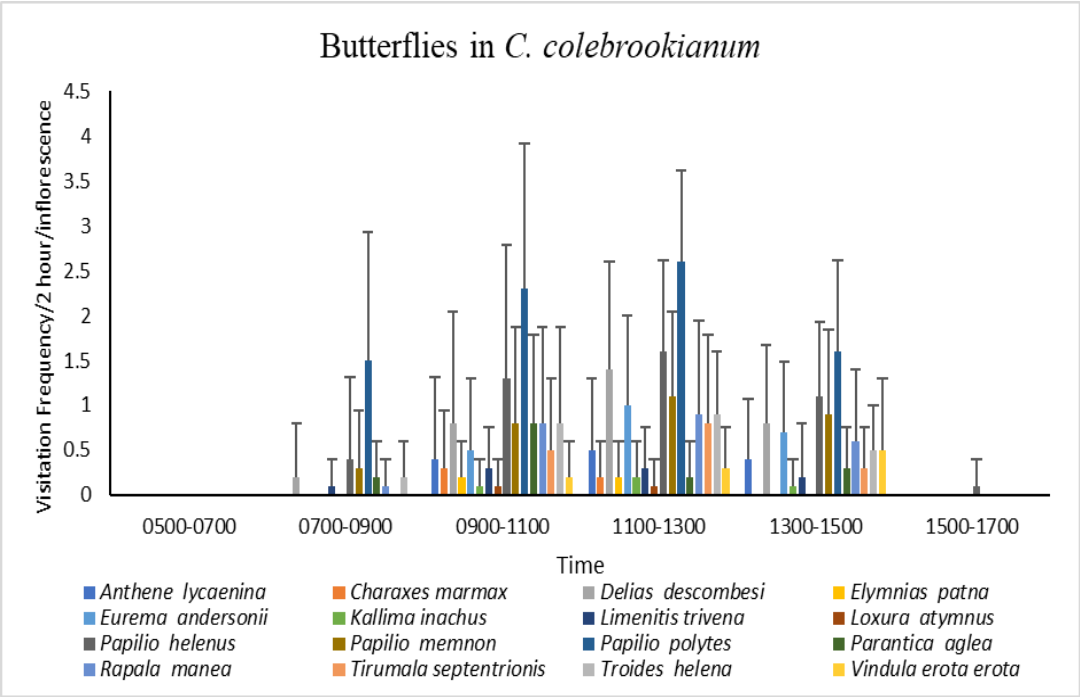


Figure 4.19 Visitation frequency of butterflies' pollinator in *C. colebrookianum*.

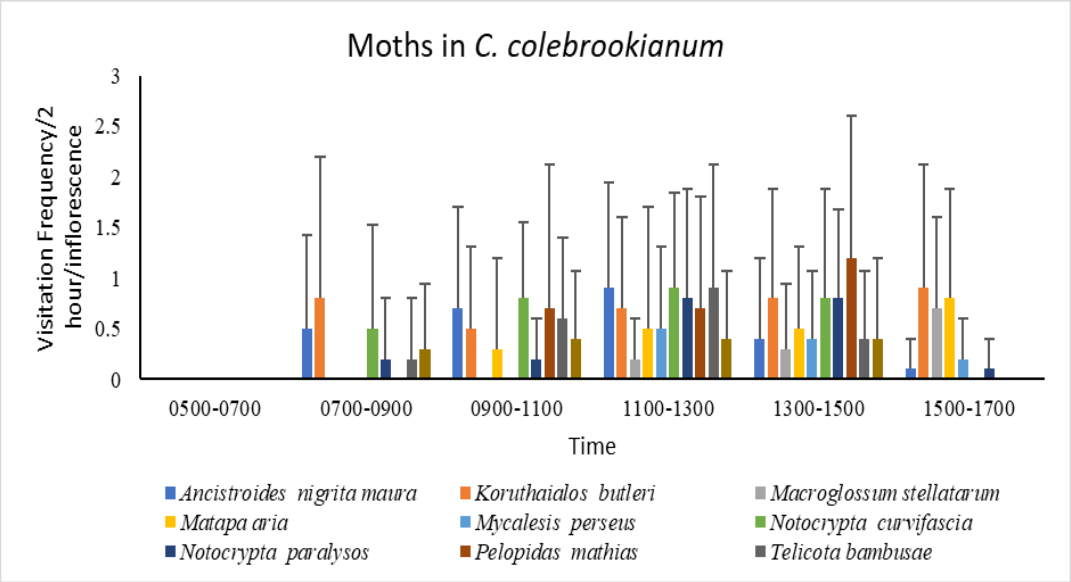


Figure 4.20 Visitation frequency of moth's pollinator in *C. colebrookianum*.

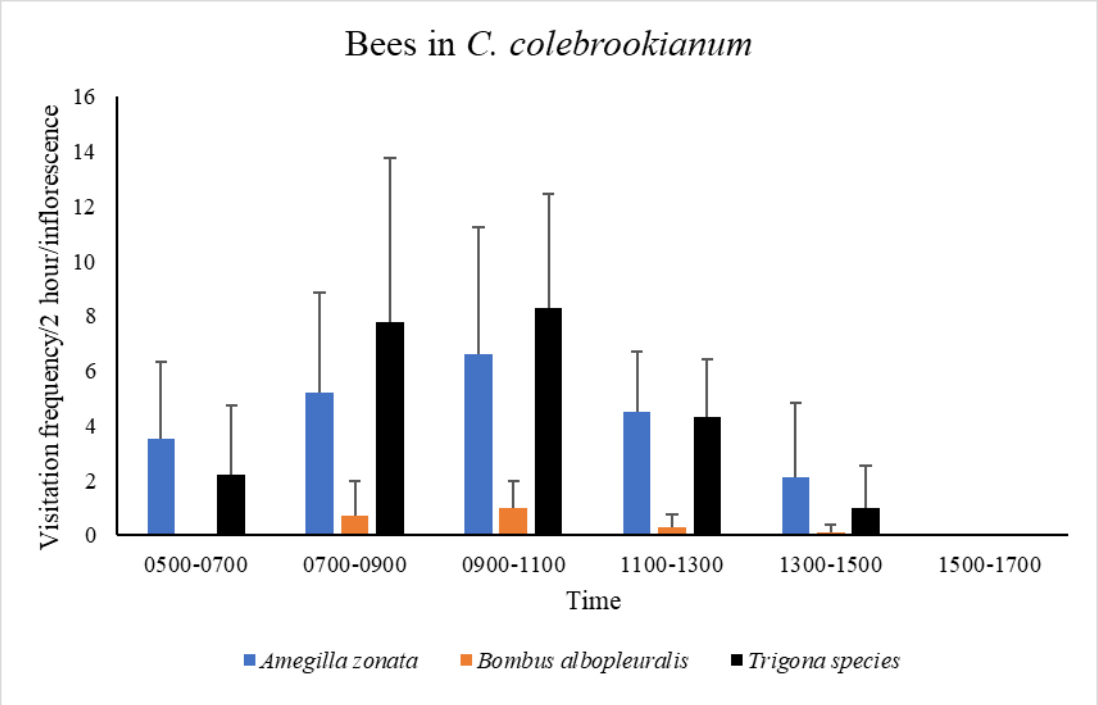


Figure 4.21 Visitation frequency of bee's pollinator in *C. colebrookianum*.

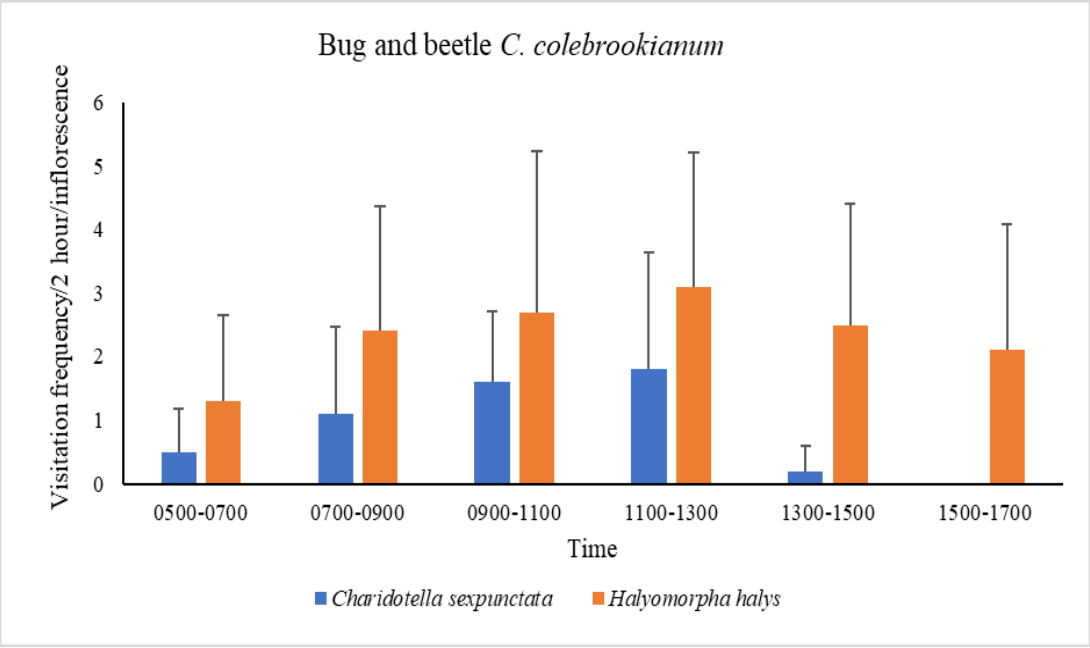


Figure 4.22 Visitation frequency of bug and beetle pollinator in *C. colebrookianum*.

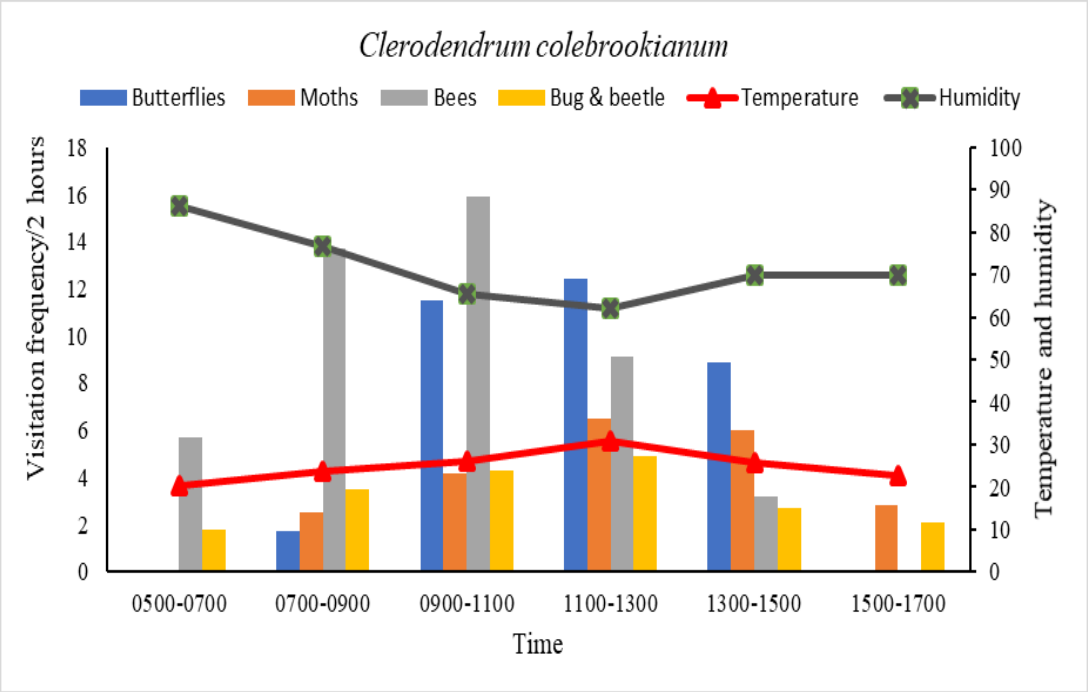
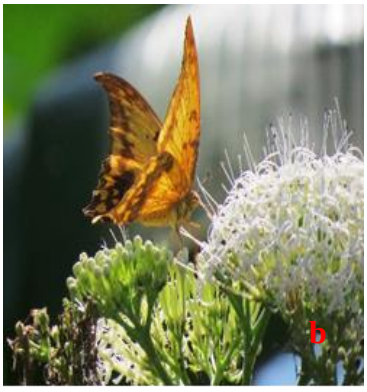


Figure 4.23 Summary of four different pollinator's visitation frequency with respect to time, temperature and humidity.


a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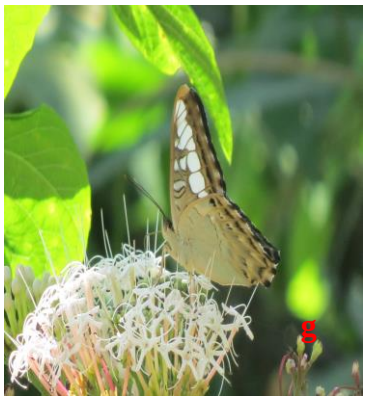
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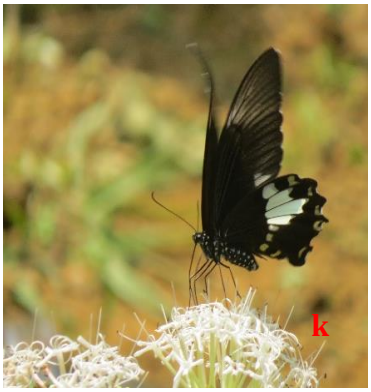
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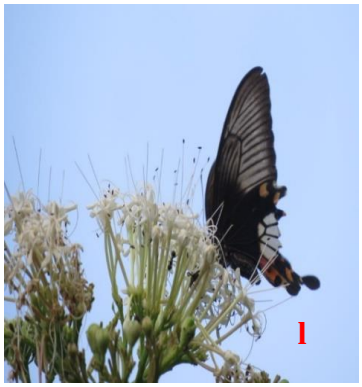
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Figure 4.24 Pollinators of *C. colebrookianum* (butterflies) (a) *Anthene lycaenina*, (b) *Charaxes marmax*, (c) *Delias descombesi*, (d) *Elymnias patna* (f) *Eurema andersonii*, (g) *Limenitis trivena*, (h) *Loxura atymnus*, (i) *Papilio helenus*, (j) *Papilio memnon*, (k) *Papilio polytes*, (l) *Papilios polytes*, (m) *Parantica aglea*, (n) *Rapala manea*, (o) *Tirumala septentrionis*, (p) *Troides helena*

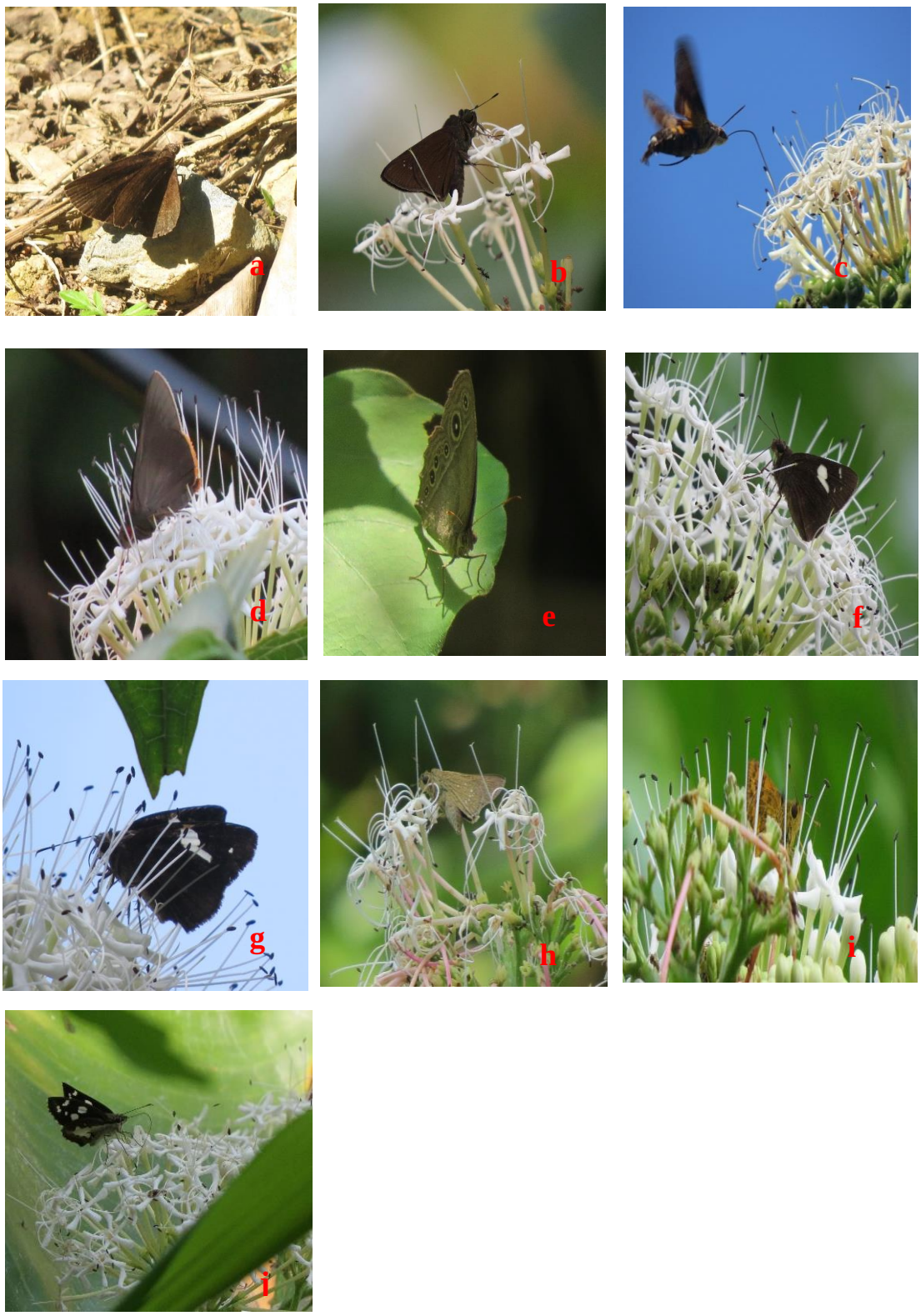


Figure 4.25 Pollinators of *C. colebrookianum* (Moths) (a) *Ancistroides nigrita maura*, (b) *Koruthaialos butleri*, (c) *Macroglossum stellatarum*, (d) *Matapa aria*, (e) *Mycalesis perseus* (f) *Notocrypta curvifascia*, (g) *Notocrypta paralysos*, (h) *Pelopidas mathias*, (i) *Telicota bambusae*, (j) *Udaspes folus*

Figure 4.26 Pollinators of *C. colebrookianum* (Bees) (a) *Amegilla zonata*, (b) *Bombus albopleuralis*, (c) *Trigona species*

Figure 4.27 Pollinators of *C. colebrookianum* (Bug and beetle) (a) *Halyomorpha halys* (b) *Charidotella sexpunctata*

4.1.5. Pollinator's availability in *Clerodendrum infortunatum*:

Lepidoptera and Hymenoptera are the insect order which was observed in the *Clerodendrum infortunatum* as floral visitors. 16 species of the order Lepidoptera have been identified as floral visitors with a maximum number of floral pollinators and 3 species of the order Hymenoptera. Butterflies were observed as the most frequently visiting pollinator of *C. infortunatum*. A total of 13 butterflies (Fig. 4.33) and 3 moths (Fig. 4.33) belonging to the order Lepidoptera were observed as floral visitors in the flower of *C. infortunatum* during the flowering period. Maximum visitation of butterflies was observed between 0700-1300 of the day, and they follow a diurnal pattern, which coincides with anther dehiscence and nectar production (Fig. 4.28). *Delias descombesi*, *Catopsilia florella*, *Papilio memnon* and *Papilio helenus* were observed as the highest visitation frequency among the butterflies (Fig. 4.28).

Moths followed the bimodal pattern, it was observed that maximum visitation of *Pelopidas mathias* and *Koruthaialos butteria* was observed during morning and afternoon hours of the day i.e., between 0900-1300 hours, whereas pollinator *Macroglossum stellatarum* was observed maximum during the evening hours between 1500-1700 hours of the day (Fig. 4.29). By using a proboscis length of 2.8 cm *Macroglossum stellatarum* collect nectar as a floral reward (Fig. 4.33 b). No pollinator was observed during the morning hours between 0500-0700 for both the species (butterflies and moths), and no floral visitor was observed in the evening hours for butterflies between 1500-1700. Nectar which is present in minute quantities, is taken as a floral reward by these pollinators.

Three bee pollinator *Apis cerana indica*, *Xylocopa species* (a) and *Xylocopa species* (b) were observed as floral visitors in the flower of *C. infortunatum* during the flowering period (Fig. 4.30). Among the three pollinators *Apis cerana indica* was observed as the most frequently visiting pollinator with proclaimed forenoon pattern; maximum visitation was observed during the morning hours between 0900-1300 of

the day (Fig. 4.30), which coincided with anthesis, anther dehiscence and nectar production of the flower.

Maximum visitation of *Xylocopa species* (a) and *Xylocopa species* (b) were observed when the stamen curled downward and the anther was fully dehisced, i.e., between 1100-1300 hours of the day (Fig. 4.30). The two species received nectar as a floral reward (Fig. 4.34 a & b). During the evening hours, no floral visitation was observed i.e. between 1500-1700.

Temperature and humidity enhance floral activity on flower and pollinator activity. The visitation frequency and activity of butterflies and moths increased with the rise in temperature, i.e., between 0900-1100 and 1100-1300 (Fig. 4.31). With the increase in temperature, the visitation frequency of the bee's pollinator increased, and maximum visitation frequency was observed between 0900-1300 (Fig. 4.31). But with the decrease in temperature around 1500-1700 hours, the visitation frequency of pollinators decreased (Fig. 4.31). All the three different groups of pollinators (butterflies, moths, and bees) positively showed (+ve) correlation at (0.01**) observed (r = 0.649, 0.928** and 0.793; p = 0.163, 0.008 and 0.060) with temperature while negative (-ve) correlation at (0.01** and 0.05*) was observed (r = -0.835*, -0.891 and -0.924**; p = 0.039, 0.017 and 0.009) with humidity (Table 4.3).

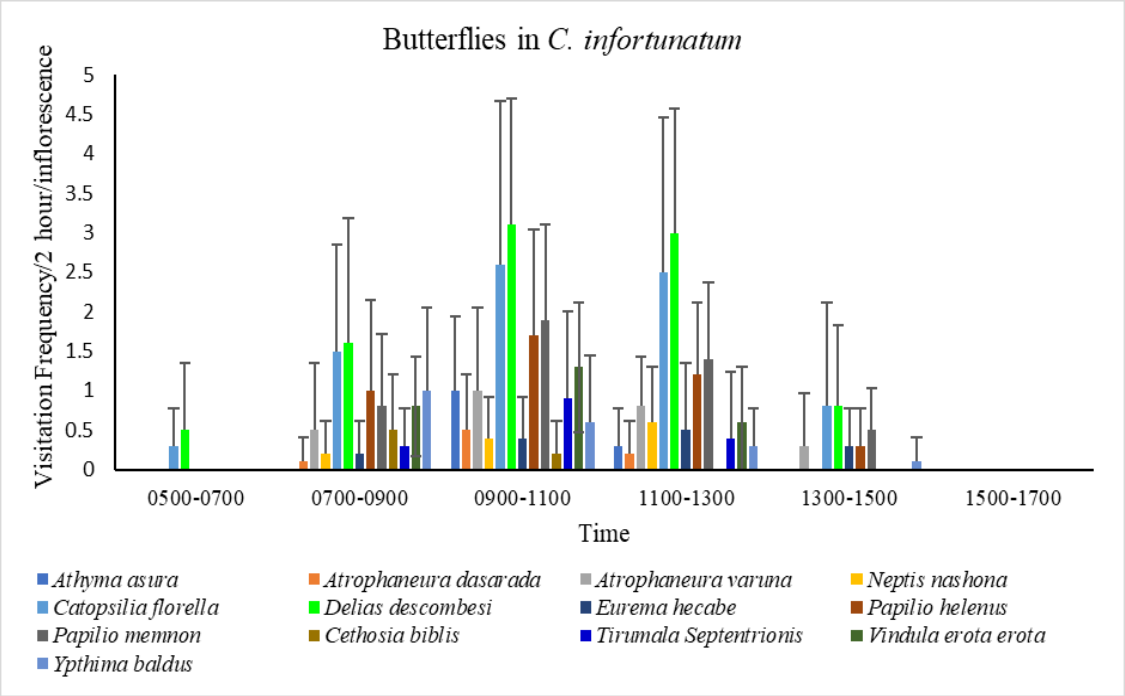


Figure 4.28 Visitation frequency of butterflies' pollinator in *C. infortunatum*.

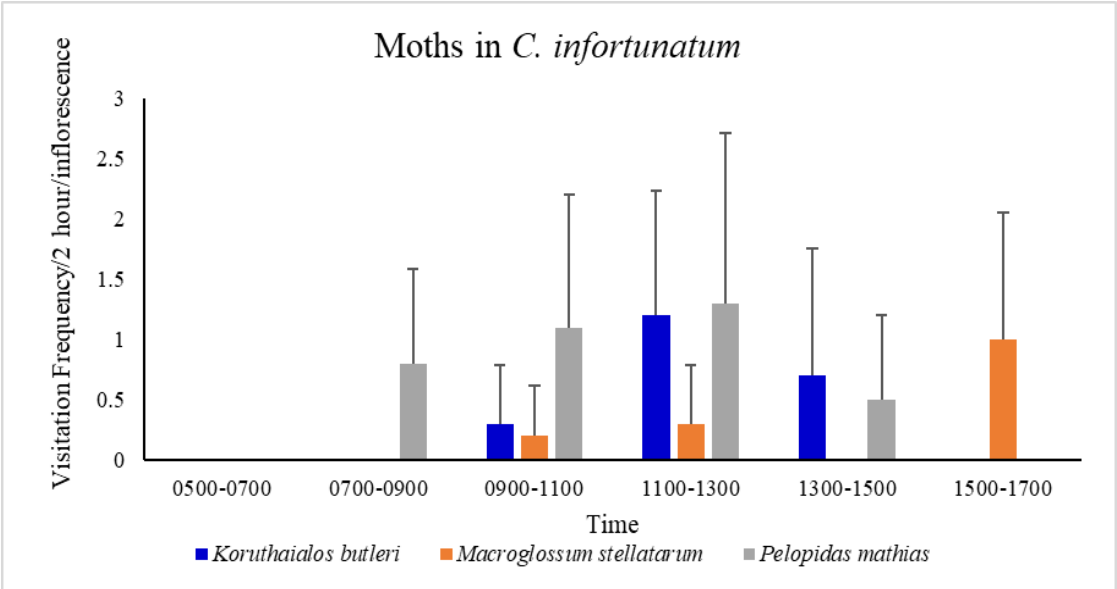


Figure 4.29 Visitation frequency of moth's pollinator in *C. infortunatum*.

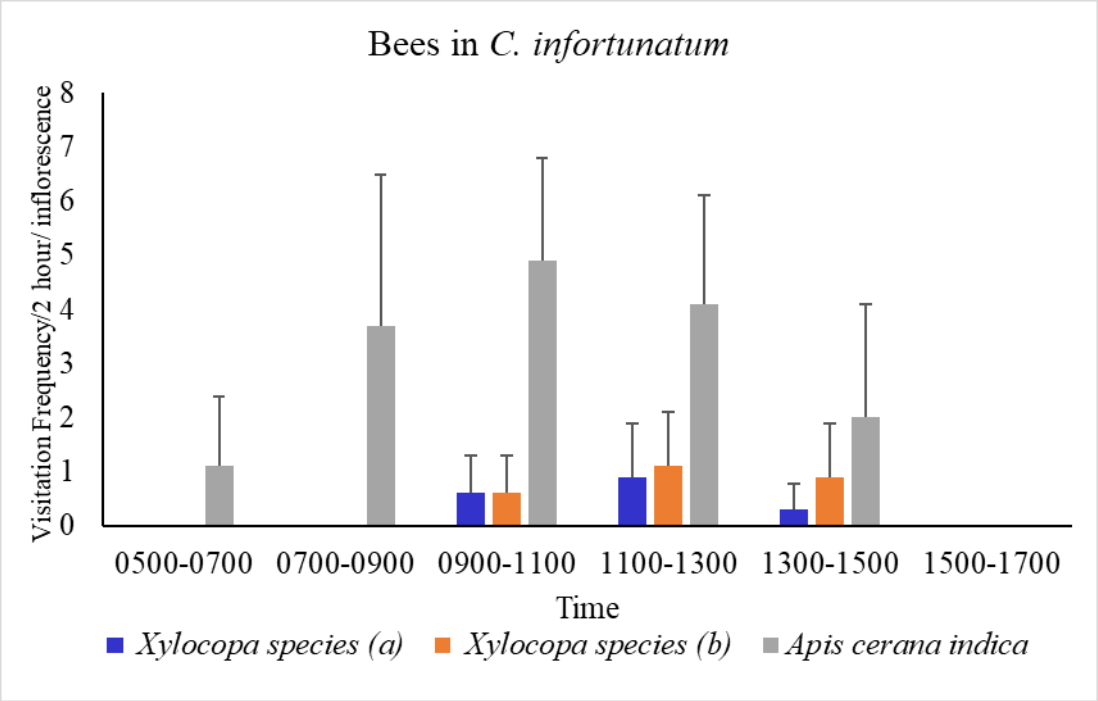


Figure 4.30 Visitation frequency of bee's pollinator in *C. infortunatum*.

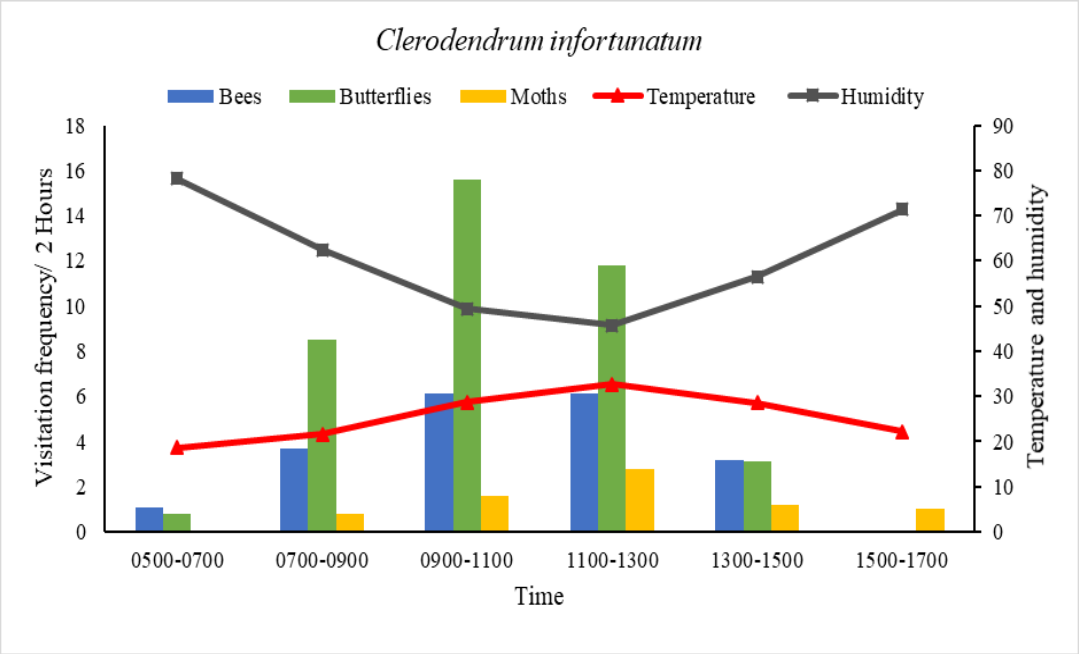


Figure 4.31 Figure: Summary of three different pollinator visitation frequency with respect to time, temperature and humidity.

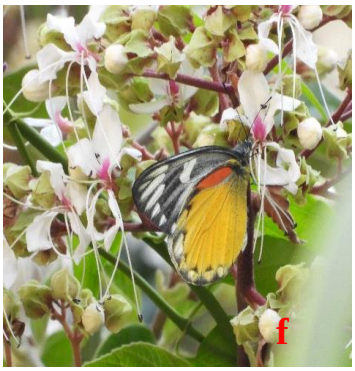

a


b

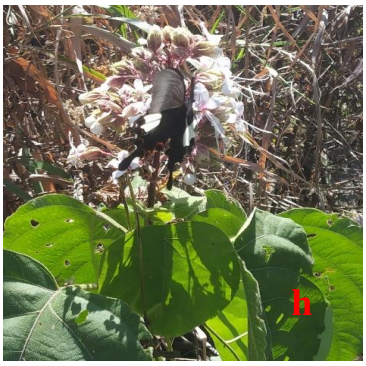

c


d


e

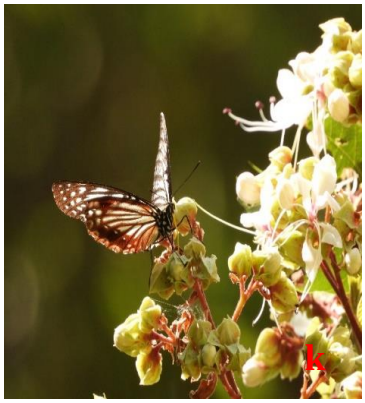

f


g


h


i


j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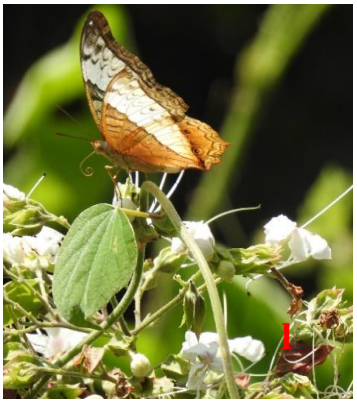
l

Figure 4.32 Pollinators of *C. infortunatum* (butterflies) (a) *Athyma asura*, (b) *Atrophaneura dasarada*, (c) *Atrophaneura varuna*, (d) *Neptis nashona*, (e) *Catopsilia florella* (f) *Delias descombesi*, (g) *Eurema hecabe*, (h) *Papilio helenus*, (i) *Papilio memnon*, (j) *Cethosia biblis*, (k) *Tirumala Septentrionis*, (l & m) *Vindula erota erota*, (n) *Ypthima baldus*

Figure 4.33 Pollinator of *C. infortunatum* (moths) (a) *Koruthaialos butleri*, (b) *Macroglossum stellatarum*, (c) *Pelopidas mathias*

Figure 4.34 Pollinator of *C. infortunatum* (bees) (a) *Xylocopa species* (a), (b) *Xylocopa species* (b), (c) *Apis cerana indica*

4.1.6. Pollinators availability in *Clerodendrum serratum*:

Lepidoptera and Hymenoptera are the insect order which is observed in the *Clerodendrum serratum* as a floral visitors. Five species of the order Lepidoptera and five species of the order Hymenoptera have been identified as floral visitors. Five butterfly species *Anthene lycaenina, Celaenorrhinus aurivittata, Hesperia sassacus, Notocrypta curvifascia* and *Pelopidas mathias* were observed as floral visitor of *C. serratum* (Fig. 4.38). Maximum visitation was observed during 0900-1600 hour (Fig. 4.35). Highest visitation frequency was observed for *Pelopidas mathias*, *Notocrypta curvifascia* and *Hesperia sassacus,* following a diurnal pattern (Fig. 4.35).

Moreover, 5 bee pollinators *Apis cerana indica, Bombus albopleuralis, Polistes spp, Xylocopa virginica* and *Xylocopa violacea* were observed as a floral visitors in the flower of *C. serratum* during the flowering period (Fig. 4.39). Among the pollinators, *Apis cerana indica* was observed as the most frequently visiting pollinator with proclaimed forenoon pattern; maximum visitation was observed during the morning hours between 0500-1100 of the day (Fig. 4.36), coinciding with anthesis, anther dehiscence and nectar production of the flower. *Bombus albopleuralis, Xylocopa species* (a) and *Xylocopa violacea* were observed to squeeze the nectar, which is present on the tip of the hairy like structure present at the base of the ovary (Fig. 4.39 b, c & e). In addition, when they have squeezed the nectar, the stamen of the flower touches the dorsal site of these three pollinators. During the evening hours, no floral visitation was observed, i.e., between 1500-1700.

Temperature and humidity enhance the floral activity of flowers and pollinator activity. The visitation frequency and activity of butterflies increase with the rise in temperature, i.e., between 0900-1300 (Fig. 4.37). With the increase in temperature and humidity, the visitation frequency of bee pollinators increased, and maximum visitation frequency was observed between 0700-0900 hours (Fig. 4.37). But with the decrease in temperature around 1500-1700 hours, the visitation frequency of pollinators decreased (Fig. 4.37).

Pollinators (butterflies and bees) positive (+ve) correlation was observed (r = 0.744 and 0.087; p = 0.869 and 0.090) with temperature, but a negative (-ve) correlation at (0.01**) was observed (r = -0.931**; p = 0.007) for butterflies and (+ve) correlation (r = 0.414; p = 0.414) for bees with humidity was observed (Table 4.4).

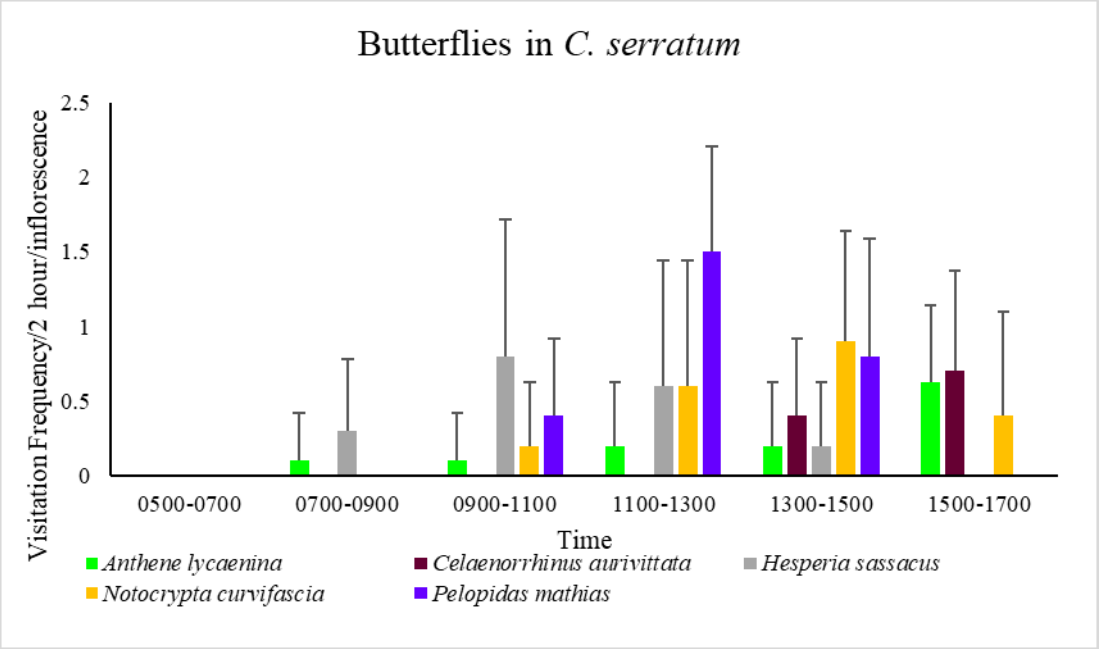

Figure 4.35 Visitation frequency of butterflies' pollinator in *C. serratum*.

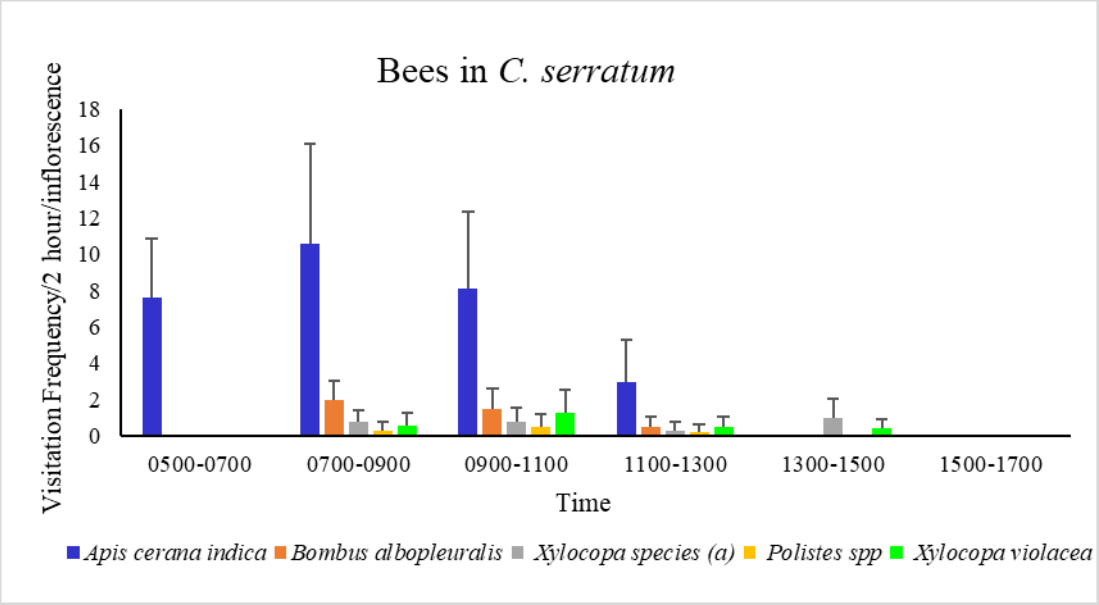

Figure 4.36 Visitation frequency of bee's pollinator in *C. serratum*.

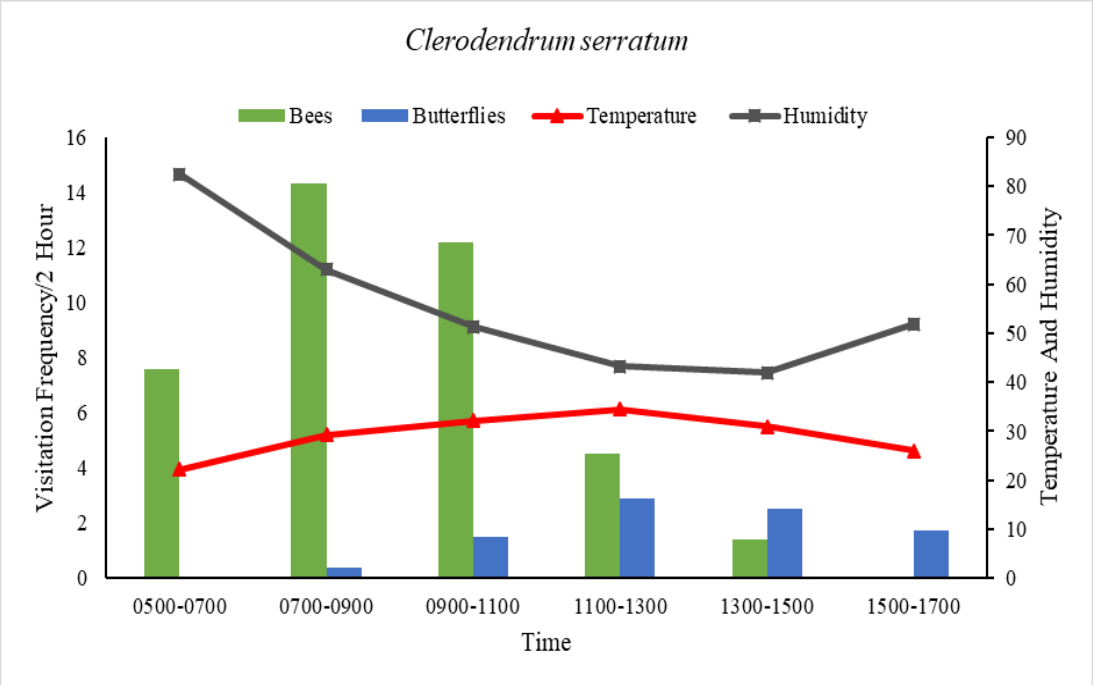

Figure 4.37 Summary of two different pollinators visitation frequency with respect to time, temperature and humidity.

Figure 4.38 Pollinator**s** of *C. serratum* (butterflies and moths) (a) *Anthene lycaenina*, (b) *Celaenorrhinus aurivittata*, (c) *Hesperia sassacus*, (d) *Notocrypta curvifascia*, (e) *Pelopidas mathias*

Figure 4.39 Pollinators *C. serratum* (bees) (a) *Apis cerana indica*, (b) *Bombus albopleuralis*, (c) *Xylocopa species* (a), (d) *Polistes spp*, (e) *Xylocopa violacea*

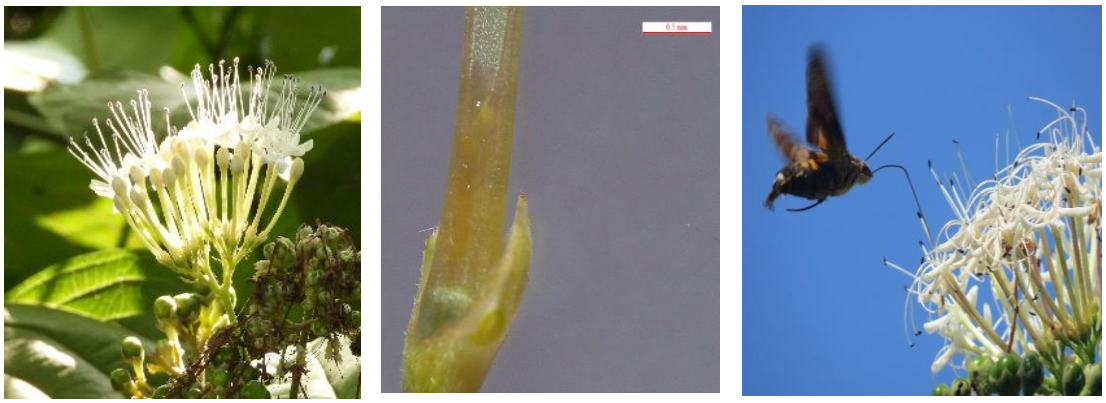

Figure 4.40: Floral adaptations to pollinator**s** in *C. colebrookianum*

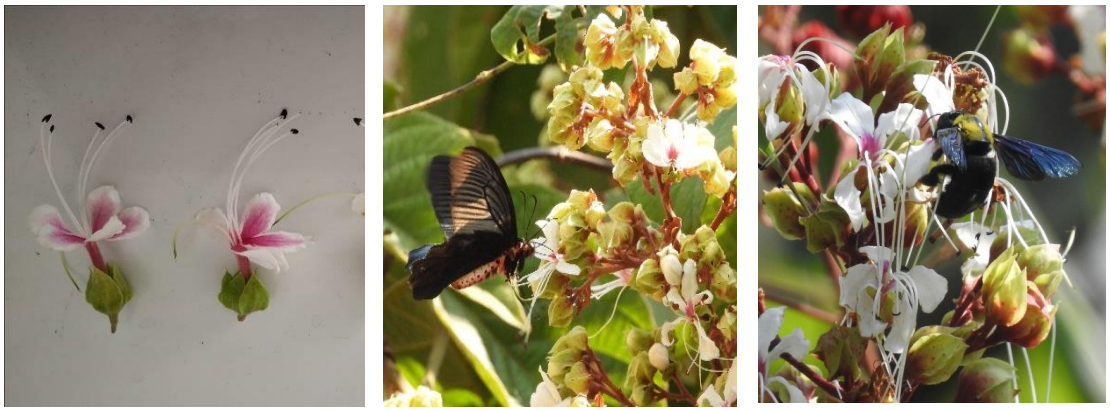

Figure 4.41: Floral adaptations to pollinators in *C. infortunatum*

Figure 4.42: Floral adaptations to pollinators in *C. serratum*

Table 4.2 Correlation coefficient between pollinators, temperature and humidity *C. colebrookianum*

Correlations							
		Butterflies	Moths	Bees	Bugs	Temperature	Humidity
Butterflies	r	1					
	p						
Moths	r	.853*	1				
	p	.031					
Bees	r	.409	.065	1			
	p	.420	.902				
Bugs	r	.813*	.668	.705	1		
	p	.049	.147	.118			
Temperature	r	.879*	.903*	.297	.877*	1	
	p	.021	.014	.567	.022		
Humidity	r	-.792	-.884*	-.150	-.732	-.863*	1
	p	.061	.019	.776	.098	.027	

*. Correlation at 0.05

Table 4.3 Correlation coefficient between pollinators, temperature and humidity *C. infortunatum*

Correlations						
		Bees	Butterflies	Moths	Temperature	Humidity
Bees	r	1				
	p					
Butterflies	r	.947**	1			
	p	.004				
Moths	r	.745	.657	1		
	p	.090	.156			
Temperature	r	.793	.649	.928**	1	
	p	.060	.163	.008		
Humidity	r	-.924**	-.835*	-.891*	-.944**	1
	p	.009	.039	.017	.005	

**. Correlation at 0.01

*. Correlation at 0.05

Table 4.4 Correlation coefficient between pollinators, temperature and humidity *C. serratum*

Correlations					
		Bees	Butterflies	Temperature	Humidity
Bees	r	1			
	p				
Butterflies	r	-.597	1		
	p	.211			
Temperature	r	.087	.744	1	
	p	.869	.090		
Humidity	r	.414	-.931**	-.823*	1
	p	.414	.007	.044	

**. Correlation at 0.01

*. Correlation at 0.05

Table 4.5 Similarity coefficient for pollinators of study tree species

	C. colebrookianum	*C. infortunatum*	*C. serratum*
C. colebrookianum	0	34.04255	14.63415
C. infortunatum	34.04255	0	20.68966
C. serratum	14.63415	20.68966	0

4.1.7. Diurnal rhythms of pollen concentrations on stigma and in air:

Clerodendrum colebrookianum and *Clerodendrum infortunatum* maximum concentration of pollen in the air was observed during 1100-1300 hours of the day (Fig. 4.43 & 4.44), while in *Clerodendrum serratum* it was observed during 0900-1100 hours of the day (Fig. 4.45). With the increase in temperature and reduction in humidity, the concentration of pollen in the air increases. Increased in time from anther dehiscence increased the concentration of pollen in the air in all three *Clerodendrum* species. Less pollen concentration was observed during the morning hours 0500-0900 (Fig. 4.43, 4.44 & 4.45), which coincides with anthesis and anther dehiscence of the three *Clerodendrum* species; low temperature and high humidity were observed during this hour. Less pollen concentration was observed during the evening hours, 1500-1700 (Fig. 4.43, 4.44 & 4.45), since no anthesis and anther dehiscence occurred during this hour and the anther is fully dehisce.

Clerodendrum colebrookianum maximum concentration of pollen on the stigma was observed during 1100-1300 hours of the day (Fig. 4.43; 4.46 a & b), but in *Clerodendrum infortunatum* and *Clerodendrum serratum*, the maximum concentration of pollen on the stigma was observed during 0900-1100 hours (Fig. 4.44, 4.46 c & d and 4.45, 4.46 e & f). During this hour's maximum visitation of pollinator and their pollination activity were high for all three *Clerodendrum* species, which helped in supplying and increasing pollen on stigma during these hours. It was observed that with the increase in temperature and reduced humidity, pollen on the stigma increases. Less pollen on the stigma was observed during morning and evening hours since fewer floral visitors for pollination during these hours in all three *Clerodendrum*. In-vivo pollen germination on the stigmatic surface of the stigma was observed in all three *Clerodendrum* species under a fluorescent microscope (Fig. 4.47 & 4.48). Aniline blue stain was used to observe the germinated pollen on the stigma with cross-pollination treatment.

Pollen on stigma and pollen in air positive correlation (0.01** and 0.05*) was observed ($r = 0.880^*$ and 0.967^{**}, $p = 0.021$ and 0.009); ($r = 0.356$ and 0.700, $p = 0.489$ and 0.121) and ($r = 0.580$ and 0.583, $p = 0.227$ and 0.225) with temperature,

but negative correlation (0.01** and 0.05*) (r = -0.889* and -0.883*, p = 0.018 and 0.021); (r = -0.958** and -0.939**, p = 0.003 and 0.006) and (r = -0.067 and -0.158, p = 0.900 and 0.764) was observed with humidity for *C. colebrookianum*, *C. infortunatum* and *C. serratum* (Table 4.6, 4.7 & 4.8).

Diurnal rhythms of pollen concentrations on stigma and in air:

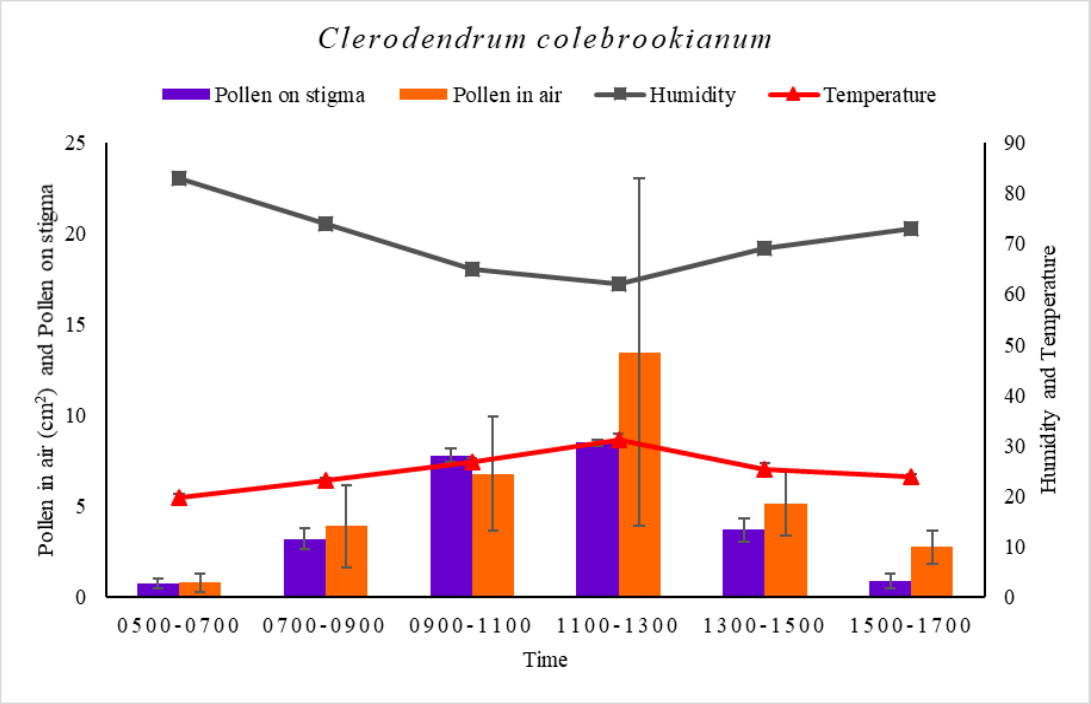

Figure 4.43 Diurnal rhythms of pollen concentrations on stigma and in air *C. colebrookianum*

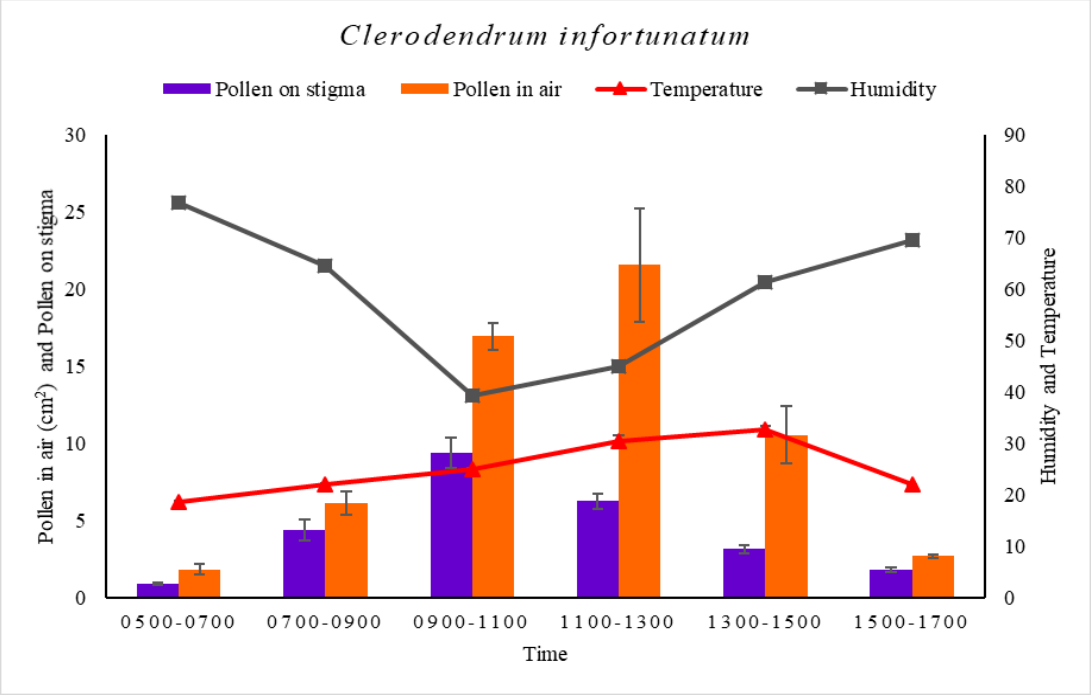

Figure 4.44 Diurnal rhythms of pollen concentrations on stigma and in air *C. infortunatum*

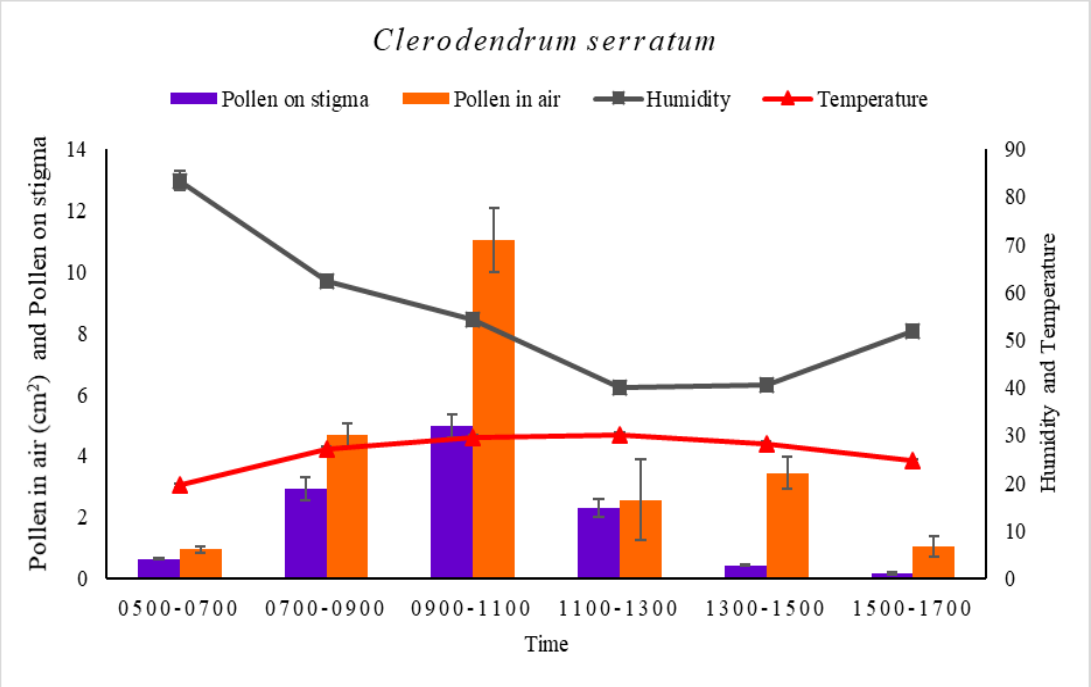

Figure 4.45 Diurnal rhythms of pollen concentrations on stigma and in air *C. serratum*

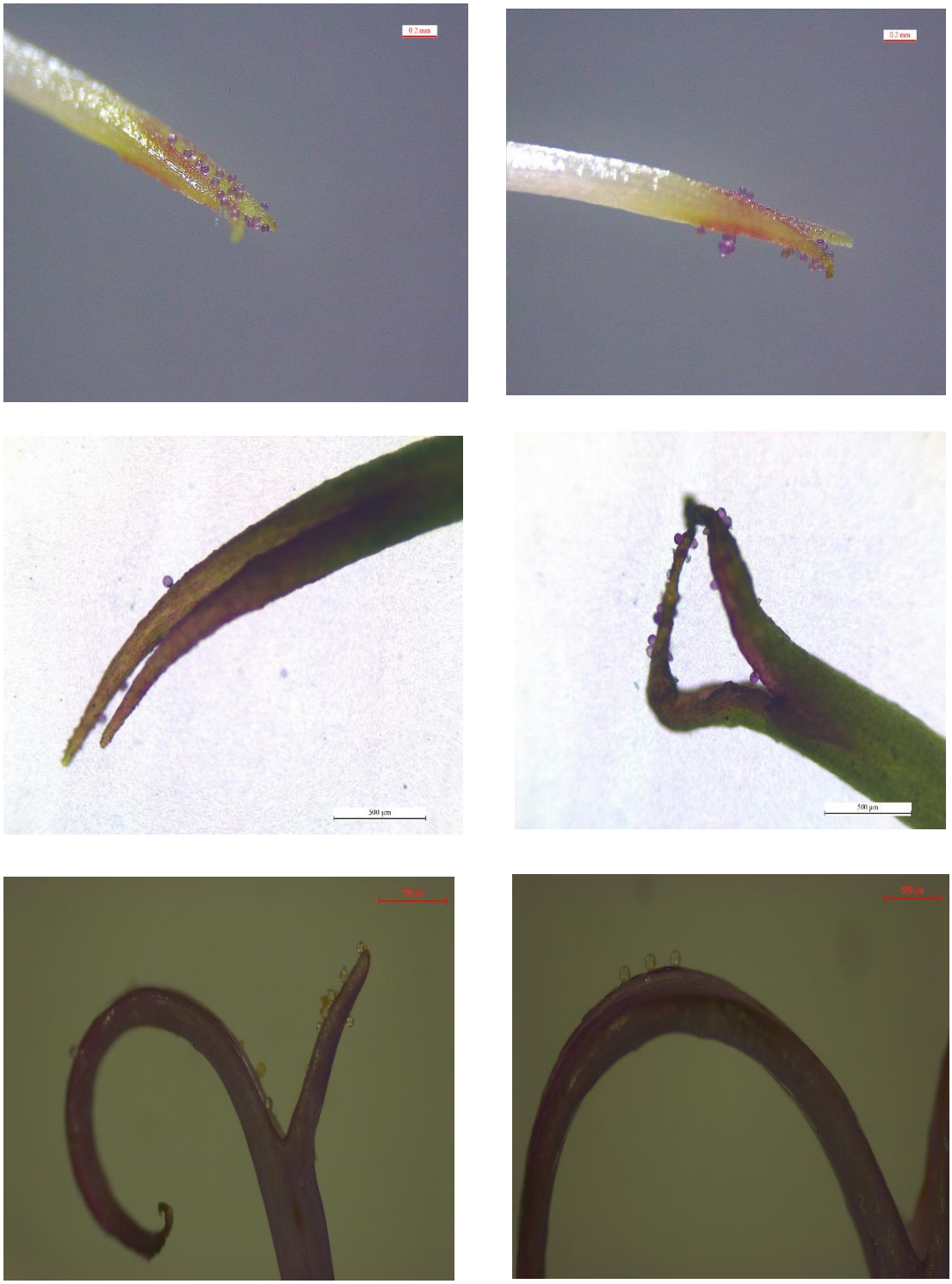

Figure 4.46 Pollen deposition on stigma of *C. colebrookianum* (a & b), *C. infortunatum* (c & d) and *C. serratum* (e & f) when exposed to pollinators.

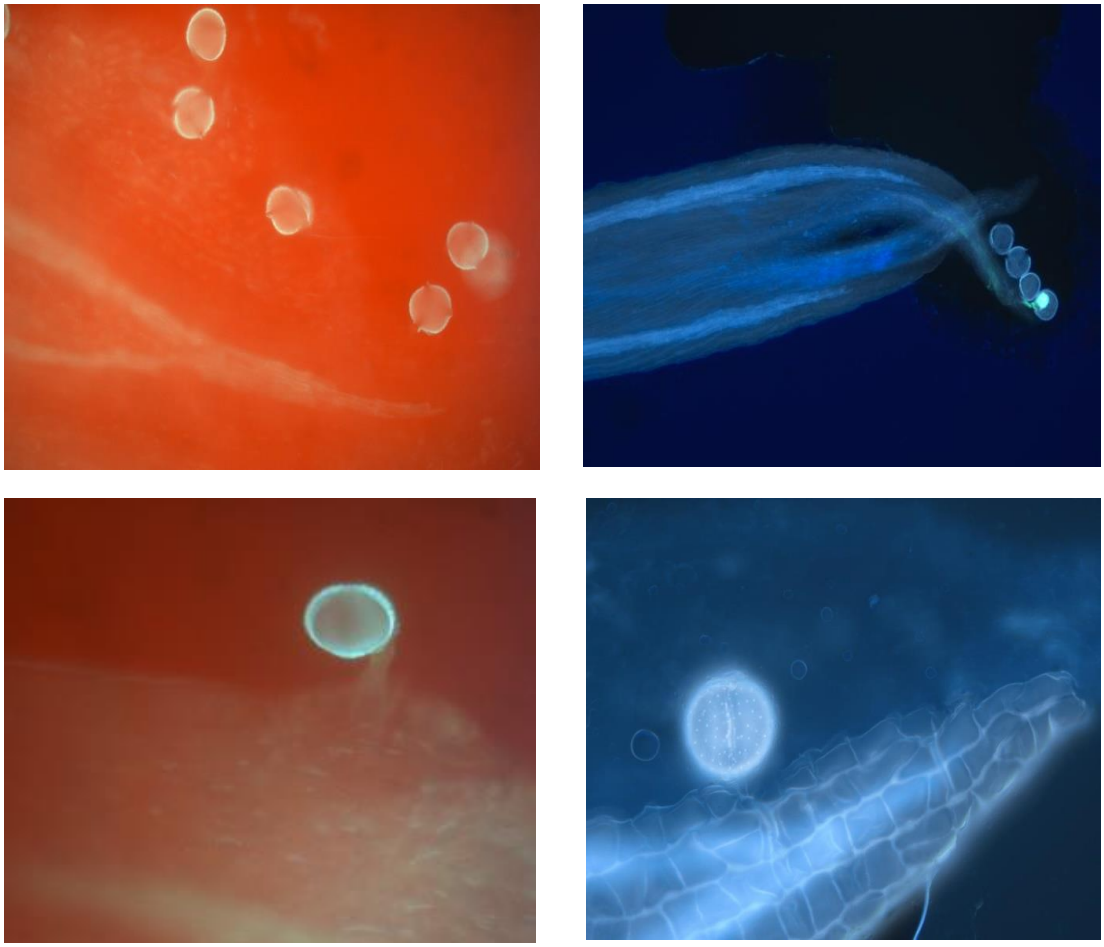

Figure 4.47 Examination of stigma in cross pollination treatment under fluorescent microscope, stigma is stained with aniline blue in *C. colebrookianum*

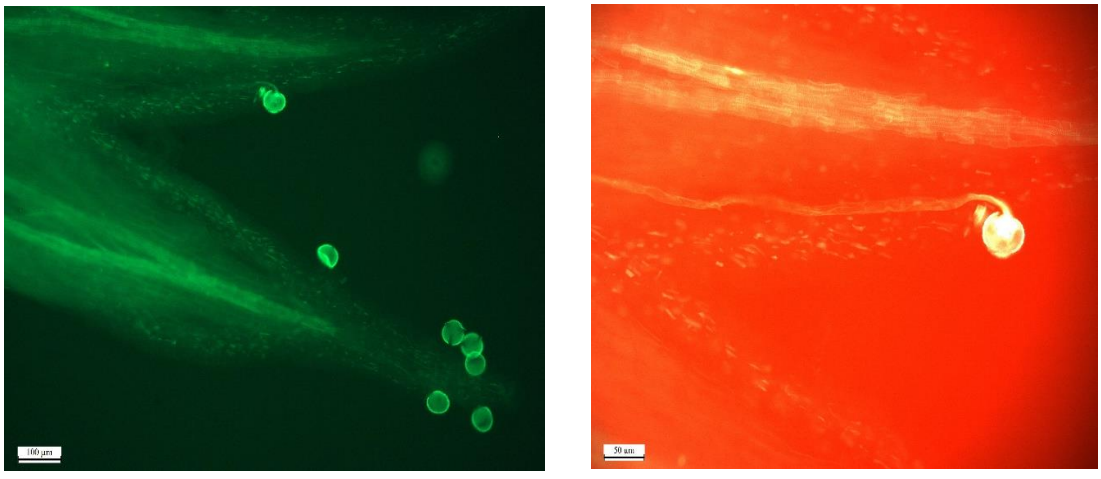

Figure 4.48 Examination of stigma in cross pollination treatment under fluorescent microscope, stigma is stained with aniline blue in *C. infortunatum*

Table 4.6 Correlation coefficient between pollen on stigma, pollen in air, temperature and humidity *C. colebrookianum*

Correlations					
		Pollen on stigma	Pollen in air	Humidity	Temperature
Pollen on stigma	r	1			
	p				
Pollen in air	r	.897*	1		
	p	.015			
Humidity	r	-.889*	-.883*	1	
	p	.018	.020		
Temperature	r	.880*	.967**	-.964**	1
	p	.021	.002	.002	

*. Correlation at 0.05

**. Correlation at 0.01

Table 4.7 Correlation coefficient between pollen on stigma, pollen in air, temperature and humidity *C. infortunatum*

Correlations					
		Pollen on stigma	Pollen in air	Temperature	Humidity
Pollen on stigma	r	1			
	p				
Pollen in air	r	.827*	1		
	p	.042			
Temperature	r	.356	.700	1	
	p	.489	.121		
Humidity	r	-.958**	-.939**	-.555	1
	p	.003	.006	.253	

*. Correlation at 0.05

**. Correlation at 0.01

Table 4.8 Correlation coefficient between pollen on stigma, pollen in air, temperature and humidity *C. serratum*

Correlations

		Pollen on stigma	Pollen in air	Humidity	Temperature
Pollen on stigma	r	1			
	p				
Pollen in air	r	.900*	1		
	p	.014			
Humidity	r	-.067	-.158	1	
	p	.900	.764		
Temperature	r	.580	.583	-.838*	1
	p	.227	.225	.037	

*. Correlation at 0.05

4.2.1 Assessment of pollen production, pollen/ovule ratio in three *Clerodendrum* species.

The average girth and average height varied with geographical locations in *C. clerodendrum* and *C. infortunatum* within the years, with slight variation between years among selected individuals of all three study plant species (Table 4.9 & 4.10). The stamen per flower was observed to be the same, i.e., 4 in *C. clerodendrum*, *C. infortunatum* and *C. serratum*. The production of flowers and pollen grains per plant in all the selected plant species substantially varied among geographical locations (Table 4.9, 4.10 & 4.11). It was observed that among the three chosen sites, the production of pollen grain per plant was more at Tanhril (medium altitude) and Sairang (low altitude) compared to Durtlang (high altitude) in *C. colebrookianum* (Table 4.9. While among selected two locations, the production of pollen grain per plant was more at Tanhril (mid-altitude) and less at Sairang (low altitude) for *C. infortunatum* (Table 4.10). Among plant species, the magnitude of pollen grain production per plant was highly varied and highest in *Clerodendrum infortunatum* (10.44-82.09 × 10^5), followed by *C. clerodendrum* (21.54-54.33 × 10^5) and *C. serratum* (45.15-53.81 × 10^5). The maximum percentage of fruit setting was recorded in C. *clerodendrum* (65.30%) at Tanhril (mid-altitude), followed by *C. serratum* (56.23%) at Hlimen (high altitude) and *C. infortunatum* (42.15%) at Tanhril (mid-altitude).

The statistical analysis revealed significant effect of plant species on flower production (p=0.0001; F=16.81), pollen grains per anther (p=0.0001; F=11.32), pollen grains per flower (p=0.0001; F=11.32) and pollen grain production per plant (p=0.0001; F=49.06) (Table 4.12). There is significant effect of geographical locations, i.e. plant populations varied in three altitudes (low, mid & high), on flower production (p=0.002; F=6.55), pollen grain production per plant (p=0.0001; F=20.53) and fruit set (p=0.0001; F=27.92) while there was non-significant effect of years on flower production (p=0.97; F=0.022), pollen grain production per plant (p=0.97; F=0.023) and fruit set (p=0.70; F=0.35) in *C. colebrookianum* (Table 4.13). There is significant effect of geographical locations i.e. plant populations in varied

two altitudes (low & mid) on flower production ($p \leq 0.001$; F=15.08), pollen grain production per plant (p=0.005; F=8.81) but non-significant effect on fruit set (p=0.264; F=1.28), while there was non-significant effect of year on flower production (p=0.79; F=0.069) and fruit set (p=0.47; F=0.51) but significant effect of year on pollen grain production per plant (p=0.005; F=8.81) in *C. infortunatum* (Table 4.14). In *C. serratum*, there is non-significant effect of year on flower production (p=0.44; F=0.61), pollen grain production per plant (p=0.34; F=0.96) and fruit set (p=0.54; F=0.37) (Table 4.15). No effect of geographical location was evaluated in *C. serratum* since the plant population was located and studied in only one high-altitude location, i.e., Hlimen.

The correlation coefficient revealed that there is a weak correlation between pollen grain per plant to fruit set percentage in *C. colebrookianum* at Sairang (low), Tanhril (mid), and Durtlang (high) altitudes. There is a significant correlation between flower/inflorescence and fruit setting percentage (p=.021) in *C. colebrookianum* at Tanhril (mid-altitude site) (Table 4.16, 4.17 and 4.18). In *C. infortunatum*, there is a weak correlation between pollen grain per plant and fruit setting percentage at Tanhril (mid-altitude), while there is a significant correlation between pollen grain per plant to fruit setting percentage (p=0.016); the number of inflorescences/plants to fruit setting/plant (p=0.016) at low altitude site Sairang (Table 4.19 & 4.20). Again in *C. serratum* there is a significant correlation between pollen grain per plant to fruit setting percentage (p=0.025); the number of inflorescences/plants to fruit setting/plant (p=0.027) at Hlimen, a high-altitude site in Mizoram (Table 4.21).

Regression analysis revealed that there is a weak to average positive relationship observed between the production of flower per plant and fruit set percentage per plant ($r^2 = 0.255$), and total pollen grain production per plant and fruit set percentage per plant ($r^2 = 0.402$) in *C. colebrookianum* (Fig. 4.49 & 4.50). In *C. infortunatum*, there is an average positive relationship observed between the production of flower per plant to fruit set percentage per plant ($r^2 = 0.457$) and total pollen grain production per plant and fruit set percentage per tree ($r^2 = 0.457$) (Fig. 4.51 & 4.52). Again, there is an average positive relationship observed between the

production of flower per plant and fruit set percentage per plant (r^2 = 0.482), and total pollen grain production per plant and fruit set percentage per plant (r^2 = 0.484) in *C. serratum* (Fig. 4.53 & 4.54). In all three-study species, four numbers of ovules per ovary were observed in the cross-section. The pollen-ovule ratio ranged from 726.3-1190.7 in *C. clerodendrum* (, 950.2-1218.9 in *C. infortunatum*, and 1300-1368.1 in *C. serratum.* For all the three-study species range value occurred within the range of 224.7-2588.0 (Table 4.22) of facultative xenogamy as per the Cruden (1977).

4.2.2. Mating system evaluation of three *Clerodendrum* species.

The evaluation of the mating system of all three-plant species of *Clerodendrum* revealed that the species are capable of being partially self-compatible. Fruit setting percentage was less in spontaneous selfing, induced by selfing, and geitonogamy in *C. colebrookianum* (24 ± 15.05, 21 ± 10.48, and 25.5 ± 13.42); *C. infortunatum* (15±10.54, 17.5±12.96 and 19±10.48) and *C. serratum* (12.5±9.78, 15.5±9.55 and 19±10.48). Nevertheless, fruit setting percentage was high in cross–artificial, cross-natural and control (open-pollinated flowers) in *C. colebrookianum* (66.5±9.73, 64.5±11.65 and 63.5.5 ± 12.70); *C. infortunatum* (43.5±9.14, 41.5±10.08 and 39.5±5.98) and *C. serratum* (52.5±11.84, 54.5±11.16 and 48.5±9.44), respectively (Table 4.23, 4.24 & 4.25). Based on the ratio of fruit set in open-pollinated to cross-pollinated, the Index of self-incompatibility (ISI) was calculated; the result showed all three *Clerodendrum* species ascertained the index value of 0.38 (*C. colebrookianum*), 0.36 (*C. infortunatum*) and 0.29 (*C. serratum*), (Table 4.26) which occurred within the range of >0.2 but <1 of partially self-compatible as per Zapata and Arroyo (1978). The value of the outcrossing index was calculated on the basis of variables such as the diameter of the flower, temporal separation of anther dehiscence and stigma receptivity and spatial positioning of stigma and anthers. All three *Clerodendrum* species scored 5 points which falls within the range >4, indicating partially self-compatible and required pollinator services for outcrossing as per Cruden, (1977).

Table 4.9 Annual production of flower, pollen and fruit setting in three different sites in *Clerodendrum colebrookianum*

Observed variables	**Sairang**		**Tanhril**		**Durtlang**	
	2018	**2019**	**2018**	**2019**	**2018**	**2019**
Average Girth (cm)	14.52±1.91	14.70±1.93	17.02±1.49	17.24±1.51	14.27±1.59	14.53±1.59
Average height (cm)	275.5±33.65	278±33.66	264.7±18.30	265.8±18.42	263.9±30.07	267.5±29.77
No. of inflorescence/plants	20.3±2.87	20.1±2.19	21.4±2.65	21.1±2.40	18.6±1.74	18.4±1.64
Flowers /inflorescence	56.2±2.48	52.9±3.44	56.4±5.70	55.7±3.41	43.9±3.23	40.3±3.78
Stamens/flower	4	4	4	4	4	4
Pollen grains/anther	1190.7±62.68	1178.1±50.86	1123.7±55.20	1114.5±49.10	756.4±28.88	726.3±30.94
Pollen grains/flower	4762.8±250.72	4712.4±203.46	4494.8±220.80	4458±196.42	3025.6±115.54	2905.2±123.79
Pollen grains/plant	5433688.008	5010647.796	5425043.808	5239353.66	2470523.424	2154263.904
No. of ovules /flower	4	4	4	4	4	4
Pollen: ovule ratio	1190.7	1178.1	1123.7	1114.5	756.4	726.3
Fruit setting (%)/inflorescence	58.84±6.59	59.09±2.78	65.30±5.93	64.06±5.97	46.73±6.54	49.17±11.09
Fruit setting (%) per plant	52.17±7.47	48.89±6.92	55.89±5.78	54.58±9.61	38.93±4.45	39.70±4.83
Seed set/fruit	2.5±0.34	2.3±0.36	2.7±0.29	2.5±0.34	2.1±0.29	2.3±0.36

Mean ± S.D. (Standard deviation)

Table 4.10 Annual production of flower, pollen and fruit setting in two different sites in *Clerodendrum infortunatum*

Observed variables	**Sairang**		**Tanhril**	
	2019	**2020**	**2019**	**2020**
Average Girth (cm)	5.5±0.40	5.69±0.41	7.41±0.60	7.88±0.62
Average height (cm)	185.6±10.02	199.9±10.78	247.2±9.48	259.9±9.86
Number of inflorescence/plants	14.2±2.11	12.1±2.02	17.8±1.52	17.9±1.52
Flowers/inflorescence	78.4±9.44	76.4±10.58	94.6±8.20	93±9.28
Stamens/flower	4	4	4	4
Pollen grain/anther	950.2±39.53	1033.7±58.80	1218.9±43.78	1119.3±48.61
Pollen grain/flower	3800.8±158.13	4134.8±235.23	4875.6±175.15	4477.2±194.47
Pollen grain/plant	4231354.624	4285099.98	8209925.328	1044632.544
No. of ovules /flower	4	4	4	4
Pollen: ovule ratio	950.2	1033.7	1218.9	1119.3
Fruit setting (%)/inflorescence	38.53±6.77	37.30±7.17	42.15±10.17	39.59±7.24
Fruit setting (%) per plant	32.02±2.41	34.17±4.24	34.83±1.72	33.73±3.43
Seed set/fruit	2.7±0.28	2.4±0.32	2.7±0.28	2.5±0.32

Mean ± S.D. (Standard deviation)

Table 4.11 Annual production of flower, pollen and fruit setting in *Clerodendrum serratum* at Hlimen

Observed variables	**Hlimen**	
	2019	**2021**
Average Girth (cm)	1.88±0.12	2.29±0.12
Average height (cm)	215.8±13.76	226.7±13.80
Number of inflorescence/plants	21.9±2.26	22.1±2.50
Flowers/inflorescence	44.9±2.43	39.2±3.14
Stamens/flower	4	4
Pollen grain/anther	1368.1±40.56	1303±37.11
Pollen grain/flower	5472.4±162.27	5212±148.45
Pollen grain/plant	5381065.644	4515259.84
No. of ovule/Flower	4	4
Pollen ovule ratios	1368.1	1303
Fruit setting (%)/inflorescence	54.12±6.62	56.23±7.98
Fruit setting (%) Per plant	49.53±2.07	50.25±2.43
Seed set/fruit	2.6±0.28	2.7±0.13

Mean ± S.D. (Standard deviation)

Table 4.12 ANOVA of the effect of plant species on number of flowers, anthers and pollen grains per tree and fruit set

Response variable and source	df	MS	F	P
Number of flowers per tree				
Species	2	2886955	16.81486	<0.0001
Number of pollen grain per anther				
Species	2	256854	11.3255	<0.0001
Number of pollen grain per flower				
Species	2	4109664	11.3255	<0.0001
Number of pollen grain per tree				
Species	2	2.64E+13	4.263384	0.018815
Fruit set				
Species	2	2873.571667	49.069669	<0.0001

Table 4.13 ANOVA of the effect of year and population on number of flowers, anthers and pollen grains per tree and fruit set *Clerodendrum colebrookianum*

Response variable and source	df	MS	F	p
Number of flowers per tree				
Year	1	56598.53	0.328115	0.568985
Population	2	940334.1	6.551888	0.002747
Year * population	2	3324.138	0.022113	0.978138
Number of pollen grain per anther				
Year	1	4489.35	0.073795	0.786853
Population	2	1144175	52.40171	<0.0001
Year * population	2	628.85	0.027411	0.972975
Number of pollen grain per flower				
Year	1	71829.6	0.073795	0.786853
Population	2	18306798	52.40171	<0.0001
Year * population	2	10061.6	0.027411	0.972975
Number of pollen grain per tree				
Year	1	1.43E+12	0.29674	0.588022
Population	2	5.87E+13	20.53468	<0.0001
Year * population	2	7.07E+10	0.023659	0.976628
Fruit set				
Year	1	3.51264	0.036641	0.848867
Population	2	1381.317	27.92677	<0.0001
Year * population	2	17.11007	0.350644	0.705826

Table 4.14 ANOVA of the effect of year and population on number of flowers, anthers and pollen grains per tree and fruit set *Clerodendrum infortunatum*

Response variable and source	df	MS	F	p
Number of flowers per tree				
Year	1	23092.83	0.069501	0.793489
Population	1	3593703	15.08043	0.000399
Year * population	1	8337.656	0.033262	0.856309
Number of pollen grain per anther				
Year	1	648.025	0.018547	0.892393
Population	1	313821.2	11.75415	0.001474
Year * population	1	83814.03	3.244097	0.080063
Number of pollen grain per flower				
Year	1	10368.4	0.018547	0.892393
Population	1	5021140	11.75415	0.001474
Year * population	1	1341024	3.244097	0.080063
Number of pollen grain per tree				
Year	1	5.83E+13	8.817264	0.005146
Population	1	1.67E+12	0.206252	0.652305
Year * population	1	1.11E+14	28.82781	4.84E-06
Fruit set				
Year	1	35.92203	0.519716	0.475375
Population	1	86.92243	1.282486	0.264533
Year * population	1	4.397114	0.06244	0.804101

Table 4.15 ANOVA of the effect of year and number of flowers, anthers and pollen grains per tree and fruit set *Clerodendrum serratum*.

Response variable and source	df	MS	F	p
Number of flowers per tree				
Year	1	68433.3	0.61701	0.442375
Number of pollen grain per anther				
Year	1	21190.05	1.261801	0.27607
Number of pollen grain per flower				
Year	1	339040.8	1.261801	0.27607
Number of pollen grain per tree				
Year	1	3.41E+12	0.960093	0.34015
Fruit set				
Year	1	22.22576	0.371343	0.549888

Table 4.16 Correlation coefficient between number of inflorescence/plant, flower/inflorescence, pollen grain/flower, pollen grain/plant, pollen: ovule ratio, fruit setting %/inflorescence and fruit setting %/plant in *C. colebrookianum* at Sairang (low altitude).

		no. of inflorescence /plant	flower/ inflorescence	pollen grain/anther	pollen grain/flower	pollen grain/plant	pollen: ovule ratio	fruit setting %/inflorescence	fruit setting %/plant
no. of	r	1							
inflorescence	p								
/plant									
flower/	r	-.218	1						
inflorescence	p	.546							
pollen	r	.479	.029	1					
grain/anther	p	.161	.937						
pollen	r	.479	.029	1.000**	1				
grain/flower	p	.161	.937	.000					
pollen	r	1.000**	-.216	.483	.483	1			
grain/plant	p	.000	.549	.158	.158				
pollen: ovule	r	.479	.029	1.000**	1.000**	.483	1		
ratio	p	.161	.937	.000	.000	.158			
fruit setting	r	.434	-.193	-.429	-.429	.431	-.429	1	
%/inflorescence	p	.210	.594	.216	.216	.213	.216		
fruit setting	r	.352	.105	.159	.159	.349	.159	.458	1
%/plant	p	.318	.773	.660	.660	.323	.660	.183	

**. Correlation at 0.01

Table 4.17 Correlation coefficient between number of inflorescence/plant, flower/inflorescence, pollen grain/flower, pollen grain/plant, pollen: ovule ratio, fruit setting %/inflorescence and fruit setting %/plant in *C. colebrookianum* at Tanhril (mid altitude).

		no. of inflorescence /plant	flower/ inflorescence	pollen grain/anther	pollen grain/flower	pollen grain/plant	pollen: ovule ratio	fruit setting %/inflorescence	fruit setting %/plant
no. of	r	1							
inflorescence	p								
/plant									
flower/	r	-.203	1						
inflorescence	p	.573							
pollen	r	-.095	.102	1					
grain/anther	p	.794	.778						
pollen	r	-.095	.102	1.000**	1				
grain/flower	p	.794	.778	.000					
pollen grain/plant	r	1.000**	-.203	-.093	-.093	1			
	p	.000	.573	.798	.798				
pollen: ovule	r	-.095	.102	1.000**	1.000**	-.093	1		
ratio	p	.794	.778	.000	.000	.798			
fruit setting	r	.046	.710*	.191	.191	.045	.191	1	
%/inflorescence	p	.899	.021	.597	.597	.901	.597		
fruit setting	r	.226	-.044	-.103	-.103	.228	-.103	-.268	1
%/plant	p	.529	.904	.778	.778	.527	.778	.455	

**. Correlation at 0.01

*. Correlation at 0.05

Table **4.18** Correlation coefficient between number of inflorescence/plant, flower/inflorescence, pollen grain/flower, pollen grain/plant, pollen: ovule ratio, fruit setting %/inflorescence and fruit setting %/plant in *C. colebrookianum* at Durtlang (high altitude).

		no. of inflorescence /plant	flower/ inflorescence	pollen grain/anther	pollen grain/flower	pollen grain/plant	pollen: ovule ratio	fruit setting %/inflorescence	fruit setting %/plant
no. of	r	1							
inflorescence	p								
/plant									
flower/	r	-.152	1						
inflorescence	p	.676							
pollen	r	-.297	-.727*	1					
grain/anther	p	.404	.017						
Pollen	r	-.297	-.727*	1.000**	1				
grain/flower	p	.404	.017	.000					
pollen	r	1.000**	-.154	-.299	-.299	1			
grain/plant	p	.000	.672	.402	.402				
pollen: ovule	r	-.297	-.727*	1.000**	1.000**	-.299	1		
ratio	p	.404	.017	.000	.000	.402			
fruit setting	r	.301	-.472	.052	.052	.316	.052	1	
%/inflorescence	p	.397	.169	.886	.886	.374	.886		
fruit setting	r	.191	.430	-.242	-.242	.201	-.242	-.044	1
%/plant	p	.598	.215	.501	.501	.577	.501	.903	

**. Correlation at 0.01; *. Correlation at 0.05

Table 4.19 Correlation coefficient between number of inflorescence/plant, flower/inflorescence, pollen grain/flower, pollen grain/plant, pollen: ovule ratio, fruit setting %/inflorescence and fruit setting %/plant in *C. infortunatum* at Tanhril (mid altitude)

		no. of inflorescence /plant	flower/ inflorescence	pollen grain/anther	pollen grain/flower	pollen grain/plant	pollen: ovule ratio	fruit set %/inflorescence	fruit setting %/plant
no. of	r	1							
inflorescence	p								
/plant									
flower/	r	-.307	1						
inflorescence	p	.389							
pollen	r	-.494	.236	1					
grain/anther	p	.147	.511						
pollen	r	-.494	.236	1.000**	1				
grain/flower	p	.147	.511	.000					
pollen	r	.975**	-.426	-.549	-.549	1			
grain/plant	p	.000	.219	.100	.100				
pollen: ovule	r	-.494	.236	1.000**	1.000**	-.549	1		
ratio	p	.147	.511	.000	.000	.100			
fruit set	r	.232	-.343	.032	.032	.262	.032	1	
%/inflorescence	p	.520	.333	.929	.929	.464	.929		
fruit setting	r	.597	.020	-.533	-.533	.541	-.533	.290	1
%/plant	p	.068	.956	.112	.112	.106	.112	.417	

**. Correlation at 0.01

Table 4.20 Correlation coefficient between number of inflorescence/plant, flower/inflorescence, pollen grain/flower, pollen grain/plant, pollen: ovule ratio, fruit setting %/inflorescence and fruit setting %/plant in *C. infortunatum* at Sairang (low altitude)

		no. of inflorescence /plant	flower/ inflorescence	pollen grain/anther	pollen grain/flower	pollen grain/plant	pollen: ovule ratio	fruit set%/inflorescence	fruit setting %/plant
no. of	r	1							
inflorescence	p								
/plant									
flower/	r	-.202	1						
inflorescence	p	.576							
pollen	r	-.110	.469	1					
grain/anther	p	.762	.171						
pollen	r	-.110	.469	1.000**	1				
grain/flower	p	.762	.171	.000					
pollen grain/plant	r	1.000**	-.202	-.110	-.110	1			
	p	.000	.576	.763	.763				
pollen: ovule ratio	r	-.110	.469	1.000**	1.000**	-.110	1		
	p	.762	.171	.000	.000	.763			
fruit	r	.034	.603	.248	.248	.034	.248	1	
set%/inflorescence	p	.926	.065	.489	.489	.925	.489		
fruit setting	r	.733*	-.437	-.421	-.421	.732*	-.421	-.552	1
%/plant	p	.016	.207	.226	.226	.016	.226	.098	

**. Correlation at 0.01

*. Correlation at 0.05

Table 4.21 Correlation coefficient between number of inflorescence/plant, flower/inflorescence, pollen grain/flower, pollen grain/plant, pollen: ovule ratio, fruit setting %/inflorescence and fruit setting %/plant in *C. serratum* at Hlimen (high altitude).

		no. of inflorescence /plant	flower/ inflorescence	pollen grain/anther	pollen grain/flower	pollen grain/plant	pollen: ovule ratio	fruit set%/inflorescence	fruit setting %/plant
no. of inflorescence /plant	r	1							
	p								
flower/ inflorescence	r	-.456	1						
	p	.185							
pollen grain/anther	r	-.254	.169	1					
	p	.478	.641						
pollen grain/flower	r	-.254	.169	1.000**	1				
	p	.478	.641	.000					
pollen grain/plant	r	.999**	-.473	-.257	-.257	1			
	p	.000	.168	.474	.474				
pollen: ovule ratio	r	-.254	.169	1.000**	1.000**	-.257	1		
	p	.478	.641	.000	.000	.474			
fruit set%/inflorescence	r	.145	.623	.056	.056	.138	.056	1	
	p	.689	.054	.878	.878	.704	.878		
fruit setting %/plant	r	.692*	-.418	.158	.158	.696*	.158	.215	1
	p	.027	.229	.662	.662	.025	.662	.552	

**. Correlation at 0.01

*. Correlation at 0.05

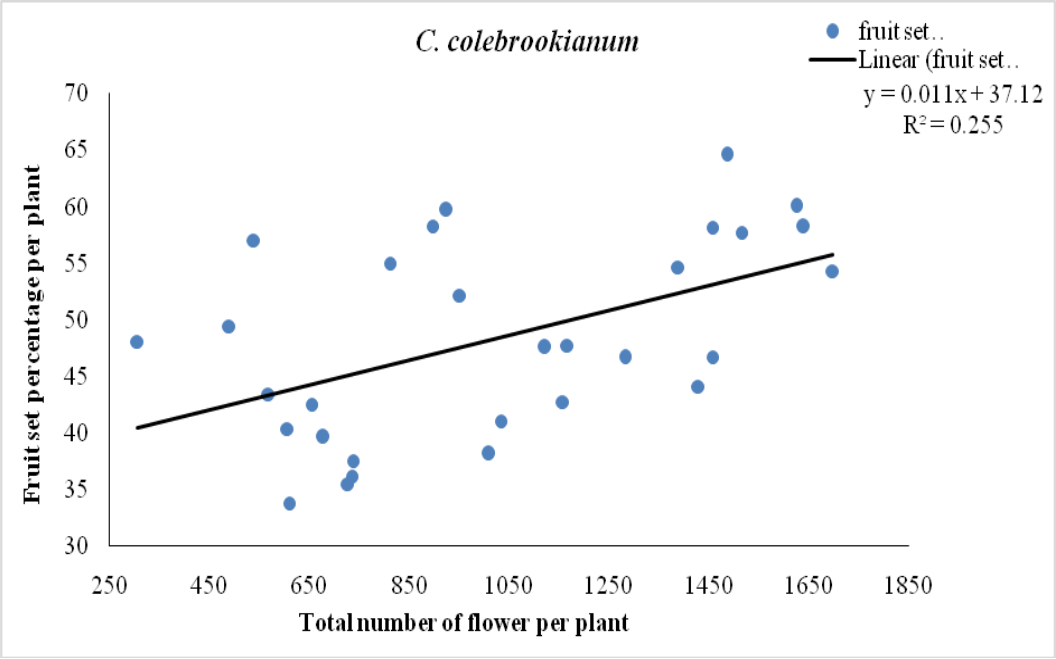

Figure 4.49 Relationship between fruit set percentage per plant and total number of flowers per plant *C. colebrookianum*

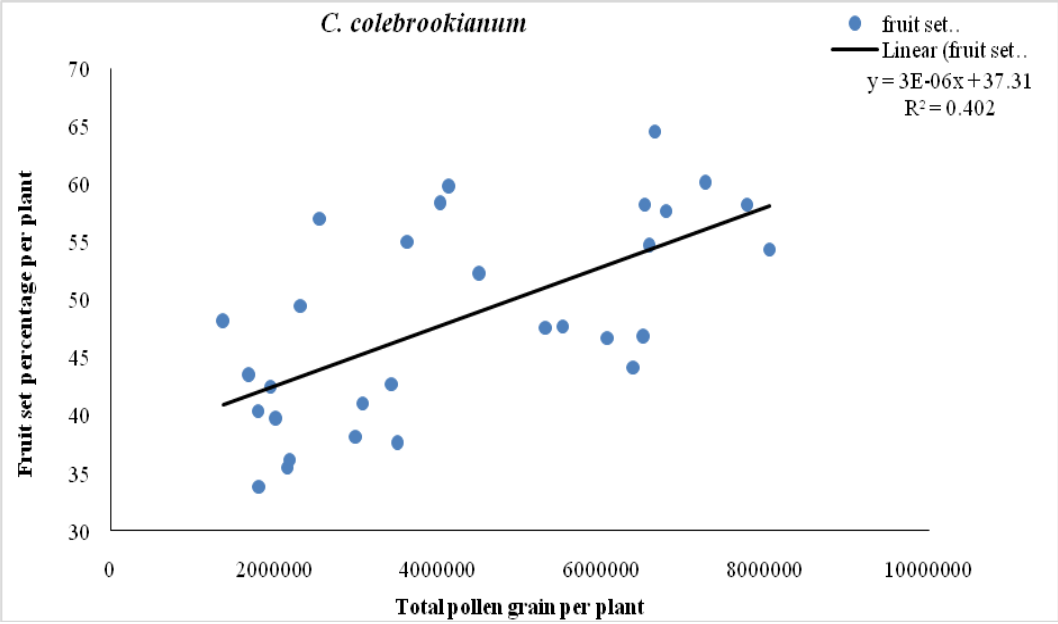

Figure 4.50 Relationship between fruit set percentage per plant and total pollen grain per plant *C. colebrookianum*

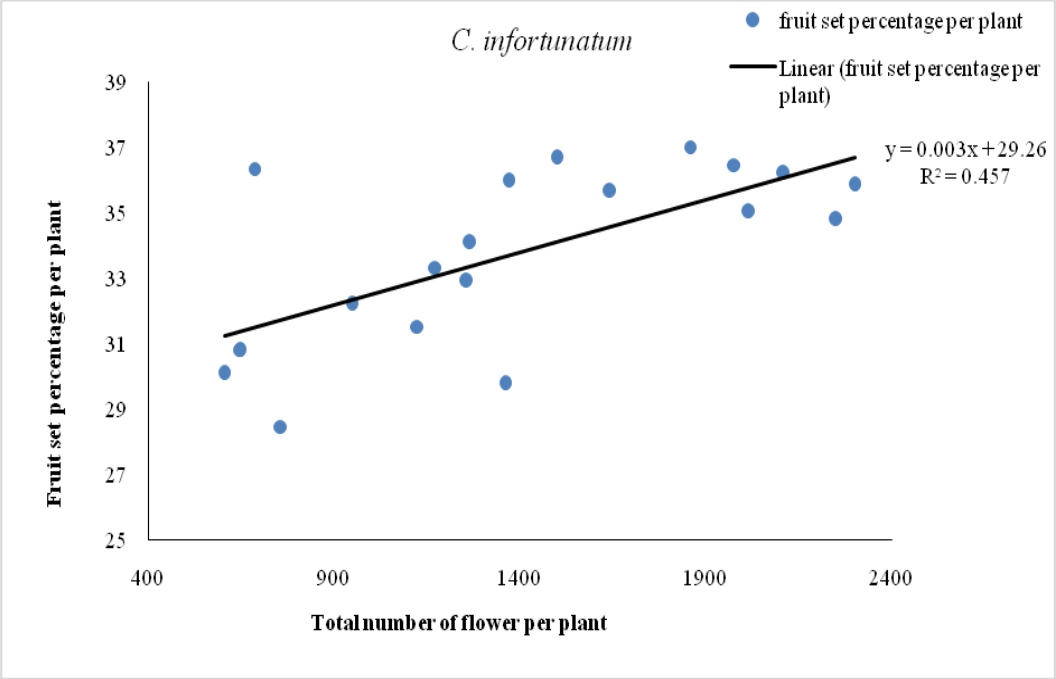

Figure 4.51 Relationship between fruit set percentage per plant and total number of flowers per plant *C. infortunatum*

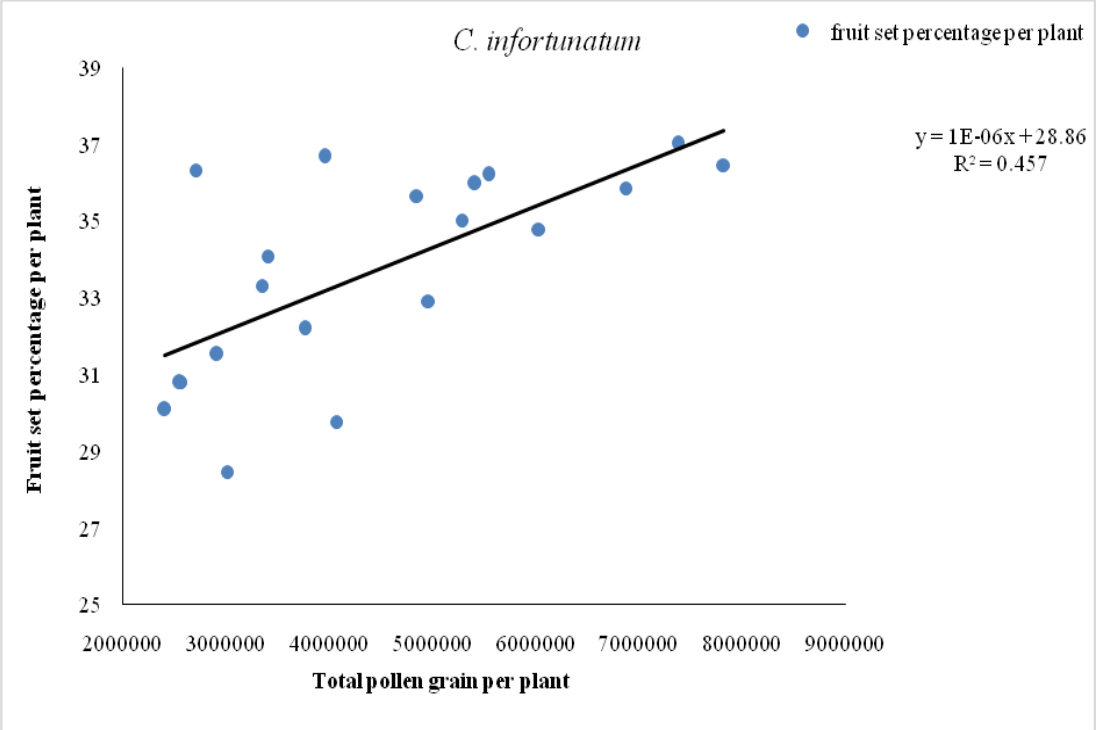

Figure 4.52 Relationship between fruit set percentage per plant and total pollen grain per plant *C. infortunatum*

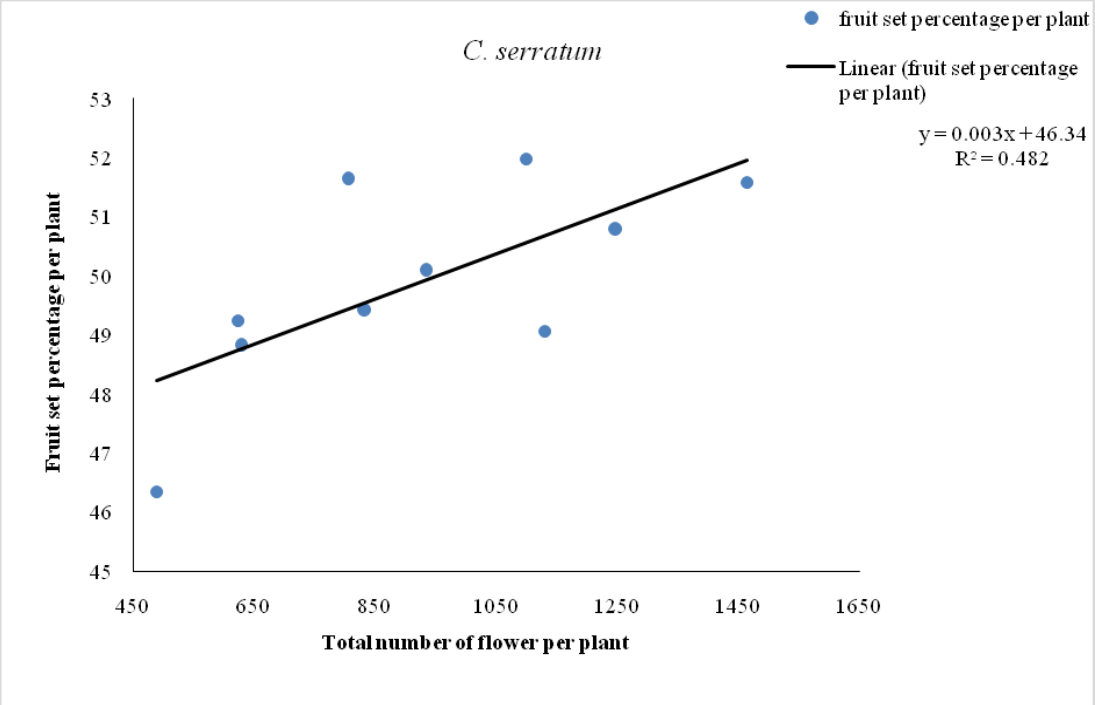

Figure 4.53 Relationship between fruit set percentage per plant and total number of flowers per plant *C. serratum*

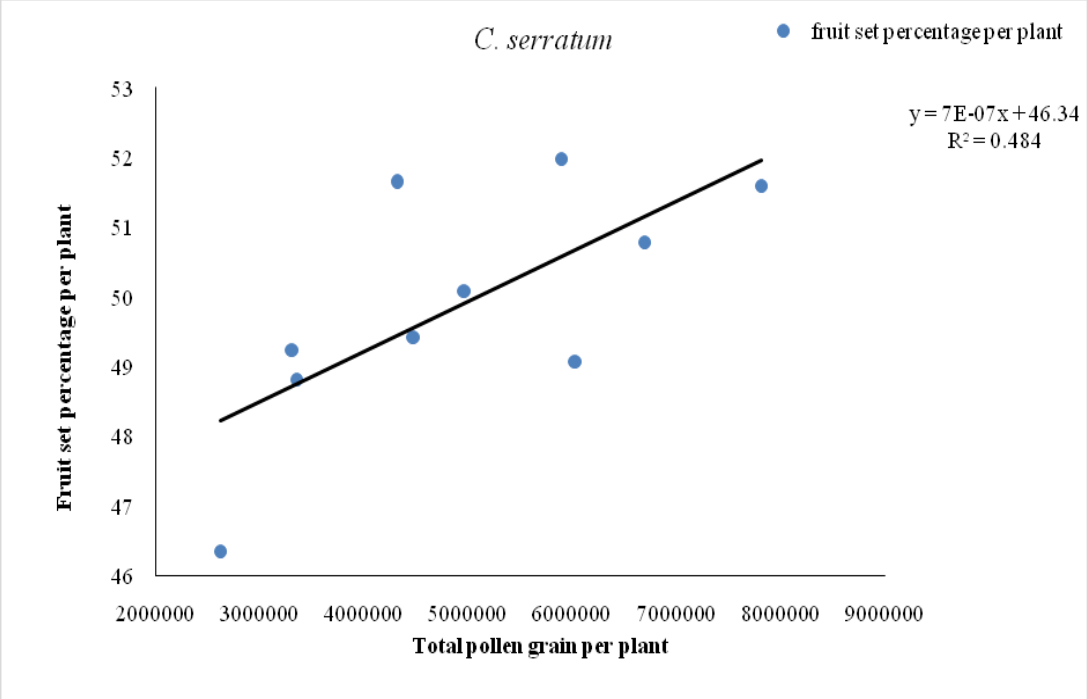

Figure 4.54 Relationship between fruit set percentage per plant and total pollen grain per plant *C. serratum*

Table 4.22 Pollen /ovule ratio of three *Clerodendrum* species.

Plant species	Pollen /ovule ratio	Type of Mating system as P/O ratio	Standard range value Cruden,1977
C. colebrookianum	726.3-1190.7	Facultative xenogamy	224.7-2588.0
C. infortunatum	950.2-1218.9	Facultative xenogamy	224.7-2588.0
C. serratum	1300-1368.1	Facultative xenogamy	224.7-2588.0

Table 4.23 Mating system evaluation in *C. colebrookianum*

Treatment	Fruit set percentage
Control	52.5 ± 11.11
Spontaneous selfing	24 ± 15.05
Induced selfing	21 ± 10.48
Geitonogamy	25.5 ± 13.42
Cross – artificial	55±10.0
Cross natural	54±11.97

Table 4.24 Mating system evaluation in *C. infortunatum*

Treatment	Fruit set percentage
Control	39.5±5.98
Spontaneous selfing	15±10.54
Induced selfing	17.5±12.96
Geitonogamy	19±10.48
Cross – artificial	43.5±9.14
Cross natural	41.5±10.08

Table 4.25 Mating system evaluation in *C. serratum*

Treatment	Fruit set percentage
Control	48.5±9.44
Spontaneous selfing	12.5±9.78
Induced selfing	15.5±9.55
Geitonogamy	19±10.48
Cross – artificial	52.5±11.84
Cross natural	54.5±11.16

Table 4.26 Index of self-incompatibility (ISI) for the three *Clerodendrum* species (Zapata and Arroyo 1978)

Plant species	ISI	Type of Mating system as per ISI
C. colebrrokianum	0.38	partially self-compatible
C. Infortunatum	0.36	partially self-compatible
C. serratum	0.29	partially self-compatible
ISI range	1 or >1	fully self-compatible
	>0.2 but <1	partially self-compatible
	<0.2 or 0.	self-incompatible

Table 4.27 Out crossing Index for the three *Clerodendrum* species (Cruden, 1977).

Variables	Point	*C. colebrrokianum*	*C. Infortunatum*	*C. serratum*
Diameter of the flower	Up to 1mm=0 1-2 mm=1 2-6mm=2 >6mm =3	3	3	3
Temporal separation of anther dehiscence & stigma receptivity	Homogamy, Protogyny=0 Protandry=1	1	1	1
Spatial positioning of stigma & anthers	Same=0 Spatially separated=1	1	1	1
Outcrossing, demand for pollinators		5	5	5
0=Cleistogamy, 1=Obligate autogamy, 2=Facultative autogamy, 3=self-compatible, >4=partially self-compatible				

4.3. Pollen viability, the effect of growth regulators on *in vitro* pollen germination with pollen longevity tests.

Pollen viability tested with TTC for the three *Clerodendrum* species (Fig. 4.60) is as follows, pollen grain viability percentages at the pre-anthesis stage (un-opened flower) are 28.57%±2.61%, 19.37%±1.73% and 18.85%±1.38% in *C. colebrookianum, C. infortunatum* and *C. serratum* respectively. While, pollen grain viability percentage at the anthesis stage (opened flower) are 71.97%±4.30 %, 81.63%±3.23 and 76.62%±2.63% in *C. colebrookianum, C. infortunatum* and *C. serratum* respectively. The above result shows that the pollen viability percent is higher at the anthesis stage (opened flower) compared to pre-anthesis (unopened flower) in all three species of *Clerodendrum* (Table 4.28).

The result of *in vitro* pollen germination in sucrose concentrations (5% and 10%), distilled water (control), with growth regulators (IAA, IBA, GA3, and Kinetin), and their varied concentrations in basal media with 10% sucrose and time outline showed a differential response in all three species *Clerodendrum*. Distilled water (control) showed a poor percentage of germination 0.85±0.23, 0.78±0.21 and 0.49±0.11 in *C. colebrookianum, C. infortunatum* and *C. serratum,* respectively, in the first 24 hours (Fig. 4.55). Later after 48 and 72 hours, there was no germination in all three *Clerodendrum* species. It showed that the presence of only moisture germination capability and viability is low for the selected three *Clerodendrum* species.

Sucrose concentrations (5% and 10%) were found to induce *in vitro* pollen germination and acted as a fundamental substrate compared to the control (distilled water). In 5% sucrose concentration, low percentage of pollen, germination was recorded with 5.19±0.60% in *C. colebrookianum,* 10.65±1.14% in *C. infortunatum* and 6.18±1.94 in *C. serratum* while at 10% sucrose concentration still low germination of 7.54±1.21 was recorded for *C. colebrookianum*, fair germination (17.92±4.93%) in *C. infortunatum* and 12.44±2.73 in *C.* serratum at the initial 24 hours. Further, the germination percentage relatively declined as time passed, i.e., at 48 hours and 72 hours (Fig. 4.55) in all three species. Additionally, it is also

observed that in all three *Clerodendrun* species, with the increment in the concentration of sucrose, the percentage of pollen germination also increased (Fig. 4.55). All the examined pant species ascribed that sucrose is a fundamental substrate for the induction of pollen germination. A significance difference was observed at ($p<0.0001$) (Table 4.29) between distilled water (control) and sucrose 5% & 10%; concentration in all three *Clerodendrum* species. There is a significance effect of time on *in vitro* pollen germination in *C. colebrookianum* and *C. infortunatum* except in *C. serratum*.

The first 24 hours had the highest levels of pollen germination, which then gradually dropped over the following 48 and 72 hours. The selected growth hormones IAA, IBA, GA3 and kinetin all had the lowest germination percentage after 72 hours in all three species of *Clerodendrum* plants at 100, 200, and 300 mg L-1 concentrations. (Table 4.30).

The highest *in vitro* pollen germination rate for *C. colebrookianum* was 52.10±5.30% in GA3 (200 mg L^{-1}), followed by 46.56±4.59% in IBA (100 mg L^{-1}), 44.48±3.26% in GA3 (300 mg L^{-1}), and the lowest, 23.37±1.67% in Kinetin (200 mg L^{-1}). The *in vitro* pollen germination rate for *C. infortunatum* was observed highest with 61.91±1.76% in GA3 (200 mg L^{-1}), followed by 48.61±1.79% in IBA (200 mg L^{-1}), 43.42±2.37% in Kinetin (100 mg L^{-1}) and least 25.45±2.89% in IAA (100 mg L^{-1}). While in the case of *C. serratum* highest *in vitro* pollen germination of 55.81±4.97% was recorded in IAA (100 mg L^{-1}), followed by 52.50±6.61 % in IAA (200 mg L^{-1}), 34.23±9.89% in GA3 (100 mg $L^{-}1$) and least 16.50±3.05% in Kinetin (100 mg $L^{-}1$) respectively.

For inducing *in vitro* pollen germination in *C. colebrookianum* and *C. infortunatum*, GA3 (200 mg L^{-1}) was shown to be the most effective growth hormone concentration, followed by IBA (200 mg $L^{-1}$and 100 mg L^{-1}) (Table 4.30). Contrarily, it was observed that IAA at (100, 200 mg L^{-1}) and GA3 (100 mg L^{-1}) were the best growth hormone concentrations for inducing *in vitro* pollen germination in *C. serratum* (Table 4.30).

Statistically, it was observed that the effects of all the treatments, viz. hormones, their concentrations, and time, on *in vitro* pollen germination of *C. colebrookianum*, *C. infortunatum*, and *C. serratum* was found to be significantly different ($p<0.05$) (Table 4.31).

The treatment of different hormones and times produced the most significant response among the treatments ($p<0.0001$), followed by the application of different hormone concentrations ($p<0.05$). *In vitro* pollen germination results showed a non-significant difference between the study plant species. (Table 4.31).

Pollen viability of all three *Clerodendrum* species decreased with increased storage durations at various temperatures -20°C, -4°C, and 6°C. The pollen viability duration of all three *Clerodendrum* species was found to be relatively longer when stored at -20°C and 6°C; viability was lost after 28 days. Additionally, it was noted that all three *Clerodendrum* species pollens stored at -4°C lost viability after 14 days of storage. As a result, *C. colebrookianum*, *C. infortunatum* and *C. serratum* all follow a similar pattern of storage conditions (Fig. 4.56, 4.57 & 4.58). For each of the three studied plant species, there is a significant difference in storage days ($p<0.0001$) (Table 4.32).

Table 4.28 Pollen viability tested with TTC for three *Clerodendrum* species

Species	*C. colebrookianum*	*C. infortunatum*	*C. serratum*
	Viable %	Viable %	Viable %
Pre anthesis (un-open flower)	28.57%±2.61%	19.37%±1.73%	18.85%±1.38%
Anthesis (open flower)	71.97%±4.30 %,	81.63%±3.23	76.62%±2.63%

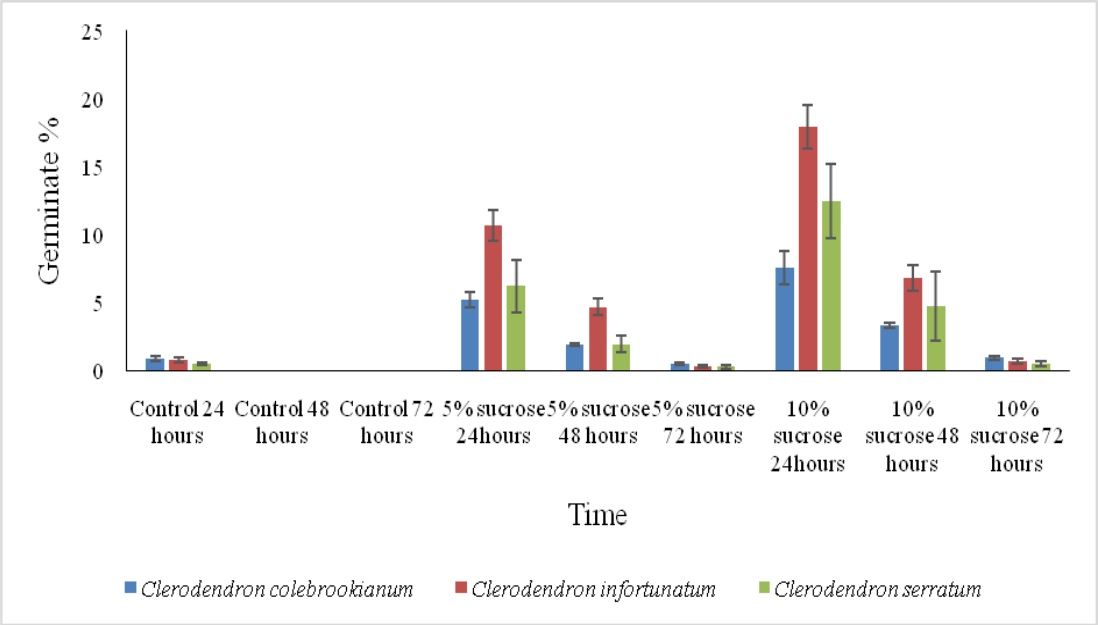

Figure 4.55 Influence of distilled water and sucrose at 5% & 10% on *in vitro* pollen germination of three *Clerodendrum* species.

Table 4.29 ANOVA for the effect distil water and sucrose 5% and 10%, concentration and time.

Response variable		df	MS	F	P
Clerodendrum colebrookianum	between distil water and sucrose 5% & 10%	2	100.94	16.69	<0.0001
	concentration	2	130.03	24.18	<0.0001
	time	2	648.13	76.54	<0.0001
Clerodendrum infortunatum	between distil water and sucrose 5% & 10%	2	511.80	17.57	<0.0001
	concentration	2	686.20	27.31	<0.0001
	time	2	3640.49	129.81	<0.0001
Clerodendrum serratum	between distil water and sucrose (5% and 10%)	2	245.54	17.44	<0.0001
	concentration	2	293.12	22.58	<0.0001
	time	2	55.01	0.99	0.375704

Table 4.30 Effect of growth hormones on *in vitro* pollen germination in two *Clerodendrum* species

Hormone	Concentration	Pollen germination %								
		C. colebrookianum			*C. infortunatum*			*C. serratum*		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
IAA (10 % Sucrose)	100 mg L^1	39.88 ±3.70	7.12 ±0.81	1.16± 0.25	25.45 ±2.89	6.00± 1.01	0.75± 0.16	55.81 ±4.97	12.28 ±2.75	1.08± 0.48
	200 mg L^1	31.80 ±1.97	4.73 ±0.61	0.42± 0.15	37.02 ±3.21	8.25± 1.39	1.46± 0.24	52.50 ±6.61	9.91± 2.08	0.54± 0.22
	300 mgL^1	26.86 ±3.08	4.64 ±0.70	0.67± 0.18	26.16 ±3.13	3.77± 0.55	0.38± 0.08	33.13 ±9.24	8.47± 2.58	0.25± 0.18
IBA (10 % Sucrose)	100 mg L^1	46.56 ±4.59	9.20 ±0.90	2.15± 0.28	34.32 ±3.30	6.83± 0.91	1.34± 0.22	27.14 ±6.69	12.13 ±3.83	0.50± 0.25
	200 mg L^1	35.87 ±2.07	6.37 ±0.58	1.01± 0.20	48.61 ±1.79	10.47 ±0.91	2.22± 0.30	35.66 ±2.90	9.24± 2.68	0.11± 0.10
	300 mgL^1	28.99 ±3.75	5.73 ±0.63	1.40± 0.23	38.27 ±4.83	9.21± 1.40	1.80± 0.24	23.47 ±9.82	6.07± 2.34	0.49± 0.06
GA_3 (10 % Sucrose)	100 mg L^1	34.02 ±1.71	5.63 ±0.66	1.25± 0.19	39.56 ±3.70	7.00± 0.88	1.63± 0.24	34.23 ±9.89	12.63 ±3.14	1.17± 0.58
	200 mg L^1	52.10 ±5.30	10.56±0.74	2.03± 0.20	61.91 ±1.76	10.44 ±1.12	1.92± 0.33	19.90 ±6.50	4.48± 2.01	0.23± 0.15
	300 mgL^1	44.48 ±3.26	6.52 ±1.16	0.88± 0.15	42.88 ±5.25	7.19± 1.01	1.69± 0.29	28.62 ±6.86	7.50± 3.16	0.77± 0.17
Kinetin (10 % Sucrose)	100 mg L^1	36.06 ±4.04	6.44 ±0.69	1.50± 0.21	43.42 ±2.37	8.87± 1.45	2.02± 0.35	16.50 ±3.05	5.48± 2.86	0.10± 0.09
	200 mg L^1	23.37 ±1.67	4.85 ±0.55	1.22± 0.18	31.68 ±2.56	5.94± 0.73	0.44± 0.12	27.53 ±6.34	11.90 ±3.06	0.51± 0.23
	300 mgL^1	28.96 ±3.08	5.90 ±0.56	1.08± 0.29	37.31 ±4.08	6.63± 0.73	1.47± 0.22	16.66 ±4.28	3.05± 1.05	0.05± 0.08

Table 4.31 ANOVA for the effect of hormone**s**, concentration, time and species

Response variable		df	MS	F	p
Clerodendrum colebrookianum	hormone	3	1548.28	11.06	<0.0001
	concentration	2	41515.14	708.19	<0.0001
	time	2	2059.96	15.81	<0.0001
	hormone*concentration	6	1032.52	7.37	<0.0001
	concentration*time	4	116.45	1.98	0.096079
	hormone*time	22	13.96	0.10	1
Clerodendrum infortunatum	hormone	3	2492.38	17.04	<0.0001
	concentration	2	48439.65	742.43	<0.0001
	time	2	2798.12	20.81	<0.0001
	hormone*concentration	6	935.69	6.39	<0.0001
	concentration*time	4	289.30	4.43	0.001651
	hormone*time	22	17.76	0.13	0.999999
Clerodendrum serratum	hormone	3	5015.83	42.29	<0.0001
	concentration	2	29885.03	384.33	<0.0001
	time	2	3122.55	29.02	<0.0001
	hormone*concentration	6	1111.34	9.37	<0.0001
	concentration*time	4	224.96	2.89	0.022243
	hormone*time	22	40.16	0.37	0.996076
Between Species		2	0.36	1.09	0.350227

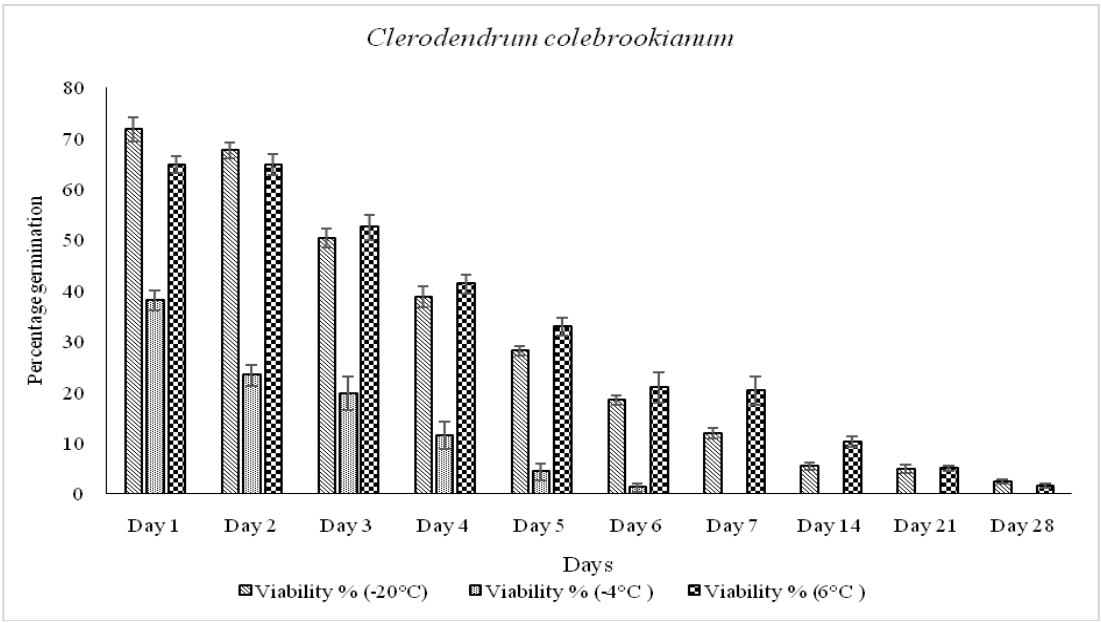

Figure 4.56 Pollen storage of *Clerodendrum colebrookianum* under different storage temperatures (-20°C, -4°C and 6°C)

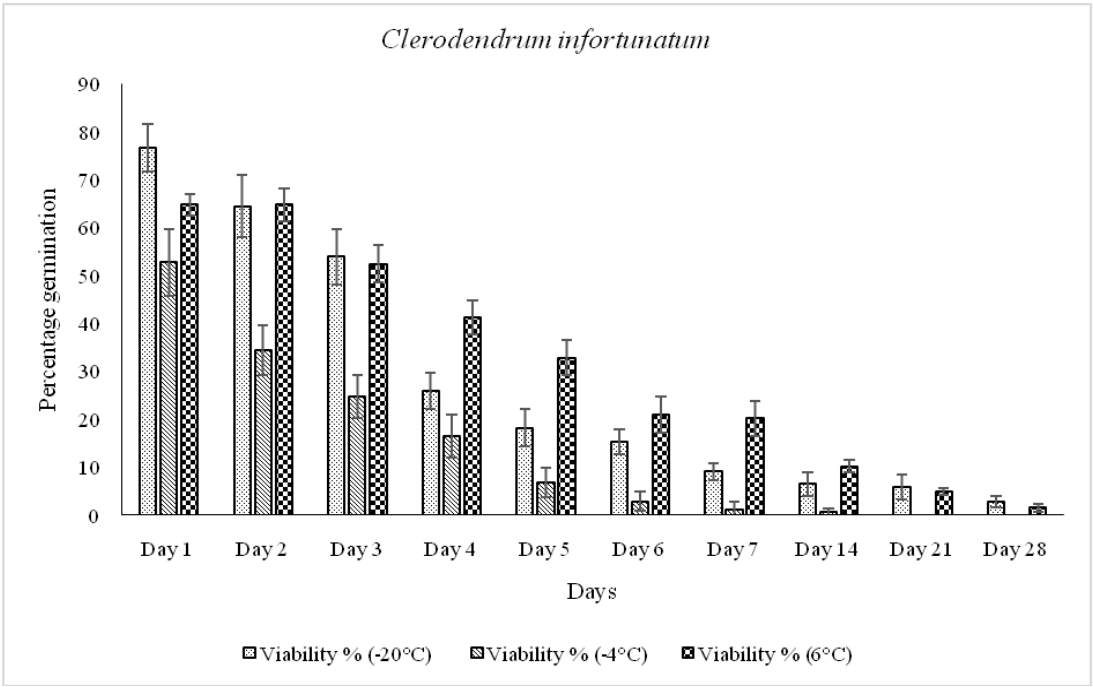

Figure 4.57 Pollen storage of *Clerodendrum infortunatum* under different storage temperatures
(-20°C, -4°C and 6°C).

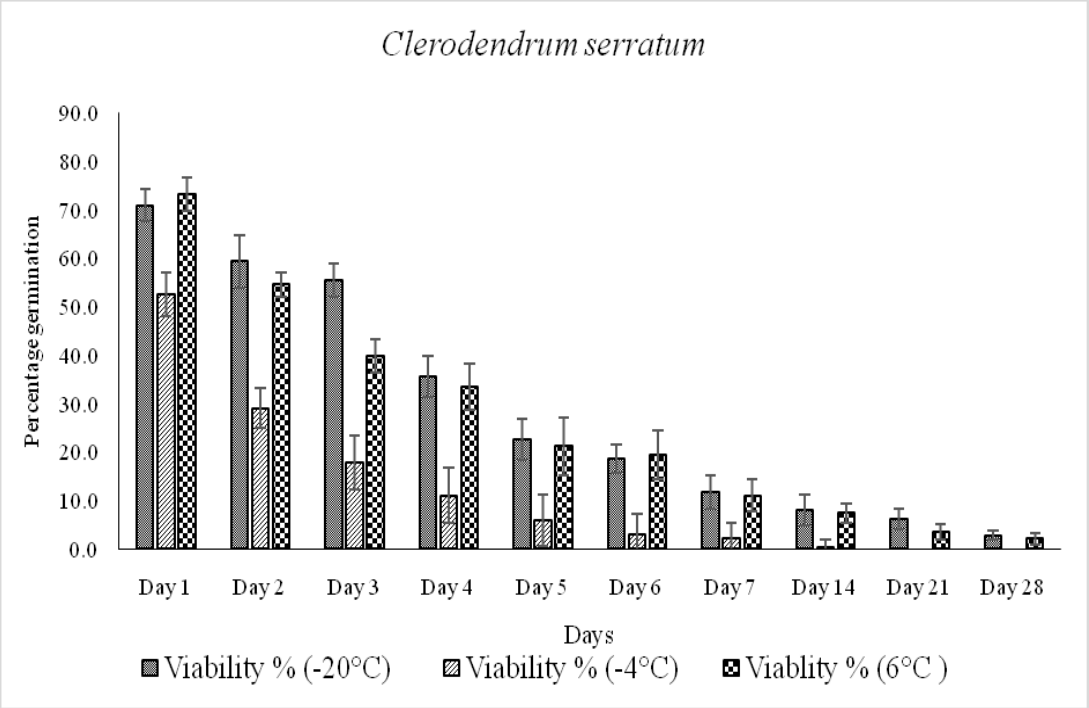

Figure 4.58 Pollen storage of *Clerodendrum serratum* under different storage temperatures
(-20°C, -4°C and 6°C)

Table 4.32 ANOVA of pollen storage with treatment and days of three *Clerodendrum* species.

Response variable		df	MS	F	p
C. colebrookianum	Temperatures	2	1455.023	3.086758	0.062048
	Days	9	1287.24199	6.353600081	0.000292681
C. infortunatum	Temperatures	2	687.1855	1.238276	0.305827
	Days	9	1588.280339	15.39339493	<0.0001
C. serratum	Temperatures	2	830.7029	1.711093	0.199683
	Days	9	1382.365705	11.87550031	<0.0001

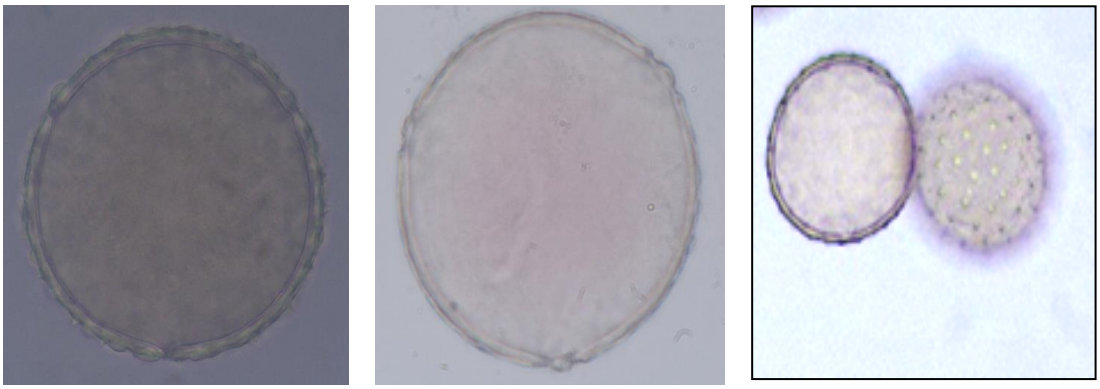

Figure 4.59 Pollen grains of (A) *C. coolebrookianum*; (B) *C. infornatum* & (C) *C. serratum* (40x)

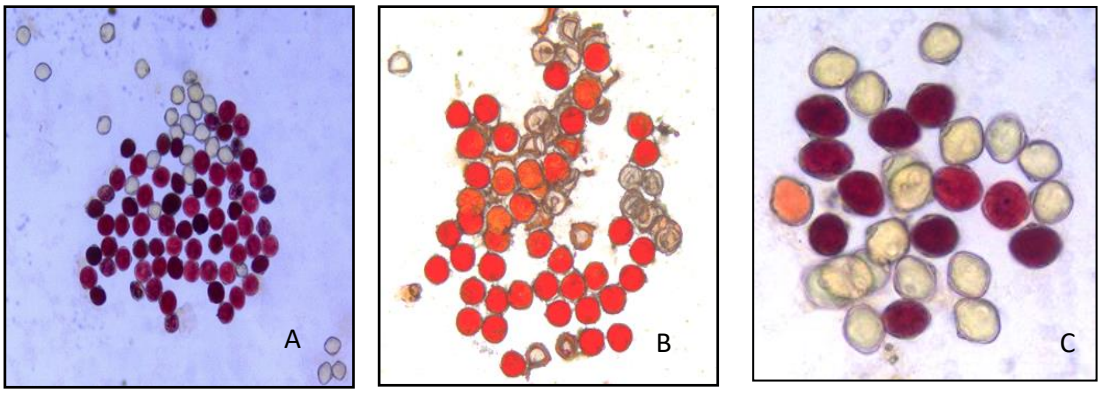

Figure 4.60 Pollen viability of (A) *C. coolebrookianum*; (B) *C. infornatum* & (C) *C. serratum* tested with TTC, red stained pollen grains are viable

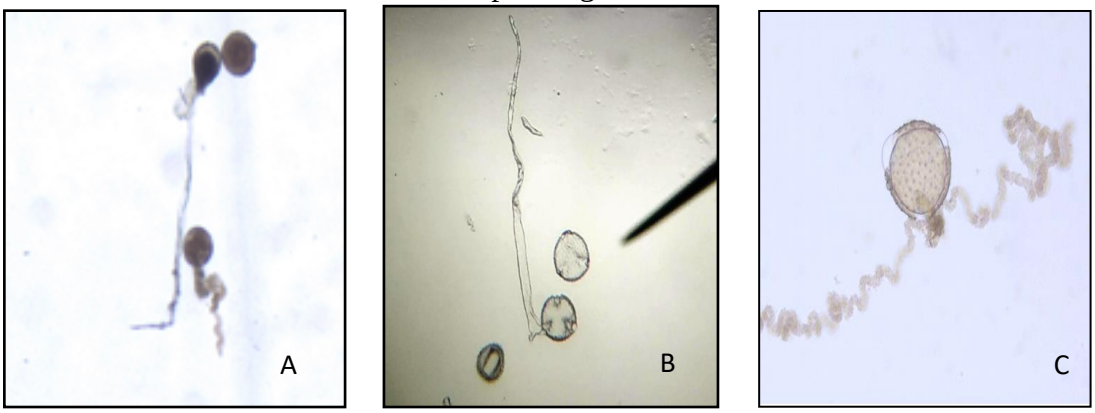

Figure 4.61 *in vitro* germinating pollens of (A) *C. coolebrookianum*; (B) *C. infornatum* & (C) *C. serratum;* if pollen tube length *is* ≥ than diameter of pollen

Chapter 5

Discussion

5.1.1. Flowering phenology

C. colebrookianum, C. infortunatum and *C. serratum* have a regular annual flowering season like other tropical flowering plant species. *C. colebrookianum* and *C. infortunatum* mostly found along roadsides, in forest edges, moist, shady places and amidst bushes habitat of North Eastern region of India (Kalita et al., 2012 and Kumar et al., 2017). As reported above the *C. colebrookianum* and *C. infortunatum* is found mostly in similar habitats in our study sites too, apart from it *C. colebrookianum* was found to grown in the local home garden of Mizoram due its traditional medicinal uses. *C. colebrookianum* flowered during July to December in the present study site. *C. infortunatum* flowered during January to April and maximum during mid-February and mid-March in Araku Valley Reserve Forest in Visakhapatnam, Andhra Pradesh (Kumar et al., 2017) while our study site flowering starts from mid of February and continues till April and the peak flowering phase occurred during March. *C. serratum* are found in forest sites at an altitude up to 1500 m and annual flowering season during the month of August to September (Patel et al., 2014 and Poornima et al., 2015). In the present study site, it was observed that *C. serratum* was found to grow at an altitude of 1140 m and flowered during March to July. It was observed that the flowering phenology varied from year to year within population of *C. colebrookianum, C. infortunatum* and *C. Serratum*. These three-plant species of *Clerodendrum* species exhibited well defined seasonality and temporal separation in annual flowering time with each other. Such separation and variation in closely related flowering plant species is of ecological and evolutionary significance. Thus, such variation in floral biological rhythms helps in avoiding pollinator's competition.

The flowers bloomed in an umbel pattern in *C. colebrookianum* while pyramid shape in *C. Serratum* and *C. infortunatum* (Kumar et al., 2017). Different reproductive developmental stages viz. budding, flowering and fruiting found to co-occur in the same inflorescence all the three study *Clerodendrum* species. Such pattern of reproductive developmental pattern was observed in *Clerodendrum inerme* (Aluri et

al., 2016). Difference in development of flowering stages among inflorescence within same plants shall helpful in promoting xenogamy and geitonogamy through floral visitors.

5.1.2. Anthesis, Anther dehiscence and Stigma receptivity

Differential pattern of anthesis was recored in *Clerodendrum* species. The flower opens during morning hours' in *C. infortunatum* (Kumar et al., 2017), *C. inerme* flowered during evening hour (Aluri, et al., 2016), *C. molle* during evening time (Mc Mullen, 2011), while *C. phlomidis* open during night time (Rohitash, 2016). During the study period it was observed that the flower opens during morning hours in *C. colebrookianum*, *C. infortunatum* and *C. Serratum*. All the three *Clerodendrum* species with the increased in temperature during morning hours it help in the developmental and maturation of flower during anthesis. Weather fluctuations such as temperature and humidity are prime importance in predicting behaviour floral development in plants. Both anthesis and anther dehiscence were significantly regulated by temperature and relative humidity (Khanduri et al., 2013). Increased in the temperature during and before the anthesis of *Allium sativum* help in the development of flower (Mayer et. al., 2015). Anther dehiscence of *Euphorbia pulcherrima* varied according to time, temperature and humidity (Vargas et al., 2017). The anthesis of the flower is more dependent to temperature (Shahbaz et al., 2021) while anther dehiscence was more strongly influenced by relative humidity (Khanduri et al., 2013; Bera et. al., 2018). Extent of anthesis i.e. the amount flower opens per unit time is played crucial role in pollination, fertilization and gene flow in plant species.

The receptivity of *C. infortunatum* observed on the next day of anthesis (Kumar et al., 2017) and receptivity of *C. indicum* observed on the third day from anthesis (Ghosh & Pal, 2017). In the present study of three *Clerodendrum* species it was observed that receptivity of stigma occurred after 3-4 days from anthesis in *C. colebrookianum* and after the next day from anthesis in *C. infortunatum* and *C. serratum*. It was observed that receptivity of stigma correlated with respect to time, temperature and humidity in all three species of *Clerodendrum*. The receptivity of

the female part of the flower is influenced by both temperature and humidity (Vargas et al., 2017; Khanduri et al., 2013). Thus, manipulating the receptivity could be of importance in promoting fertilization and fruit set. In Douglas fir, receptivity of stigma was extended by artificial overhead water-cooling treatment (Sk Lai et al., 2010).

In our present finding all the three *Clerodendrum* species exhibited distinct protandous, dichogamous and herkoganous maturation of male reproductive part i.e. stamen first and maturation of female later with physical and temporal separation of male and female during anthesis of flower (Fig. 4.4, 4.10 & 4.16). The stigma is receptive only for one day all the three species. In *C. infortunatum*, *C. inerme* and *C. serratum* is reported to be protandous, herkogamous and dichogamous (Singh et al., 2012; Aluri et al., 2016 and Mukhopadhyay & Quader, 2022). Flower with typical adaptations of herkogamy and dichogamy becomes potential donor to conspecific flowers and at the same time the donor flower itself become a site for pollen reception (Waines & Hegde, 2003). Since the anthesis, anther dehiscence, and receptivity differed significantly within the same inflorescence and at population in all the three-study species will influence pollination, mating system and potential gene flow at population level.

5.1.3. Flower pollinators

During the present study, it was observed that all three species of *Clerodendrum* are visited by moths, butterflies, bees, ants, and bugs that belong to the order Lepidoptera, Hymenoptera, and Hemiptera. As the anthesis begins, the floral features such as flower color, nectar, pollen, and odour/fragrance of the flower attract different pollinators. The order Lepidoptera and Hymenoptera are the primary floral visitors in all three *Clerodendrum* species, which include moths, butterflies, and bees. As pollen is an essential source of proteins and lipids for many insects, thus all three-plant species of *Clerodendrum* are attracted by many insect pollinators.

C. colebrookianum with morning anthesis, white corolla, unpleasant order, landing platform, and floral tube with deep-seated concentrated nectar appeared to adapt toward Lepidoptera (butterflies and moths). *C. infortunatum* morning anthesis, with

the white corolla, distinct nectar guide, sweet odour, landing platform, and floral tube with deep-seated nectar, appeared to adapt toward both Lepidoptera (butterflies and moths) and Hymenoptera (bees), while *C. serratum* exhibited morning anthesis, with the purple corolla, pubescent nectar guide, landing platform, and short floral tube, minute droplet nectar secreted on hairy nectar guide appeared to adapt toward more Hymenoptera (bees) and less to Lepidoptera (butterflies and moths).

According to Subba and Solomon (1997), papilionoid butterflies are the only pollinators of *C. infortunatum* with morning anthesis; they noticed that pollination took place as a result of butterfly wings striking anthers and stigma. Aluri et al. (2016) reported that *C. inerme* with evening anthesis, long white corolla, hairy interior, and strong fragrance appeared to attract hawk-moth *Macroglossum gyrans* as the main pollinator. And he further mentioned that this plant species is visited by insect orders belonging to Lepidoptera and Hemiptera as pollinators. According to Guddeti (2014), the characteristic butterfly pollination of *C. infortunatum* is caused by the non-promiscuity of floral rewards to other foragers. She added that the development of the floral isolating mechanism is a crucial prerequisite for this pollination syndrome.

Wadhwa & Sihag (2012) reported that *Rauvolfia serpentina*, Lepidopterans, and Hemipterans were observed as pollinators. They concluded that all these pollinators species were not equally and sufficient pollinators for this crop. In our finding, the floral adaptation of the flower *C. colebrookianum* and *C. infortunatum* is highly suited for Lepidopterans, while the floral adaptation of the flower *C. serratum* is suited for Hymenopterans apart from other groups of floral visitors, seems to assist in playing a role in the pollination.

Proboscis length is a crucial organ of Lepidoptera for harvesting nectar from flower *C. colebrookianum* and *C. infortunatum*, while harvesting nectar, landing and sitting posture (Zhang et al., 2010b) of insect play a crucial role in pollen transfer. While harvesting nectar from the flower, the head of the insect is pressed against the floral tube of the flower, resulting in contact with the flower's reproductive organs. Cruden et al. (1983) reported that the flowers pollinated by the insect order

Lepidoptera are rich in nectar sugar concentration ranging from 15-25%. From our findings, the two species, *C. colebrookianum* and *C. infortunatum* are pollinated mainly by the insect order Lepidoptera, and the flower is also rich in nectar sugar concentration ranging from 18-23%.

Butler & Johnson (2020) reported that the wing of butterfly help in the pollination of *Scadoxus multiflorus*, a South African Amaryllidaceae plant species. He observed that the umbel inflorescence flower shape of *Scadoxus multiflorus* gives a perfect landing platform for the butterfly species and helps facilitate pollination. The striking of butterflies and moths' wings helps pollinate *C. colebrookianum* and *C. infortunatum.* The presence of pollen on the wings, proboscis, and legs of butterflies and moths and the deposition of pollen on the stigmatic surface prove that they help in the pollination of the flower during their visitation. The elongated filament and styles with umbel and pyramidal inflorescence shape with compact flower arrangement provide a perfect landing platform for the butterflies and moths pollinator, which supports that *C. colebrookianum* and *C. infortunatum* is well adapted to Lepidoptera for pollination. The presence of deep-seated nectar in *C. colebrookianum* and *C. infortunatum* was very little about (0.5-1µl and 3-4 µl respectively), promote and force butterflies and moths move from one flower to another flower to quench their thirst for nectar, during this movement, they help in pollen transfer which leads to cross-pollination. Whereas *C. serratum* they don't show uniform inflorescence shape, which does not provide a proper landing position for butterflies and moth's pollinator, and the nectar are present in the form of the minute droplets (Fig. 4.7); this shows that *C. serratum* is not well adapted to Lepidoptera for pollination.

Bees often have dense body hairs, long or short proboscis, and pollen baskets (Wojtaszek & Maier, 2014). Observation showed that the flower of *C. colebrookianum* was visited by *Amegilla cingulata, Bombus albopleuralis,* and *Trigona carbonaria*. *C. infortunatum* was visited by *Apis cerana indica, Xylocopa micans* and *Xylocopa virginica;* pollen grains, sweet odour, nectar guide, and deep-seated nectar attracted the bee's pollinator. However, these

pollinators are symmetrically not fit for deep-seated nectar from the floral tube since their proboscis length is shorter than the floral tube length. The above finding supports that *C. colebrookianum* and *C. infortunatum* flower are not well adapted for bees.

While the floral tube length of *C. serratum* flower is relatively short; bees' pollinators *Apis cerana indica, Bombus albopleuralis, Polistes spp, Xylocopa virginica* and *Xylocopa violacea* can easily be squeezed out the nectar, which are present in the form of minute droplets in pubescent nectar guide by using their proboscis. Aluri et al. (2016) observed *Xylocarpa* and *Anthophora* bee species bite the corolla and suck out the nectar from the plant species *C. inerme*. Witter et al. (2015) noted that during pollen collecting, bees touched the *Brassica napus* flower's reproductive organs and landed on the corolla. From our field observation, the lip of the *C. serratum* flower gives a perfect landing platform for the bee's pollinator to sit and squeeze out the nectar. While sitting on the lip and squeezing out the nectar from the flower the dorsal site of the bee pollinator touches the curve/bending filament and style which help in the cross pollination of flower since the nectar are present in the form of droplets in less quantity pollinator move from one flower to another flower for the same. Hu et al. (2022) reported that bee pollinators are attracted to flowers with red and yellow filaments. From our result, it can be said that *C. serratum* flowers with purple colour filament are visual attractiveness to bee visitors for pollination. The above result supports that *C. serratum* is well adapted to the insect order Hymenoptera (bees). High vibration and clinking onto the flower by bees increased pollen released (Pereira & Vallejo, 2022). A similar finding was observed for the bee's pollinator in *C. colebrookianum*, *C. infortunatum*, and *C. serratum*. This bee's pollinator collects pollen through vibration with a pollen basket, which is present on the hind tibia body parts of bees.

In addition to nesting locations, floral phenology and nectar volume also influence insect flower visits. According to Faheem et al. (2004), the number of insect pollinators was influenced by the size, shape, colour, volume of the nectar secreted, the number of flowers that bloom, and pollen content. From our findings, it can be

said that the white and purple colour flowers of *C. colebrookianum, C. infortunatum,* and *C. serratum* attract a greater number of insect pollinators. Duara & Kalita, (2013) reported that the inflorescence with blue-yellow flower colour attracts Hymenoptera, Diptera, and Lepidoptera. However, from our finding, it can be said that inflorescence with white and purple flower colour attract Lepidoptera, Hymenoptera, Coleoptera, and Hemiptera.

Rianti et al. (2010) reported the effectiveness of insect pollination using basic data such as flowering periods, flower nectar volume, and environmental factors in the seed set of *Jatropha curcas*. He observed that insect diversity is not influenced by temperature, light intensity, and wind velocity. The contrary result might be due to the site's location and anthropogenic activity, which might reduce the insect visitation on a flower. From our diverse insect study, we found the highest number of individuals is from Hymenoptera (Fig. 4.19), and the highest species numbers (diversity) was from Lepidoptera (Fig. 4.20). *C. colebrookianum,* was observed to be visited by insect order, belonging to Hemiptera (bugs), but these floral visitors seem to be of minor importance. Though floral features adapted to relatively primitive group pollinators (Lepidoptera), but seems to be highly important in conserving huge diversity of Lepidoptera pollinators since moths and butterflies show solitary nature, don't preserve food materials like social bees, they seem to be more prone to biotic and abiotic stress factors or climate change.

Kumar et al. (2017) reported that *C. infortunatum* is visited by bees, ants, thrips, and butterflies during the day but is mainly pollinated by papilionid and pierid butterflies. Begum et al. (2014) mentioned that *C. infortunatum* is an important source of nectar for certain butterflies such as the Papilionid, *Papilio polytes*, the Nymphalid, *Danaus chrysippus,* Pierids, *Eurema blanda, E. hecabe,* and the Lycaenid, *Zizinaotis*. In all three species of *Clerodendrum* presence of nectar in flower was observed, the presence of nectar in flower attracts; the nectar foraging insect species of the order Hymenoptera and Lepidoptera. Two ant species *Tapinoma melanocephalum* and *Trichomyrmex destructor*, were reported as floral visitors of *C. infortunatum* as nectar larceny and herbivory (Mukhopadhyay & Quader, 2018).

Raju & Kumar (2016) reported that *C. inerme* flowers are rich in nectar, so they attract pollinators such as hawk-moth (*Macroglossum gyrans)*, bees (*Xylocopa* and *Anthophora),* and butterflies. Aluri et al. (2016) reported that pollinator hawk moth, *Macroglossumgyrans*, bees *Xylocopa pubescens* and *Anthophora bicincta*, and butterflies *Pareronia valeria, Danaus genutia* and *Borbocinnara* were observed to pollinate on *C. inerme*, pollen and nectar are received as a floral reward. Gautam, (2012) reported that *C. splendens* were pollinated by *Xylocopa* (carpenter bee), *Eumenes* sp.(wasp) and *Componotus campestris* (black ant).

The presence of nectar guide was observed in *C. infortunatum* and *C. serratum*, which attracts the insect species of the order Hymenoptera. *Clerodendrum* flower pollinators frequently make inter-floral visits from one flower to another since the amount of nectar present in the flower is very low. The morphological character of flowers in all three *Clerodendrum* species differs, such as flower color, presence of nectar, nectar guide, and pollen attracts different groups of insect pollinators. Carpenter bees, *Xylocopa* species, butterflies, *Papilio* species, and the hawk-moth *Macroglossum* spp. pollinate *C. trichotomum*, according to Sakamoto et al. (2012a). According to Primack et al. (1981), *C. inerme* never receives visitors during the day but receives one huge hawk-moth during the dusk hours. As it is reported that *C. trichotomum* and *C. inerme* is visited by hawk-moth, during the study period it was observed that *C. colebrookianum* and *C. infortunatum* is visited by hummingbird hawk-moth *Macroglossum stellatarum* during the evening hours of the day. It was observed that the hummingbird hawk-moth sucked only the flower's nectar with the help of its long proboscis. The anthesis of the *C. colebrookianum*, *C. infortunatum*, and *C. serratum* is in the morning followed by the release of pollens from anther, so the pollinator's visits and pollination mainly occurred from morning to afternoon through different pollinating agents.

A study by Mizusawa et al., 2014 reported that *Clerodendrum izuinsulare* and *C. trichotomum* were visited by diverse insect floral visitors. *C. trichotomum* and *C. izuinsulare* were reported to share common pollinators, diurnal hawk-moths, bees,

swallow tails and nocturnal hawk-moths (Miyake & Inoue, 2003), and they also mention that pollen transfer might happen between the two species, but hybridization does not occur. During our study period, visitation of common pollinators were observed among the three *Clerodendrum* (Table 4.5) species. Among the butterflies, *Anthene lycaenina* was recorded as a common floral visitor in *C. colebrookianum* and *C. serratum*. And among *C. colebrookianum* and *C. infortunatum Delias descombesi, Papilio helenus, Papilio memnon, Tirumala septentrionis*, and *Vindula erota erota* were recorded as common floral visitors. The hummingbird hawk-moth (*Macroglossum stellatarum*) was recorded as a common floral visitor *C. colebrookianum* and *C. infortunatum*. *Pelopidas mathias* was recorded as common moths floral visitors who visited all three *Clerodendrum* species. And among the bee pollinator, *Bombus albopleuralis in C. colebrookianum* and *C. serratum; Xylocopa virginica* and *Apis cerana indica in C. infortunatum* and *C. serratum* were observed as commom pollinator. The flowering seasons of all three *Clerodendrum* are quite different, but they start flowering one after another. Sharing of common pollinators among three species of *Clerodendrum* species might be important from an evolutionary and hybridization point of view. Butler et al. (2020) observed pollen deposition on the butterflies' wings when pollinating on the flower of *C. infortunatum*. The result of pollen storage from three *Clerodendrum* (Fig. 4.56, 4.57 & 4.58), i.e., pollen is viable for up to 28 days; this support that pollen which was attached to the body part of the pollinator might pollinate the flower during floral visitation this might lead to cross pollination among the species.

Both pollination and subsequent fertilization play an essential role in successful fruit set and subsequent fruit development in plants with seeded fruit (Zhang et al., 2010a). Pollination plays an important role in the deposition and transfer of pollen on the stigmatic surface of a flower (Cheung, 1996). During the study period, it was observed that pollen deposition on the pollinator's body part and on the stigmatic surface after insect visits in open flower in all three *Clerodendrum* species proves that pollen deposition on the stigmatic surface (Fig. 4.46) of flower take place during the insect visits leads pollination. Pollen germination and tube growth *in-vivo* in

many plant species depend on the density of pollen deposited on the stigmatic surface (Chen et al., 2000). All three *Clerodendrum* species are visited by a significant number of insect pollinators; the movement and visitation by insect pollinators in flower increased the pollen density and deposited sufficient pollens on the stigmatic surface of the flower (Fig. 4.46). *In-vivo* and *in vitro* pollen germination of *Pyrus pyrifolia* was reported by Zhang et al. (2010a), and they also mention an increase in pollen density, increased pollen tube growth, and higher pollen germination rate. In the present study, *in vivo* and *in vitro* pollen germination for all three *Clerodendrum* species (Fig. 4.47, 4.48 & 4.61) was observed. The concentration of pollen on the stigma and in the air was recorded as high during noon time between 2 pm for *Tectona grandis* (Khanduri, 2012); he also observed 4-8 pollen grains per stigma for fruit development. During our study period, it was observed that pollen on the stigma and in the air was recorded highest after anthesis for all three *Clerodendrum* species between 0900-1300 hours (Fig. 4.43, 4.44 & 4.45).

Increased fruit set production in cross–artificial, cross-natural, and control treatment support high pollen density and allows for a higher frequency of ovule fertilization. An increase in pollen number increased the rate of fruit set production. Varieties of biotic and abiotic (insect and wind) mechanism help in the transportation of pollen on the stigma of a flower (Edlund et al., 2004). Aerial pollen concentration depends upon pollen production, transportation, and pollen uptake into the air by wind, temperature, and humidity. Low pollen concentration in the air was reported when the relative humidity was high (Erkara, 2008). Our finding supports that pollen concentration varies high in the afternoon hours when the relative humidity is low and less pollen concentration during morning and evening hours. Change in wind direction, temperature and humidity change the concentration of pollen in the air throughout the day. The accumulation of pollen on the stigmatic surface of the flower and on the glass slides at hourly intervals reflects the amount of pollen released into the air. Many authors have reported that pollen in the air cause seasonal pollen allergy (Garcia, 2017), and pollen concentration varies from season to season (Schramm et al., 2021). Different concentrations of *Clerodendrum* pollen during the

study period support that this pollen might cause pollen allergy during that particular flowering season. The pollen content in the air varies due to climatic and weather conditions (Velasco et al., 2015). Our finding supports that temperature and humidity influence the content of *Clerodendrum* pollen (Table 4.6, 4.7 & 4.8) in the air. Increased pollen content in the air during the daytime with an increase in temperature. This pollen might go along with the wind flow and help in reproductive success. In addition, this pollen might also go along the wind flow and add to the pollen allergy.

5.2.1. Flower and pollen production

Fluctuations in the weather throughout time may be the cause of seasonal variations in the amount of pollen, flowers and inflorescences produced (Table 4.9, 4.10 & 4.11) (Ribeiro et al., 2005; Khanduri et al., 2015b). The results support that with the variation in the geographical area, there is a variation in climatic conditions in all the selected sites, and the production of the flower, pollen, and fruit set varied in all three *Clerodendrum* species between years and geographical locations. Pollen production per anther differs between the two populations; the data showed some inter-population variability that may be related to differences in environmental conditions affecting pollen production (Delph et al., 1997 and Guardia & Belmonte, 2004). Annual variation in pollen production between the years in *Ambrosia artemisiifolia* is associated with an increase in temperature (Ranpal et al., 2022). Ziello et al. (2012) and Ranpal et al., (2022) reported the effect of pollen production at higher and lower elevations might be the effect of temperature. During the study period, it was observed that the production of flowers and pollen was recorded high at a lower elevation than higher elevation (Table 4.9 & 4.10).

The variation in pollen production per flower in different plant species and even in closely related species belonging to the same family is attributed to male reproduction variables (Garcia et al., 2020). Pollen productivity data is valuable in selecting male mates in plants (Vankin et al., 2003). It has been widely reported that the amount of pollen produced in flowers is a genetically determined trait that can

vary greatly between species and between cultivars or types (Anton & Denisow, 2018).

Pollen production varies greatly between individuals of the same species, across growing seasons and is not a continuous characteristic. It is strongly influenced by external factors (Alonso et al., 2013). From the result of this study, it can be said that the environmental conditions and flowering seasons for all three *Clerodendrun* species are different. It supports that pollen, flower, and fruit production vary according to the seasons and differ with climatic conditions in all three *Clerodendrum* species. Low temperature, drought stress, and soil conditions also have an impact on pollen production (Garcia et al., 2020). The quantity of flowers and inflorescence on a plant, as well as the environment in which it grows, directly correlate with pollen production. Additionally, there is a direct correlation between fruit set output and the number of flowers and pollen grains produced by each tree. Hidalgo et al., (1999) reported that the density of flowers (flower per plant) has an effect on pollen production further; he also mentioned that the distribution of flowers over the crown is affected by environmental and genetic factors. Our finding supports that at low and mid elevations, *Clerodendrum* flowers have high flowering density and produce more pollen than at higher elevations, and a greater number of flower inflorescence per plant was observed at mid and lower elevations in the present study sites. Large and high-quality pollen production acts as potential donors in plant mating success. Flowers with high pollen production are visited by a great number of pollinators which makes reproduction successful (Khanduri et al., 2015a). Our finding support that all three *Clerodendrum* are visited by a great number of pollinators (Fig. 4.28, 4.29 & 4.30). This proves that all three *Clerodendrum* flowers have high pollen production, which helps for successful reproduction.

Hidalgo et al., (1999) reported the shape of the crown and the radius of the tree affects pollen production. The height of a tree varied with the variation in elevation. It was observed that the tree with good height produced a greater number of flowers and fruits. Production of flowers and fruit was more in large trees than smaller trees. Our finding supports that plant height for *Clerodendrum* species which were found at

low and mid-altitude, was taller produced more branch numbers, a high amount of flower, and pollen grains than those found in higher elevations.

The production of pollen is related to the length and size of the anther (Bhowmik & Datta, 2013). Different research has reported the correlation between the size of the anther and the quality of pollen grains (Mondal et al., 1992). During our present study, no correlation was established between the anther size and pollen production among the species.

According to Rojo et al. (2015), *Olea europaea's* production of flower branches, inflorescences, flowers, and pollen fluctuates from year to year depending on the timing of the pre-flowering period as well as environmental conditions such temperature, rainfall, elevation, and exposure to the north or south. Aguilera & Valenzuela, (2012) reported, changes in the microclimate have an impact on the development of *Olea europaea* fruiting branches, inflorescences, flowers, and pollen grains production. Khanduri, (2012) reported the onset and end of *Tectona grandis* flowering, as well as the amount of pollen grains and fruit produced by each tree, varies throughout the year. The reproductive phenophases and the production of flowers and production of pollen vary from year to year for *Lagerstroemia speciosa* (Khanduri, 2014). Flower production, pollen production, fruit production, and seed production of *Schima wallichii* vary from year to year (Khanduri et al., 2013).

Pollen production significantly affects reproductive success in plants (Khanduri et al., 2015a). Low pollen production can negatively affect fruit and seed set in distantly located conspecific individuals at a population level. In *Taxus canadensis* seed set was correlated with pollen and ovule production (Allison, 1990). The relationship between pollen availability and fruit and seed production is crucial in gene flow among populations of wind and animal-pollinated taxa (Allison, 1990 and Khanduri et al., 2015b). A large amount of pollen grain production in outcrossing plant taxa is attributed to compensation loss due to the uncertain fate of pollen grains during pollen transfer. Pollen transfers are affected by a variety of biotic (pollinators) and abiotic (rainfall, temperature, humidity, etc.). In the present

site, high amounts of rainfall, humidity, and dense canopy covers are impeding factors for pollen transfer. Naturally, less than 1% of pollen produced is transported conspecifics stigma (Minnaar et al., 2019). Henceforth, pollen grains produced a much higher magnitude than ovules in xenogamous plant species (Cruden, 2000). Magnitude pollen production per flower is significantly related to the number of ovules to be fertilized (Jürgens et al., 2002). Wind-pollinated plants produce a huge amount of pollen grains, and high pollen production in tropical plants is related to pollinator limitations. Thus, pollinator limitation acts as a selective for huge pollen production outcrossing plant species (Cunha et al., 2022).

Apart from the role of pollen production in mating success, pollen production and seasonal variability could be useful in apiculture and aerobiological monitoring. By knowing the pollen production of a plant, we can estimate the number of pollen grains that will present in the air ambient for that particular season; this will add knowledge to the aerobiological data, which are crucial in allergy (Ghitarrini et al., 2017). Even the pollen production capability of the plant could be utilized in choosing appropriate plant species as food sources for bees in apiculture, since *C. coolebrokianum* is also planted in a home garden in Mizoram and other states of North east India hence could a valuable plant species for aforesaid.

5.2.2. Pollen ovule ratio (P/O):

A facultative xenogamy type of mating system was reported for *C. infornatum* (Kumar et al., 2017) since pollen ovule ratio falls at the range between 244.7-2588. Pollen ovule ratio of *C. splendens* is 540:1 (Jai, 2010). During our study period all three *Clerodendrum* species show facultative xenogamy type of mating system as per pollen ovule ratio (Table 4.27). McMullen, (2011) reported *C. mole* to show a facultative xenogamy type of mating system. A high ovule number per flower is crucial when pollinators are limited by biotic and abiotic factors. The higher magnitude of pollen production compared to ovule production per flower reflects *Clerodendrum* species reproduction could be limited by pollinators.

5.2.3. Mating system

Open pollination, autonomous autogamy, facilitated autogamy, and facilitated cross-pollination of *C. mole* shows no difference in fruit and seed production, but cross-pollination showed higher fruit and seed set production (McMullen, 2011). *C. infornatum* (Kumar et al., 2017 and Mukhopadhyay & Quader, 2022) recorded a higher percentage of fruit and seed set percentage in xenogamy and lesser fruit and seed set the percentage at open pollination and geitonogamy, but no fruit formation was observed with spontaneous autogamy and facilitated autogamy. *(*Sakamoto et al., 2012a) reported *C. trichotomum* show less fruit and seed set percentage with self-pollination and a higher percentage of fruit and seed set with cross-pollination. Aluri et al., (2016) and Raju & Kumar, (2016) reported *C. inerme* showed high fruit and seed set percentage with xenogamy and lesser fruit and seed set percentage with open pollination and geitonogamy. *C. viscosum* (Liza et al., 2010), *C. splendens* (Jai, 2010) and *C. splendens* (Rohitash, 2018) show fruit set when the flower is cross-pollinated, but when bagged, no fruit set was observed, which indicates self-incompatibility breeding system. *C. trichotomum* and *C. izuinsulare* show higher fruit set when it is outcrossing but fewer fruit set with self-pollination and open-pollination (Mizusawa et al., 2014).

During the study period, it was observed that the flower was found to set fruit at different test levels of treatment. Cross artificial, cross natural, and control shows higher fruit set percentage but spontaneous selfing, induced selfing, and geitonogamy show less fruit set percentage (Table 4.23, 4.24, 4.25). From this result, it can be said that a partially self-compatible type of mating system is recorded in all three *Clerodendrom* species (Table 4.26). Therefore, pollinator plays an important role in supplying pollen on the stigma of the flower and helping in the fertilization of the ovary to form fruit and seed. The lower fruit setting percentage after the treatment suggests that there is an occurrence of pollen limitation in flowers. Reduced supply of pollen and blocking of pollinators in flower causes pollen limitation, which leads to less fruit setting in plants.

Birds are engaged in the fruit or seed dispersion process in the *Clerodendrum* genus, according to Wheeler et al., (1992). The fruits of *C. laevifolium* are likely dispersed by birds, according to Keng (1990). *C. macrostegium* is spread by fruit-eating birds, according to Lorence & Flynn (1997). Solomon & Rajendra (2016) stated that *C. inerme* fruits are dispersed by birds such as *Acridotheres tristis, Corvus splendens, Corvus macrorhynchos* and *Turdoidescaudatus*.

During our study, there was no observation recorded with the dispersion of fruit and seed by birds, and there is no previous study found on the dispersal of fruit and seed for these three *Clerodendrum* species. In all three *Clerodendrum* species, the fruit is a drupe with a fleshy mericarp. After ripening, the fruit's color turns purple, and the colour of the calyx, which is green during the flowering period, turns red during the ripening phase for *C. colebrookianum* and *C. infortunatum*.

5.3. Effect of growth regulators on *in vitro* pollen germination with pollen longevity tests.

The TTC test was found to be a reliable test for evaluating pollen viability at various flowering phases, such as pre-anthesis and anthesis, to differentiate between alive and dead pollen in *C. colebrookianum*, *C. infortunatum* and *C. serratum*. All three *Clerodendrum* species showed a colour difference between viable pollen stained with red and non-viable pollen without a stain (Figs.). Using TTC, it is possible to distinguish between alive and dead pollen by colour *Jatropha curcas* (Abdelgadir et al., 2012), *Prunus armeniaca* (Yaman &Turan, 2021), *Bursera* hybrids (Rico & Reyes, 2019), and *Fraxinus excelsior* (Buchner et al., 2022). The TTC test for pollen viability was reported by Yang et al. (2021) to be effective for *Amomum villosum* and *Amomum longiligulare*. All three *Clerodendrum* species were found to have a high percentage of viable after anthesis (Table 4.28), which is similar to the results for *Jatropha curcas* (Abdelgadir et al., 2012), *Passiflora cincinnata, Passiflora edulis, Passiflora edmundoi, Passiflora galbana, Passiflora gibert*ii and *Passiflora suberosa* (Soares et al., 2013). Before anthesis, pollen for *Leonurus cardiaca* had low viability and germination percentages, according to Shekari et al. (2016b). For artificial pollination and breeding, the quality of the pollen is frequently correlated with its

viability (Dafni & Firmage, 2000). In the pollination, fertilization and breeding of *Passiflora sp*., choosing the right anthesis stage for noticeably viable pollen grains is crucial. (Soares et al., 2013), improved banana varieties (Soares et al., 2015) and *Leonurus cardiaca* (Shekariet al., 2016b).

In vitro experiments with pollen assist to simulate the *in vivo* environment of pollen tube germination on the pistil. Sucrose increases pollen germination and tube growth, thereby increasing the amount of nutrients in the culture medium (Lin et al., 2017). The right amount of sucrose is necessary for pollen germination because it provides nutrition, osmotic balance, and essential carbon energy (Dong & Beckles, 2019). The germination of pollen grains may be hindered by high sugar content (Lin et al., 2017). Unbalanced osmotic pressure may cause pollen germination within the same culture and media changes (Youmbi et al., 2015). All the three *Clerodendrum* species showed a significant difference in pollen germination and tube growth depending on the sucrose concentration. The pollen germination in all the three *Clerodendrum* species was improved by increasing the sucrose concentration from 5% to 10%. (Fig. 4.55). The influence of sucrose content on the percentage of pollen germination was also seen in *Cunnighamia lanceolata* (Fragallah et al., 2019), *Psidium guajava* (Sarkar et al., 2018), *Impatiens balsamina* (Patel & Mankad, 2014), and *Leonurus cardiaca* (Shekari et al., 2016 a). In *Momordica subangulata*, a low sucrose concentration causes low pollen germination, but sucrose concentration of more than 10% produces a significant amount of pollen germination (Naik et al., 2016). Between treatments and incubation periods, the pollen germination rates differed considerably. At 24 hours, pollen germination rates were higher and lower at 48 hours (Table 4.30). The findings demonstrated that the pollen grains of all the three species of *Clerodendrum* require just 24 hours to start growing for germination (Fig.); a comparable effect was also seen for *Prunus laurocerasus* (Sulusoglu & Cavusoglu, 2014); *Cunnighamia lanceolata* (Fragallah et al., 2019); and *Spathodea campanulata*, *Bauhinia purperea* and *B. racemosa* (Sanjay et al., 2016). The effectiveness of pollen germination media depends on the rate of pollen germination.

Pollen germination was regulated by all the four growth hormones, although at different rates (Table 4.30 and Fig 4.58). Different hormone concentrations lead to different percentages of germination (Table 4.30). The pollen germination in *C. colebrookianum* and *C. infortunatum* was accelerated by an increase in GA3 concentration; as a result, a high concentration of GA3 is essential for pollen germination. *Prunus dulcis* (Maita and Sotomayor, 2015), *Acca sellowiana* (Xiong et al., 2016), and the functioning male flower of the pomegranate all showed comparable outcomes (Engin & Gokbayrak, 2016). However, the germination percentage reduced at higher concentrations of (GA3), i.e. at 200 mg L^{-1} and 300 mg L^{-1}, but it increased for the plant species *C. serratum* at lower concentrations of (GA3), at 100 mg L^{-1} (Table 4.30). Similar results have been seen for Pistacia vera and strawberry pollen on (GA3) (Voyiatzis & Paraskevopoulou-Paroussi, 2002; Acar et al., 2010). In some species, (GA3) application and concentration can either stimulate or hinder pollen germination and pollen tube growth i*n vitro* or *in vivo* (Acar et al., 2010). Amylase, acid phosphatase and β-glucosidase activities are all promoted by GA3. Amylase and acid phosphatase enzyme leaching is accelerated to promote pollen germination (Sanjay et al., 2016). GA3 significantly increased the germination of pollen in *Momordica charantia*, *Spathodea campanulata* and *Vitis vinifera in vitro* (Gokbayrak & Engin, 2015; Sanjay et al., 2016). Gibberellins (GA3) are endogenous plant growth regulator hormones that play a significant role in many aspects of plant development for a variety of species; application of (GA3) aids in the establishment of pollen tube development in-vivo or *in vitro* (Singh et al., 2002).

By regulating the growth of stamens and ovaries, boosting egg cell maturation and generating embryonic axial polarity and polar development, indole-3-acetic acid (IAA) plays a significant role in plant sexual reproduction (Abdelgadir et al., 2012). IAA either directly or indirectly enhances pollen germination and tube growth of *Nicotiana tabacum* L. *in vitro* (Chen & Zhao, 2008). IAA induces pollen tube development in pistils (Chen & Zhao, 2008). The findings indicate that IAA promotes pollen germination and tube growth for *C. serratum* species (Table 4.30). IAA pollen germination percentage and tube growth considerably higher at 100 mg L^{-1} and 200 mg L^{-1} compared to IAA at 300 mg L^{-1}. Pollen germination and tube

growth are inhibited by the increased concentration of IAA, 300 mg L^{-1}. Additionally, it is possible to state that the application of IAA at a suitable concentration can speed up pollen germination and tube growth, whereas IAA at a larger concentration can slow down pollen germination and tube growth.

Different concentrations of Kinetin influence pollen germination and tube growth (Manonmani & Mekala, 2016 and Marchioretto et al., 2019). *C. colebrookianum* and *C. infortunatum* species, pollen germination was inhibited by high Kinetin concentration (Table 4.30), but pollen germination and tube growth were promoted by lower Kinetin concentration. According to Dziurka et al. (2019) and Usman et al. (2022), a low Kinetin concentration in the plant enhances regeneration, increasing the efficiency of doubled haploid synthesis; this finding supports our findings. Pollen germination and tube growth of *Prunus dulcis* are said to be influenced by Kinetin (Maita & Sotomayor, 2015). At different hormone concentrations, such as 100 mg L^{-1}, 200 mg $L^{-1}$and 300 mg L^{-1}, it was found that Kinetin stimulates pollen germination and tube growth for *C. serratum* (Table 4.30). Kinetin shows the least impact on pollen germination and tube growth for *C. serratum* (Table 4.30). Similar findings were obtained for *Prunus dulcis* (Sotomayor et al. 2012). They also observed that Kinetin had the least impact on pollen germination.

IBA and IAA at higher concentrations decreased pollen germination in *C. colebrookianum*, whereas they increased pollen germination in *C. infortunatum* at higher concentrations (Table 4.30). Therefore, *C. colebrookianum* and *C. infortunatum* species necessitate the right concentrations of IAA and IBA (Table 4.30). According to Li et al. (2015), IBA enhanced *in vitro* pollen germination in four Hibiscus species; also report for *B. purpurea* and *B. racemose* (Sanjay et al., 2016); Litchi chinensis (Zeng et al., 2018) and *Actinidia deliciosa* pollen (Marques, 2018). Abdelgadir et al. (2012) reported, optimal pollen germination and tube growth require an appropriate IAA concentration; this conclusion supports that of *C. colebrookianum* and *C. infortunatum* as well. Additionally, Kovaleva et al. (2005) observed that pollen germination was reduced at high IAA concentrations while enhanced at low IAA concentrations. IAA increased pollen germination in male

Petunia hybrida plants *in vitro* via activating K+ channels to regulate osmoregulation (Kovaleva et al., 2016).

IBA was found to regulate pollen germination and pollen tube elongation for the plant species, *C. serratum* (Table 4.30). IBA activates the catalytic activity of peroxidase, an enzyme essential for pollen germination and pollen tube growth (Qiu et al., 2021). IBA shows less pollen germination and tube growth at concentration 100 mg L^{-1} and 300 mg L^{-1}, and at concentration 200 mg L^{-1} IBA shows good pollen germination and tube growth for *C. serratum*. From the result, it can be said that a decrease or increase in the concentration of hormone also had an adverse effect on pollen germination and tube growth; appropriate concentration is required for pollen germination and tube growth. IBA promotes pollen germination and tube growth on *Litch chinensis* (Zeng et al., 2018), *Salix lapponum* (Pogorzelec et al., 2015), *Bauhinia purperia* and *Bauhinia* (Sanjay et al., 2016), *Torreya grandis* (Aihua et al., 2001) and also for other different plant species.

In the three species of *Clerodendrum*, IAA, GA3, IBA and Kinetin influenced and regulated *in vitro* pollen germination. However, their response to the concentrations of growth hormones in three specific *Clerodendrum* species varied, indicating that the three *Clerodendrum* species' pollen grains responded differently to the various growth hormones.

For plant breeding, particularly in asynchronous flowering species, and for germplasm exchange, long-term pollen storage is crucial. Lifespan of pollen varies from minutes to months and from plant species to species. In order to sustain pollen grains' viability over an extended period of time for making crosses between two varieties or species that flower at various periods, it is practical to examine and standardize storage conditions. According to the results of pollen storage, pollen grains of three *Clerodendrum* species stored (Fig. 4.56., 4.57 and 4.58) at different temperatures (-20°C, -4°C and 6°C) for varying lengths of time (0-28days), it was observed that the pollen viability decreased with an increase in the interval of time after storage at different temperatures. This is because pollen's metabolic activity is influenced by temperature and time (Du et al., 2019). All three *Clerodendrum*

species had considerably different pollen viability percentages while stored at -20°C, -4°C, and 6°C. The vitality of pollen is influenced by temperature and humidity (Sidhu, 2019 and Du et al., 2019). In order to perform species hybridization between *C. colebrookianum* (as flowering occurred between July and December) and *C. infortunatum* (flowering occurred between February and April), as well as between *C. infortunatum* and *C. serratum* (flowering occurred between March and July), *C. serratum* and C. *infortunatum* it was necessary to screen an appropriate temperature through additional experimentation to extend the longevity of pollen viability up to 70 days.

Fraxinus excelsior pollen grains can be stored for a longer period of time at temperatures of -20°C and -80°C than at 4°C; the viability of stored pollen grains could be exploited to solve a critical issue with ash dieback disease through a future breeding programme (Buchner et al., 2022). *Juniperus communis* pollen grains can be kept effectively with significant pollen viability at -20 °C as compared to -4 °C (a tree with sparse seed sets due to pollen limitation in naturalistic environments). The stored pollens were used for pollen supplementation studies to improve seed germination in the tree species (Kormutak et al.,2021). Therefore, pollen storage is important in pollen management and can be a useful tool for breeders to overcome challenges related to variations in flowering time, pollen shedding, stigma receptivity, and pollen limiting in controlled pollination trials. Additionally, research on the long- and short-term viability and storage of pollen may aid in the creation of crosses between individuals from different ethnic subpopulations that are growing in geographically distinct regions and have adapted to biotic and abiotic gradients in in order to improve desirable characteristics.

Chapter: 6

Conclusions:

Anthesis, anther dehiscence & stigma receptivity of the flower are associated and liable with the variation in climatic factors such as temperature and humidity in all three-study species of *Clerodendrum*. A distinct herkogamy i.e. physical separation of stamens and stigma, and dichogamy i.e. temporal separation in maturation of male (anther dehiscence) and female (stigma receiptivity) reproductive parts and mainly protandry i.e. maturation of stamens well before stigma receiptivity was observed all three species of *Clerodendrum*. Variation in anthesis, anther dehiscence and stigma receptivity among inflorescences of plants and spatial (herkogamy) and temporal (dichogamy) variation in male and female reproductive parts of flowers in all three species of *Clerodendrum* are important adaptations in floral biology features for promoting xenogamy and geitonogamy.

Distinct floral adaptions were observed in *C. colebrookianum, C. infortunatum* and *C. serratum*. White colour flowers with floral tube length (2.7 cm) with deep seated minute quantity rich nectar is well adapted to hummingbird hawk-moths, moths and butterflies due to their synchrony with nectar harvesting organ proboscis and its length in *C. colebrookianum*. Proboscis is almost similar to *C. colebrookianum* floral tube length i.e. 2.8 cm in hummingbird hawk-moths. White colour flower with distinct light pink coloured nectar guides and floral structures are adapted for bees and floral tube length of (1.9 cm) with deep seated minute nectar is well synchronous and adapted to hawk moth, moths and butterflies. Therefore bees, moths and butterflies are important pollinators for *C. infortunatum*. Light purple flower with distinct pubscent nectar guides, landing platform, shallow nectar in form of minute droplets is well adapted to carpenter and bumble bees compared to butterflies due to their synchrony with flower in *C. serratum*.

Diversity i.e. number of species (species richness) of butterflies and moths floral visitors are high compared to bees in *C. colebrookianum*. Butterflies and moths are ascertained as important pollinators based on their visitatation and synchrony with floral adaptation in facilitating pollen transfer in *C. colebrookianum*. Again, the

species richness of butterflies and moths are high in *C. infortunatum* compared to bees. However, both Lepidoptera (butterflies and moths) and Hymenoptera (Carpenter bees) were ascertained as important pollinators on the basis of visitation frequency and pollinators synchrony with floral features of *C. infortunatum*. But in *C. serratum* both bees and butterflies are found to be equally rich. Nevertheless, bumble bees and carpenter bees were found to be most important pollinators due synchrony with floral adaptations, foraging behaviour and visitation frequency. Floral visitors followed a diurnal pattern in all three species of *Clerodendrum*. Increase in temperature during day time enhanced pollinator activity on flower. Temperature positively correlated with insect floral visitors while humidity is negatively correlated with pollinator's visits in all three species of *Clerodendrum*. Concentration of pollens in air in nearby to flower and deposition of pollen grains on stigma are highly correlated and followed diurnal pattern. Maximum concentration in air and deposition on stigma was found between 0900 to 1400 hours coincided high visitation frequency of pollinators compared to early morning and evening hours. Thus, the rate and quantity of pollinator's visitation in the study plant species is linked with deposition of pollen on stigma. Lepidoptera and large solitary bees are most vulnerable to climate change compare to social bees due to long-term fluctuations in resource availability, because they do not store food or switch diets henceforth *C. colebrookianum*, *C. infortunatum* and *C. serratum* could be a valuable plant species for butterflies, moth and bees conservation. Based on mating system evaluation, index of self incompatibility and out crossing index mating system of all three plant species of *Clerodendrum* was found to be partially self-compatible.

Pollen production revealed that each species has significant differential production capacity. There is significant difference for flower and pollen production among populations but insignificant inter annual variability was recorded. The production of flower and pollen was recorded high at lower elevation than higher elevation. Pollen production was significantly positively correlated with fruit settings in different years.

TTC staining test is suitable to determine the viability of pollen grains for all three *Clerodendrum* species. Anthesis stage pollen grains would be valuable in future breeding and hybridization experiments for the plant species due to their high viability. Moisture and sucrose were found to be initiating factors for pollen grain developments. Pollen grains germination under *in vitro* conditions exhibited a differential response to different growth hormones and their concentrations and time with respect to *Clerodendrum* species. For inducing *in vitro* pollen germination in *C. colebrookianum* and *C. infortunatum* during the first 24 hours of incubation, GA3 (200 mg L^{-1}) and IBA (200 mg L^{-1}) were found to be the most effective growth hormone concentrations. IAA was found to be the most effective growth hormone for stimulating *in vitro* pollen germination in the case of *C. serratum*, followed by IBA, GA3 and Kinetin in the first 24 hours of incubation. The application of different hormones and time give the most significant response among the treatments ($p<0.0001$), followed by the application of different hormone concentrations ($p<0.05$). *In vitro* pollen germination varied non-significantly between the plant species. All three *Clerodendrum* species pollen grains sustained at a viable state for up to 28 days. Therefore, pollen from all three species of *Clerodendrum* should be gathered at the anthesis stage for short-term storage of pollen grains that will be useful for use in future pollination, supplementing, hybridization and breeding experiments among the three *Clerodendrum* species.

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Ph.D. Topic: Pollination biology of three *Clerodendrum* species in a tropical forest of Mizor

List of Publication

1. Pollen storage, viability and effect of growth hormones on *in vitro* pollen germination in two medicinal plants (*Clerodendrum colebrookianum* Walp. and *Clerodendrum infortunatum* L.) of the tropical moist forest of North-east India. *Journal of Applied and Natural Science*. 14(3), 999-1008 (2022).
2. Pollen viability and in vitro pollen germination of *Clerodendrum serratum* (L.) Moon: A valuable medicinal plant. Science and Technology. 10(2): 64-70 (2022)
3. Flower-Pollinator Interactions in Liana (*Caesalpinia cucullate* Roxb.) in a Tropical Rain Forest of Mizoram. *Indian Journal of Ecology*. 49(3): 703-710. (2022)
4. Three new distributional records of orchids from Mizoram, India – *Bulbophyllum lopalanthum* J.J.Verm., Schuit. & de Vogel, *Cymbidium bicolor* Lindl. And *Zeuxine flava* (Wall. ex Lindl.) Trimen. *International Journal of Ecology and Environmental Sciences*. 2 (3): 263-266 (2020).

Paper (s) presented on Workshop/Seminar

1. Pollination Biology of *Clerodendrum colebrookianum* Walp a Valuable Ethno – Medicinal Plant of North East India. National seminar on conservation and sustainable use of mrdicinal and aromatic plants. Organized by Department of Forestry, Mizoram University, Aizawl Mizoram
2. Pollen Viability and *in vitro* Pollen Germination of *Clerodendrum colebrookianum* Walp a Valuable Ethno – Medicinal Plant of North East India. International Conference on Chemistry & Environmental Sustainability. Organized by Department of Chemistyr, Mizoram University, Aizawl Mizoram
3. *In vitro* pollen germination of *Clerodendrum serratum* (L.) Moon: A valuable medicinal plant. National Seminar (Hybrid Mode) on "Strengthening environmental Health: Role of Society, Science & Technology". Organized by Department of Rural Technology and Social Development

Seminars/ Symposia/ Course/ Workshop Attended

1. 12th Annual conservation of Association of biotechnology and Pharmacy (ABAP) & International conference on biodiversity, Environment and human health: Innovation and emerging trends (BEHIET)- 2018.
2. National workshop on Statistical tools in Biomedical research. 23/11/2018 organised by Bioinformatics Infrastructure Facility, Department of Biotechnology, Mizoram University.
3. NRDMS-DST orientation programme on Geospatial technologies. 24-26 July, 2019 Department of forestry, MZU.
4. High- End Workshop on Quality Control and Standardization of Herbal Raw Materials of Value Addition and sustainable Development in North East. 16-21 September 2022.

Working experience

1. Project fellow in "Pollination, pollen germination and stigma biology of *Clerodendron colebrookianum* Walp. a valuable ethnomedicinal plant of North East India using Foldscope". (2018-2019).
2. Junior project fellow in "Characterization and value-added products of Garcinia genetic resources for rural livelihood improvement in Mizoram". (2020).

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Three new distributional records of orchids from Mizoram, India – *Bulbophyllum lopalanthum* J.J.Verm., Schuit. & de Vogel, *Cymbidium bicolor* Lindl. And *Zeuxine flava* (Wall. ex Lindl.) Trimen

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Abstract
Three new reports of orchids have been identified during a field survey in Mizoram *viz. Bulbophyllum lopalanthum* J.J.Verm., Schuit. & de Vogel *Cymbidium bicolor* Lindl. And *Zeuxine flava* (Wall. ex Lindl.) Trimen. Taxonomic description of the species have been given with their classification along with photographs of each.

Keywords: Mizoram, new reports, orchids

1. Introduction
Orchids are well known worldwide for their distinctive flowers and constitute the largest flowering plant family. Orchid species can easily be recognized for their unique characteristics which play an important role in their life history and pollination (Adams and Lawson, 1993; Weston, *et al*., 2005) [1, 21]. Epiphytic orchids are mainly found growing on trees and shrubs in subtropical and tropical regions while terrestrial orchids prefer temperate and Mediterranean grasslands and forests (Arlott, 1978; Dressler, 1993; Ramirez, *et al*., 2007) [2]. More than 26000 species have been recorded from the orchidaceae family around the world (WCSPF, 2019).
Conservation of orchid species is crucial because they have considerable value in ornamental and medicinal markets. De and Medhi (2014) [6] have emphasized the conservation of rare and endangered orchids in north-eastern India by introducing approaches to remote sensing and GIS surveys to conserve genetic resources and by using biotechnological tools to promote disease resistance and high yield. Some endangered orchid species have already undergone major population declines (Cribb *et al*., 2003) [5] and, based on information such as the IUCN red list (IUCN, 2020) and policy recovery plans (e.g., Australian Government, 2019). Work on orchid conservation must concentrate on population montoring, species distribution and ecology, rather than the current trend in genetic diversity, propagation techniques and taxonomy (Wraith *et al*. 2019) [22]. A study conducted in Indonesia stressed that conservation efforts are very much needed at the local orchids are endangered by illegal harvest and not the local populace (Broto *et al*., 2020) [4].
Many researches have contributed to new distributions of orchid species around the World. Gogoi *et al*. (2015) [10] reported 398 specific and 6 intraspecific taxa belonging to 102 genera. Eria merguensis was reported from India by Singh *et al*. in 2013 and *Eria carinata* was identified as a new record for Mizoram by Roy *et al*. 2012 [18]. Karuppusamy *et al*. 2013 [13] discovered a new record of *Eria exilis* in southern India. Kumar *et al*. 2013 [8] and Panday *et al*. 2014 also identified seventeen new records of orchid I their study in Mizoram. Mao *et al*. (2009) [16] highlighted the wealth of northeast India with some orchid species being threatened in the region. Youssef *et al*. (2017) [23] recorded two new orchid species for the flora of Iraq: *Anacamptis papilionacea* (L.) R.M. Bateman, Pridgeon & M.W. Chase and *Dactylorhiza romana* (Sebast.) Soó. A new record of *Cymbidium bicolor* for Nagaland was identified by Deb *et al*. 2017 [7]. Zeuxine flava was also added to the plant wealt of Manipur by Devi *et al*., 2019 [8]. A recent study by Engels *et al*. 2020 [9] in south Amazon, Brazil led to identification of a new species *Mormodes matogrossensis*.

2. Materials and Methods
Field surveys were conducted by the authors throughout the Wildlife Sanctuaries and protected areas of Mizoram from 2016 onwards and have led to the discovery of three new orchid species in the state viz. *Bulbophyllum lopalanthum J.J. Verm., Schuit. & de Vogel, Cymbidium bicolor Lindl.* And *Zeuxine flava (Wall. ex Lindl.) Trimen*. A brief taxonomic description, distribution and phenology is provided.

3. Taxonomic treatment
3.1 *Bulbophyllum lopalanthum* J.J.Verm., Schuit. & de Vogel, Phytotaxa 166: 104 (2014); *Sunipia grandiflora* (Rolfe) P.F.Hunt, Kew Bull. 26: 184 (1971); *Ione grandiflora* Rolfe, Bull. Misc. Inform. Kew 1908: 413 (1908).
Description: An epiphytic herb1-2cm tall. Pseudobulbs 1 – 2 cm apart connected by small woody rhizomes, ovoid, less than 2 cm in size. Leaves solitary, notched at apex, oblong, 5 – 7 cm long, 1 – 2 width; Single flowered, purple colored, nerved with cream white. Lip dark purple, oblong.

Common Name: The lone bulbophyllum
Flowering: October – December
Ecology: In an open branch under light shade
Distribution: Himalaya to China (SE. Yunnan) and Indo-China
Specimen examined: Tawizo, Tawi Wildlife Sanctuary, Aizawl, Mizoram, Alt. 1596m, Lat. 23°33'46.6" N and 092°57'18.1" E. 15.12.2017. C. Remlalpeka & Kalidas Upadhyaya 01206.

3.2 *Cymbidium bicolor* Lindl. Gen. Sp. Orchid. Pl.: 164 (1833).
Description: A monopodial plant 20-50 cm long, epiphytic orchid found on a tree trunk. Stem fleshy with sheaths covered rhizome. Pseudobulbs ovoid, 4-8cm; Inflorescence arching, raceme, Sepals and petals pale yellow; flowers 2.5-4cm across, reddish-brown colored with white edge. Leaf green in color with a length of 30-40 cm and width 1-2 cm.
Common Name: The two colored cymbidium
Flowering: March – April
Ecology: Found in the branch of trees in large clusters, medium shade
Distribution: Himalaya to China (SE. Yunnan) and Indo-China
Specimen examined: Chhawrtui village, Champhai District, Mizoram, Alt. 1222m, Lat. 23°30'24.2" N and 093°04'21.9" E. 24.03.2017. C. Remlalpeka & Kalidas Upadhyaya 01203.

3.3 *Zeuxine flava* (Wall. ex Lindl.) Trimen, Syst. Cat. Fl. Pl. Ceylon: 90 (1885); *Monochilus flavus* Wall. Ex Lindl. Gen. Sp. Orchid. Pl.: 487 (1840); *Zeuxine aurantiaca* Schltr. Repert. Spec. Nov. Regni Veg. 19: 377 (1924).
Description: A terrestrial orchid 25 – 30 cm tall. Fleshy pseudostem 5 – 10 cm long, nodes enclosed in sheaths; rhizome creeping. Leaves 2 or 3 at nodes, oblong, 6 - 7 cm in length and 2 – 3 in width. Flowers yellow 0.2-0.4, sepals greenish yellow, ovate-lanceolate, obtuse. Petals oblong, obtuse Lip yellow oblong, curved at the base, winged.
Common Name: The Yellow Zeuxine
Flowering: December – February
Ecology: Under light to medium shade, top soil with leaf litters
Distribution: China (SW. Yunnan) to Indo-China
Specimen Examined: Tanhril, Aizawl, Mizoram, Alt. 812m, Lat. 23°44'07.8" N and 092°39'52.1" E. 06.03.2019. C. Remlalpeka & Kalidas Upadhyaya.

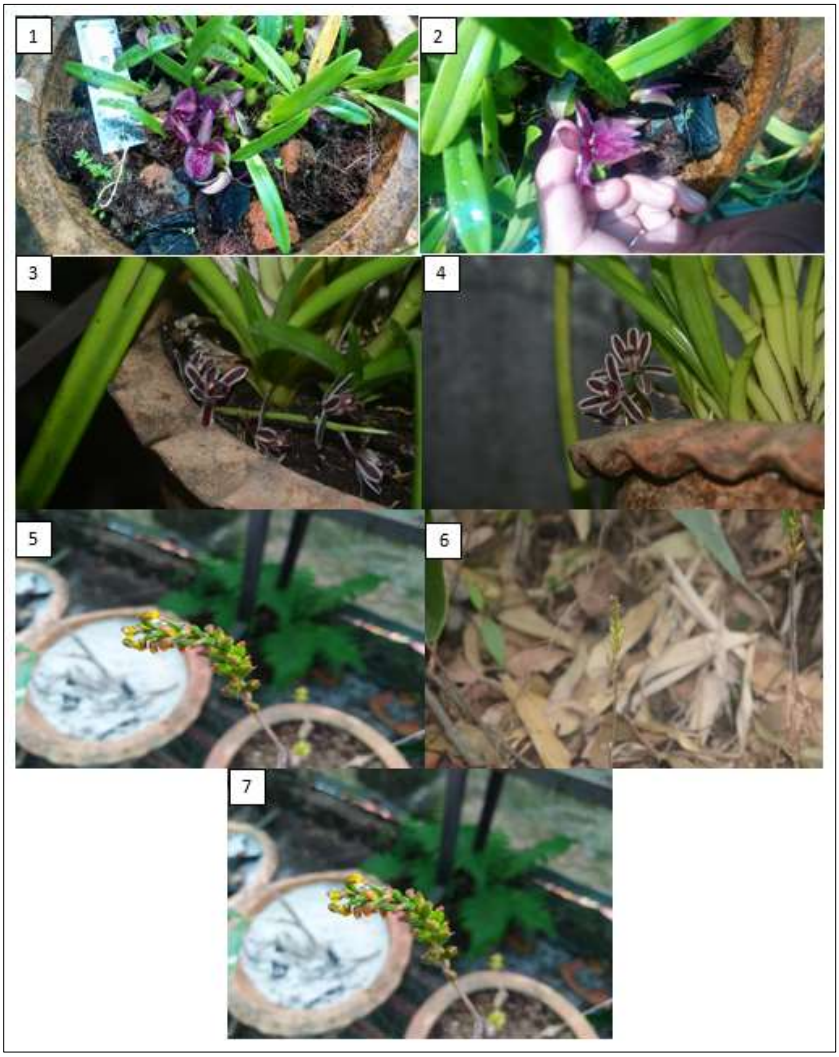

Fig 1 and 2: *Bulbophyllum lopalanthum* J.J.Verm., Schuit. & de Vogel; 3 and 4 - *Cymbidium bicolor* Lindl. 5, 6 and 7 - *Zeuxine flava* (Wall. ex Lindl.) Trimen.

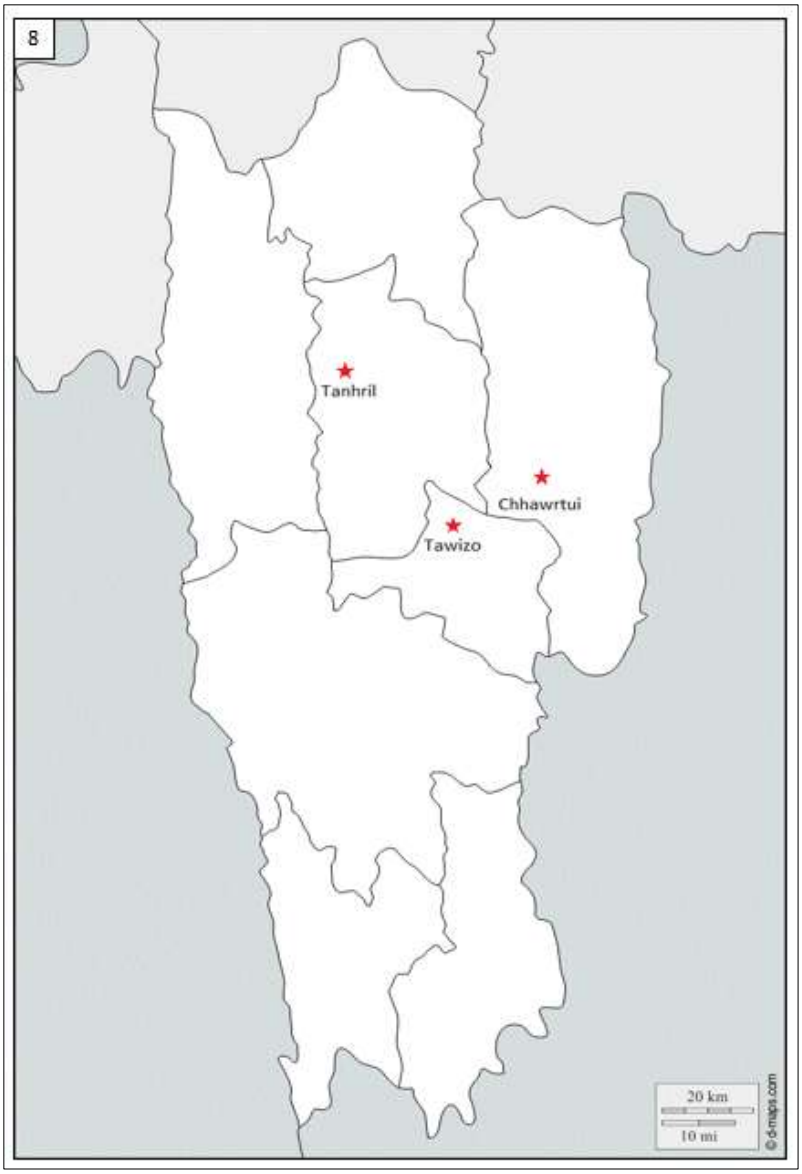

Fig 2: Map of Mizoram showing location of collection sites.

4. Conclusion

From the findings made during the field survey, it is evident that there is potential for more distribution of identified orchids within the state. Population study of orchids is a vital process which can help in conservation of threatened species. Past researches have yielded great results in identifying many new species and their distribution throughout the World. Further studies in the future is required as there is untapped resource of the flora and could aid in enhancing the present wealth of the state. The recent studies have shown lesser records around the Globe which could indicate that apart from smaller chances of findings, there could also be a population decline as well. Thus, field surveys in search of new findings along with the evaluation of current population and distribution of the orchid resources is needed for adding to the richness of the flora and their conservation as well.

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Pollen viability and in vitro pollen germination of Clerodendrum serratum(L.) Moon: A valuable medicinal plant

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Pollen Viability and *in vitro* Pollen Germination of *Clerodendrum Serratum*(L.) Moon: A Valuable Medicinal Plant

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Abstract—*Clerodendrum serratum* (L.) Moon is a valuable medicinal plant used in traditional medicines. Due to its overexploitation, the plant population is swiftly declining from its wild habitat. The present study on pollen biology was conducted to support the efforts for its propagation and conservation. The pollen outlay, viability, *in vitro* pollen germination, and pollen storage under varying temperatures were performed using standard methodology. The number of pollen grains per anther was recorded 1368.1±40.56, and the viability of pollen with 2,3,5-triphenyl tetrazolium chloride (TTC test) ranges from 76.62% in the anthesis stage (open flower) with to 18.85% pre-anthesis stage (unopen flower). Sucrose and moisture initiated pollen germination under *in vitro* conditions. A significant difference was observed between distilled water and sucrose 5% &10% ($p<0.0001$). Different growth hormones, i.e. {Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Gibberellic Acid (GA3), and Kinetin} exhibited differential influence on pollen germination and tube growth at different concentration, i.e., 100, 200 and 300 mg L-1. Basal culture media supplemented with Indole-3-Acetic Acid (IAA) at 100, and 200 mg L-1 concentration was found to be most suitable in inducing pollen germination and tube growth in *C. serratum*. Maximum pollen germination and tube growth were observed in the first 24 hours and decreased with time increased. Among the treatments, hormone and time gave higher significant responses ($p<0.0001$), followed by concentrations of hormones ($p\leq0.0023$). *C. serratum* pollen grains could be stored and remain viable for up to 28 days at -20°C without any preservative under lab conditions.

Keywords: Medicinal Plants, Pollen Viability, *in vitro*, Pollen Germination, Growth Hormones

INTRODUCTION

C. serratum is an important ethnomedicinal plant. The plant species is native to the forest areas of tropical and warm temperate regions of Africa and Southeast Asia regions of India, Malaysia, and Sri Lanka (Patel, 2014). Its parts, such as roots and leaves, are used to treat various human ailments in traditional systems of ethnomedicine across geographical regions. In Assam, the tender leaves extract and leaves juice of *C. serratum* are used in treating helminthic diseases, dysentery, and seasonal fever, while the fruit is utilized for dietary purposes (Yadav *et. al.* 2018). The leaves and roots of *C. serratum* are used to treat malaria, febrile and cathartic infections, fever, cephalgia, and snake bites in the Western Ghats (Raviraja, 2005). The root of *C. serratum* used for the treatment of asthma in Andhra Pradesh (Savithramma *et al.* 2007). The root of *C. serratum* contains sapogenins, D-mannitol, and stigmasterol, while the leaves contain high amount of flavonoids and phenolic acids (Apana *et al.* 2021).

The medicinal values of *C. serratum* caused unlimited exploitation from its native habitat. Limited planting and

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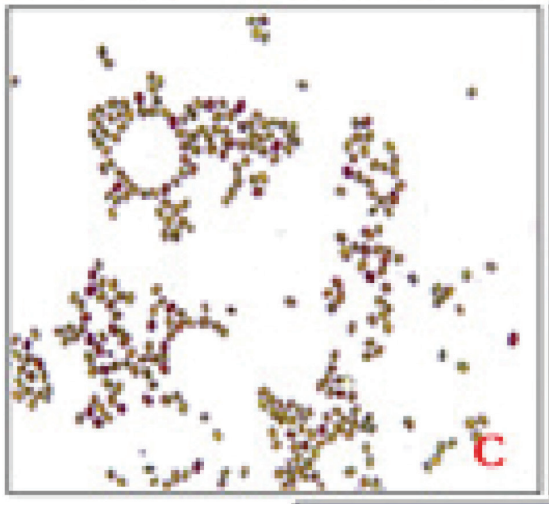
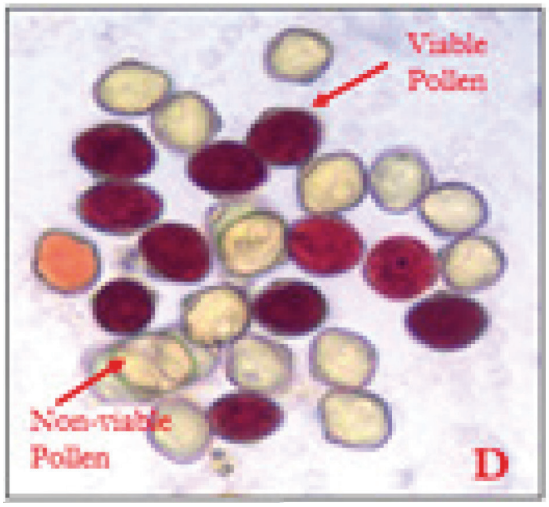
propagation attempts lead to the fast depletion of wild stocks. In addition, deforestation and low seed germination of *C. serratum* are also causal to the reduction of the natural plant population (Sharma *et al.* 2009). The Chhattisgarh Medicinal Plant Board has reported *C. serratum* as threatened species (Upadhyay and Koche, 2015). *C. serratum* enlisted as a vulnerable and endangered plant species in India (Apana *et al.* 2021; Ved *et al.* 2003).

It is crucial to study pollen biology, such as pollen viability, storage, and *in vitro* pollen germination of such valued medicinal plants to achieve the goals of its propagation, utilization, and conservation. The functional quality of pollen, such as viability and vigor, is vital in pollen-stigma interaction, incompatibility, fertility, storage, plant breeding, and improvement (Shivanna, 2019). Henceforth, it is critical to evaluate and standardize *in vitro* pollen germination conditions and its physiological requirements, such as moisture, sugars, amino acids, elemental nutrients, and growth regulators (Sanjay *et al.* 2016). Pollen grains need to be stored and preserved under artificial conditions so that they can be used to fertilize, especially among asynchronous flowering individuals within the genotype, for genetic improvement and hybridization between the early and late flowers (Martinez-Gomez *et al.* 2002). The study's primary objectives were to discern the pollen outlay, viability of pollen, effect of growth hormones, time on *in vitro* pollen germination, and its pollen storage under laboratory conditions in *C. serratum*.

MATERIALS AND METHODS

The study was carried out for the medicinal plant species *C. serratum*. The plant samples were collected in 2019 from Hlimen forest area (23° 40' 29" N, 92° 43' 17.95" E, 1140 above mean sea level), district Aizawl, Mizoram. Sample collection was done during the peak flowering from March to July 2019. Five individual plants of the species were selected randomly from the study site. Ten flowers of two different flowering stages, i.e., pre-anthesis (un-open flower) and anthesis (open flower), were collected from the selected individual plants to check the viability of pollen. Mode of anther dehiscence was observed by magnifying hand lens. The samples are collected in the morning between 7 to 9 am. 2, 3, 5-triphenyltetrazolium chloride (TTC) was prepared in sucrose solution (0.5 % TTC). One to two drops of TTC solution were put on the clean micro slide, and one anther was put on these drops; with the help of a needle, the anther was crushed, and the outer cover of the anther was removed. Then, the drops were carefully covered by a cover glass without trapping air and were incubated in the dark for 1 hour. After an incubation period, pollen grains were observed under a light microscope at a magnification of (5 X and 10 X), and the pollen grains which turned red were counted as viable (**Fig. 3 C & D**).





Four different growth regulators, viz. Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Gibberellic Acid (GA_3), and Kinetin were used to evaluate the effect on *in vitro* pollen germination. Each growth regulator was prepared at a concentration of 100, 200, and 300 mg L^{-1}. Freshly opened flowers were collected during morning hours (7-9 am) for *in vitro* pollen germination from different individuals of both the plant species from the study site. The method

given by Brewbaker and Kwack, (1963) was used to assess the influence of sucrose, IAA, IBA, GA_3, and Kinetin against the control (distilled water) on *in vitro* pollen germination. The sucrose concentration for pollen germination was prepared at 5 and 10 %. The experiment was blocked in time with five replications in a randomized complete block design (Tuinstra and Wedel, 2000). The fresh anther was crushed onto germination media in cavity slides, distributed homogenously, and placed in a room condition. The cavity slides were maintained at room temperature. After inoculation, the germination cavity slides were observed and recorded at an interval of 24, 48, and 72 hours using a light microscope. Pollen grains were observed, and then the germinated pollen grains were characterized when the pollen tube length was greater than the diameter or equal to the diameter of the pollen grain (Tuinstra and Wedel, 2000). One-way ANOVA was performed by MS Excel package, 2016 to analyze the effect of hormones, sucrose concentrations, time, and species on *in vitro* pollen germination.

Fresh flower anthers were used for pollen storage under laboratory conditions. The pollen grains were stored at three different temperatures, viz. -20°C, -4°C, and 6°C. The viability of the stored pollen was checked regularly at the interval of 24 hours for seven days using 0.5% 2,3,5-triphenyltetrazolium chloride(TTC), and after 7 days, the viability was checked at an interval of 7 days, i.e., at 14 days, 21 days and 28 days respectively until the viability was observed. The viable pollen grains were observed under a light microscope at a magnification of (5 X and 10 X), and the pollen grains which turned red were counted as viable.

RESULTS

The total number of pollen grains per anther recorded was 1368.1±40.56. The number of anther per flower and ovule per flower was recorded as four, and a longitudinal slit mode of anther dehiscence was observed (**Table 1**). The viability test with TTC observed that the viability percentage was high at the anthesis stage (open flower), i.e., 76.62%±2.63 %, then pre-anthesis (un-open flower) 18.85%±1.38% (**Table 2 and Fig. 3**). The recorded result of *in vitro* pollen germination with distilled water (control), sucrose (5% and 10%), and growth regulators exhibited a differential reaction with varied concentrations of growth regulators and sucrose in the time outline. In distilled water (control), pollen grains showed a poor percentage of germination (0.49±0.11) in the first 24 hours later; after 48 and 72 hours, no pollen germination was recorded. Pollen germination recorded in 10% sucrose concentration was 12.44±2.73 while in 5% sucrose concentration it was lesser 6.18±1.94 at the first 24 hours, later the germination percentage gradually declined with the time passed, i.e., at 48 hours and 72 hours (**Fig. 1**). A significant difference was observed between distilled water and sucrose concentrations at 5% & 10% ($p<0.0001$), but no significant difference was observed between the time intervals (**Table 5**).

All the experimentally used plant hormones in the study, viz. IAA, IBA, GA_3, and Kinetin with 10% sucrose in the growth media have resulted in better *in vitro* pollen germination in first-time intervals of 24 hours in 100, 200, and 300 mg L^{-1}. IAA, recorded for the maximum percentage of germination at the initial stage, the scored value was 55.81, 52.50, and 33.13 %. IBA and GA_3 have shown fair *in vitro* pollen germination in 100, 200, and 300 mg L^{-1}; the observed value recorded are 27.14, 35.66, and 23.47(IBA) and 34.23, 19.90, and 28.62 (GA_3) while relatively Kinetin has scored the lowest in *vitro* pollen germination in 100, 200 and 300 mg L^{-1} as followed 16.50, 27.53 and 16.66 respectively. As the time passed, the pollen germination rate also decreased, i.e., at 48 and 72 hours in all the growth hormones (Table 3). Statistically, the response of all the treatments to *in vitro* pollen germination of *C. serratum* was found to be significantly different ($p<0.05$). It was also observed that among the treatments, hormone and time gave a higher significant response ($p<0.0001$), followed by the hormone concentration application ($p<0.05$) (**Table 4**).

Table 1: Outlay of Pollen in *C. Serratum*

Species	Pollen/Anther	Anther/ Flower	Pollen/Flower	Ovule/ Flower	Mode of Anther Dehiscence
Clerodendrum serratum	1368.1±40.56	4	5472.4±162.27	4	Longitudinal slit

Table 2: Pollen Viability Tested with TTC for *C. serratum*

	Viable %
Pre anthesis (un-open flower)	18.85±1.38
Anthesis (open flower)	76.62±2.63

Table 3: Effect of Growth Hormones on *in vitro* Pollen Germination in *C. Serratum*

Hormone	Concentration	Pollen Germination %		
		24 hours	48 hours	72 hours
IAA (10% Sucrose)	100 mg L^{-1}	55.81±4.97	12.28±2.75	1.08±0.48
	200 mg L^{-1}	52.50±6.61	9.91±2.08	0.54±0.22
	300 mg L^{-1}	33.13±9.24	8.47±2.58	0.25±0.18
IBA (10% Sucrose)	100 mg L^{-1}	27.14±6.69	12.13±3.83	0.50±0.25
	200 mg L^{-1}	35.66±2.90	9.24±2.68	0.11±0.10
	300 mg L^{-1}	23.47±9.82	6.07±2.34	0.49±0.06
GA3 (10% Sucrose)	100 mg L^{-1}	34.23±9.89	12.63±3.14	1.17±0.58
	200 mg L^{-1}	19.90±6.50	4.48±2.01	0.23±0.15
	300 mg L^{-1}	28.62±6.86	7.50±3.16	0.77±0.17
Kinetin (10% Sucrose)	100 mg L^{-1}	16.50±3.05	5.48±2.86	0.10±0.09
	200 mg L^{-1}	27.53±6.34	11.90±3.06	0.51±0.23
	300 mg L^{-1}	16.66±4.28	3.05±1.05	0.05±0.08

Results are expressed as Mean ± SEM

Table 4: ANOVA for the Effect of Hormones, Concentrations and Time

Plant Species	Response Variables	df	MS	F	p
C. *serratum*	hormones	3	5015.83	24.94	<0.0001
	concentrations	2	1569.22	6.35	0.0023
	time	2	2798.12	11.67	<0.0001

Table 5: ANOVA for Control (Distilled Water), Sucrose 5% and 10% Concentration and Time

Plant Species	Response Variables	df	MS	F	p
C. serratum	between control and sucrose (5% and 10%)	2	245.54	17.44	<0.0001
	time	2	55.01	0.99	0.375704

Table 6: ANOVA for between Pollen Storage Temperatures (-20 °C, -4 °C and 6 °C) and Days

Plant Species	Response Variable	df	MS	F	p
	between temperatures	2	830.7029	1.71	0.1996
	between days	9	1382.365705	11.87	<0.0001

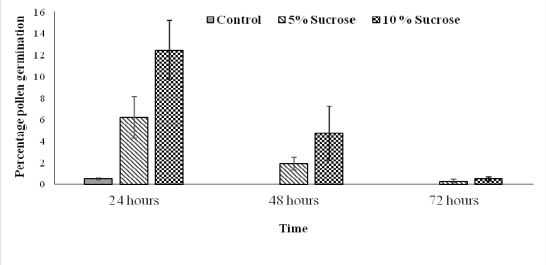


Fig. 1: Influence of Sucrose Concentrations on *in vitro* Pollen Germination in *C. serratum* species

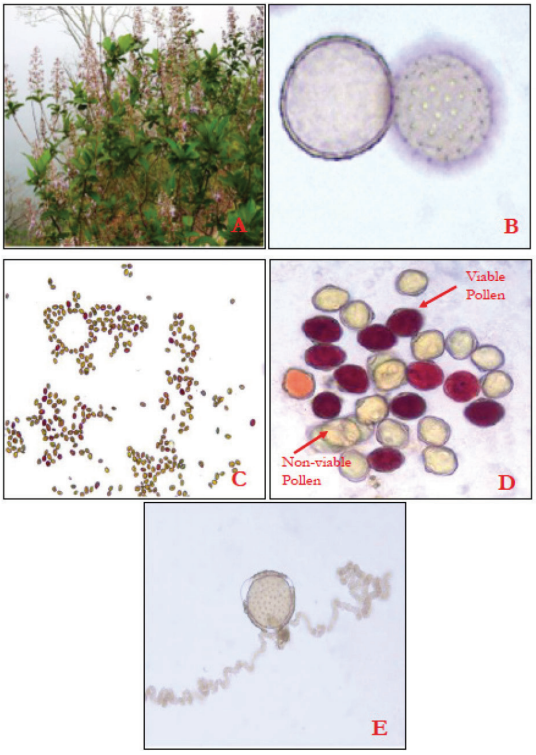


Fig. 3: (A) Habit of C. *Serratum*; (B) Microscopic View of Pollen Grains (40x); (C) & (D) *in vitro* Pollen Viability Test with Tetrazolium Salt (TTC) 5x & 20x (E) *in vitro* Pollen Germination (20x)

Pollen viability percentage of *C. serratum* decreases with the increase of storage duration, i.e., at -20°C, -4°C, and 6°C storage conditions. It was observed that pollen grains that are stored at -20°C and 6°C showed a longer viable duration. It was also observed that the pollen grains kept under -4°C

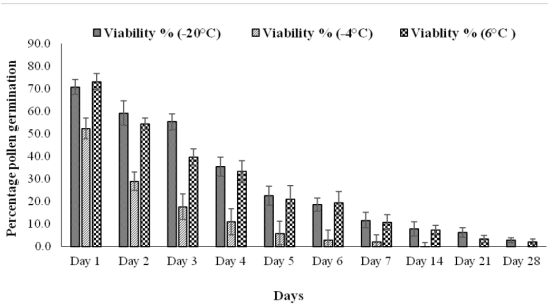


Fig. 2: Pollen Storage of *C. Serratum* Under Different Storage Temperatures viz. (-20°C, -4°C and 6°C)

lost their viability within 14 days (**Fig. 2**). Pollen loses its viability after 28 days. There is significant difference between storage days ($p<0.0001$) (**Table 6**).

DISCUSSIONS

The percentage viability at the time of anthesis (just opened flower) exceeds 75 while at the pre-anthesis stage (unopened flower) was low and less than 30 %. Hence, the TTC test was found to be effective in differentiating between viable and non-viable pollen. Similarly, a high percentage of pollen viability was recorded during the anthesis stage in *Jatropha curcas* flowers (Abdelgadir *et al.* 2012). Apart from the pollen viability test, it is further suggested for *in vitro* pollen germination studies (Shivanna, 2019). Though pollen grains of *C. serratum* showed low pollen germination in the control condition (i.e., distilled water), it simultaneously revealed the requirement of moisture for pollen germination and pollen tube formations (Sulusoglu and Cavusoglu, 2014). It is scientifically crucial to reproduce and decipher the conditions inducing germination and pollen tube growth during pollen-pistil interactions.

Pollen germination and pollen tube growth occurred among different sucrose concentrations, with relatively high germination at 10% sucrose concentration compared to 5% in the plant species. An increase in sucrose concentrations favored pollen tube growth and germination (Zhang and Huang, 2009), also recorded in *Cunnighamial lanceolata* L. (Fragallah *et al.* 2019). Sucrose plays a vital role during pollen germination and pollen tube formation as it acts as a source of energy (Lin *et al.* 2017). Sucrose balanced and maintained the osmotic pressure of pollen internally and externally and also preserved the vitality of pollen. Plant

species require different ideal sucrose concentrations for pollen germination (Liu *et al*. 2013).

IAA at concentrations of 100 mg L^{-1} and 200 mg L^{-1} was found to be most effective in inducing pollen tube germination under *in vitro* conditions. The higher concentration at 300 mg L^{-1} lowered the pollen germination and tube growth. Hence, applying IAA at an appropriate concentration can improve the pollen germination rate and tube growth in *C. serratum*. IAA plays a vital role in plant sexual reproductions, in the development of stamens and ovaries, egg cell maturation, induces embryonic axial polarity, and promotes pollen germination and tube growth of *Nicotiana tabacum* L. *in vitro* directly or indirectly (Abdelgadir *et al*. 2012; Chen and Zhao, 2008). IBA and GA_3 exhibited a differential rate of pollen germination and tube growth; from the result, it can be inferred that a decrease or increase in hormone concentration regulates pollen germination and tube growth. IBA promoted pollen germination and tube growth in *Litchi chinensis* (Zeng *et al*. 2018), *Salix lapponum* (Pogorzelec *et al*. 2015), *Bauhinia purpurea and Bauhinia variegata* (Sanjay, *et al*. 2016), *Torreya grandis* (Aihua *et al*. 2001). The application of (GA_3) helps to develop pollen tube growth *in vivo* or *in vitro* (Singh, *et al*. 2002). A similar result has also been observed on (GA_3) for strawberry pollen (Voyiatzis and Paraskevopoulou-Paroussi, 2002) and in *Pistacia vera* L. (Acar *et al*. 2010). Kinetin was observed to have the least effect on pollen germination and tube growth for *C. serratum*. A similar observation for *Prunus dulcis* was recorded where Kinetin has a relatively minor impact on pollen germination.

Pollen grains are sensitive to temperature, and if not stored properly, they may lose their viability quickly. Different species require different specific temperatures to store pollen for long-term viability. Knowledge of pollen storage and the viability of a particular plant species can help us supply pollen for breeding purposes, especially among the asynchronous flowering individuals of the plant species. Different storage temperatures (-20°C, -4°C and 6°C) showed different viability periods for the sample species, with 28 days' maximum viability. Viability of pollen can be maintained for 30 days and 20 days at -20°C and at 4°C for tea varieties such as Baojing Gold tea, Yabukita, Fuding white tea, and Foxiang (Lei *et al.* 2020). Successful fruit setting after cross-pollination of pollen which are stored for 1 year of species *Lilium lancifolium, L.* 'Raizan' and *L.* 'Casa Blanca' were reported (Rhee *et al.* 2003). Appropriate storage temperature is required to know the storage time of pollen for every particular species, which will help to establish a pollen gene bank for breeding purposes for the plant species.

CONCLUSION

TTC staining test is suitable to determine the viability of pollen grains for *C. serratum*. Anthesis stage pollen grains will be valuable in future breeding and hybridization experiments for the plant species due to their high viability. Moisture and sucrose were found to be initiating factors for pollen grain developments. Pollen grains germination under *in vitro* conditions exhibited a differential response to different growth hormones and their concentrations and time. IAA was found most suitable growth hormone in inducing *in vitro* pollen germination, followed by IBA, $GA_{3,}$ and Kinetin in the first 24 hours of incubation. Pollen grains could be stored for up to 28 days at -20 ºC, for their future scientific utilities in artificial pollination and breeding.

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Research Article

Pollen storage, viability and effect of growth hormones on *in vitro* pollen germination in two medicinal plants (*Clerodendrum colebrookianum* Walp. and *Clerodendrum infortunatum* L.) of the tropical moist forest of North-east India

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Abstract
Clerodendrum colebrookianum Walp. and *Clerodendrum infortunatum* L. are well known for their medicinal uses in treating various human ailments traditionally. Pollen biology study was done in both plant species to decipher pollen viability, *in vitro* pollen germination, and pollen storage in varied temperature conditions. Pollen viability tests was done by 2, 3, 5-triphenyl tetrazolium chloride (TTC test) for which pollen grains were collected at the anthesis stage that ascertained high viability, ranged between 71.97%±4.30 % in *C. colebrookianum* and 81.63%±3.23 in *C. infortunatum*. *In vitro* pollen germination was conducted under different hormones (IBA, IAA, GA3, and Kinetin) with concentrations of 100 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1}. Highest germination percentage of 52.10±5.30% and 61.91±1.76% in GA3 (200 mg L^{-1}) was the most suitable growth hormone concentration for inducing *in vitro* pollen germination in both *Clerodendrum* species. Statistically, the response of all the concentrations of treatments, sucrose, and hormones, with their time on *in vitro* pollen germination of *C. colebrookianum and C. infortunatum* was found significantly different ($p<0.05$). In contrast, non-significant differences were recorded for *in vitro* pollen germination between the medicinal plant species. Pollen storage under temperature gradient conditions exhibited a similar trend in the viability for both *C. colebrookianum, and C. infortunatum, i.e.,* the pollen remained viable up to 28 days at -20°C and 6°C, respectively. This study will be helpful in future breeding, hybridization, and conservation efforts for both the *Clerodendrum* species.

Keywords: *Clerodendrum colebrookianum,* Hormones, Medicinal uses, Pollen germination

INTRODUCTION

Genus *Clerodendrum* (Family: Lamiaceae) is a well-known genus for its medicinal uses in indigenous systems of medicines for treating various diseases across cultural landscapes. *C. colebrookianum* and *C. infortunatum* L. are valuable and popular medicinal plant resources in the genus. *C. colebrookianum* is a vulnerable flowering shrub (Gogoi and Nath, 2021). *C. colebrookianum* is a medicinal food plant, and its leaves are used as a vegetable. Local people of the Northeast region of India commonly use the leaf vegetable of *C. colebrookianum* as a home remedy for high blood pressure. It is highly valued in the treatment of natural antihypertension, antidiabetic, hepatoprotective, and sedative properties (Das *et al.,* 2015 and Arya *et al.,* 2018). The traditional practitioners in Mizoram commonly used its leaves as a cardioprotective against diarrhoea and dysentery and as anti-colics for infants (Lokesh and Amitsankar, 2012). Local inhabitants of Assam, Arunachal Pradesh, Manipur, and Nagaland used leaves extract and its decoction for abdominal pain, dizziness, gastric disorders, dysentery treatment, cough, skin diseases, and anthelmintic (Kalita *et al.*, 2012; Murtem

and Chaudhry, 2016; Jamir and Tsurho, 2016; Yadav *et al*., 2018 and Kshetri *et al*., 2022). Leaves were reported to possess clerosterol, colebrin, sitosterol, octacosanol, daucosterol, and fatty acids (Yang *et al.,* 2000).

C. infortunatum is a shrub medicinally useful in relieving thirst and burning sensation, foul orders, and blood diseases (Rej *et al.,* 2014). Leaf extracts are effective against scorpion sting, pain reliever, and act as expectorant and vermifuge, while bark juice relieves abdominal pain and indigestion (Nandi and Lyndem, 2016). In homeopathy, it is used as remedial medicine for diarrhoea and fresh wounds (Helen *et al*., 2021). *C. infortunatum* is reported to have several pharmacological properties, viz. anthelmintic, anticonvulsant, analgesic, wound healing, antioxidant, anticancer, antimalaria, and antifungal activities (Bhattacharjee *et al.,* 2011 and Saha *et al*., 2018). *C. infortunatum* also contains saponin, diterpene (Clerodin), triterpene (lupeol), steroid (β-sitosterol), flavonoids, glycerides of stearic acid, linoleic acid, and lignoceric acid (Bhattacharjee *et al.,* 2011).

Compared to the medicinal importance of *C. colebrookianum* and *C. infortunatum*, there are minimal studies on its propagation (Mao *et al.,* 1995) and reproductive biology. Pollen viability, germination, and storage are essential aspects of reproductive biology and breeding of a plant species, as viable and fertile pollen is critical for efficient sexual plant reproduction. The pollen's viability and vigor determine the pollen quality rate, which is crucial in artificial pollination and inbreeding experiments for understanding sterility and hybridization (Shivanna, 2019). Proper germination and growth of pollen grains are essential for fertilization, fruit, and seed development (Shivanna and Rangswamy, 2012). *in vitro* pollen germination is used significantly on a variety of pollen frameworks (Hao *et al.,* 2022). The study of pollen germination is vital in plant developmental biology. It can provide abundant knowledge on the nutritional and physiological requirements of pollen germination and its growth (Shivanna and Rangswamy, 2012). A linear relationship between pollen viability and pollen germination was observed, and in numerous plant species, it has got direct correlation with fruit and seed set (Abdelgadir *et al.,* 2012; Mesnoua *et al.,* 2018 and Shivanna, 2019).

The storage of viable pollen under controlled conditions is valuable in breeding programs, genetic preservation, artificial fertilization, and self-incompatibility (Shivanna, 2019). The life span of pollen varies significantly with plant species and storage conditions (Mesnoua *et al.,* 2018). Organic solvents, refrigeration, freeze-drying, and cryopreservation are distinctive strategies for pollen storage (Sidhu, 2019). The duration of viable pollen storage can be expanded by regulating temperature, relative humidity, and storage atmosphere (Mesnoua *et al.,* 2018; Jaskani and Naqvi, 2017).

Looking into the economic medicinal importance of *C. colebrookianum* and *C. infortunatum*, the present study was conducted to discern the influence of growth regulators and time on *in vitro* pollen germination and pollen storage for its application in future breeding and conservation program.

MATERIALS AND METHODS

Two species of *Clerodendrum*, viz. *C. colebrookianum* and *C. infortunatum* (Family: Lamiaceae) were selected at Tanhril village (23° 44' 15" N, 92° 39' 44" E, 748 m asl) in the district Aizawl, Mizoram. The study was conducted during the year 2019. In *C. colebrookianum*, flowering occurred between July to December (2019), while in *C. infortunatum,* it occurred between February to April, 2019 at the forest site.

Freshly opened flower samples at the anthesis stage were collected in the morning (6-9 am) from five individuals growing 100 m apart from each other from the forest site for experiments. Unopened flowers (just prior to anthesis) and just opened flowers were chosen to check the pollen viability. 0.5 percent 2, 3, 5-triphenyl tetrazolium chloride (TTC) prepared in the sucrose solution was used to check the pollen viability. A small number of pollen grains were suspended in the TTC (0.5%) solution and were covered with cover galas. Slides were incubated in darks for 60 minutes. After the incubation period, the preparation was observed under a light microscope (5 X & 10 X); pollen grains stained red are counted as viable (Shivanna and Rangaswamy, 2012).

Brewbaker and Kwack's (1963) basal medium and method was used for *in vitro* pollen germination studies. 5% and 10% sucrose concentrations were used to analyze the effect of sucrose against control (distilled water). Growth regulators, viz., Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Gibberellic Acid (GA_3), and Kinetin and their concentrations of 100, 200, and 300 ppm were supplemented in the basal medium to check their effects on *in vitro* pollen germination. A randomized complete block design with five replications blocked in time was used in the experiment (Tuinstra and Wedel, 2000). With the help of a needle, pollen grains from fresh anthers were put on germination media in the cavity slides. The cavity slides were placed in room conditions; average temperature (26.35±0.98) and average humidity (79.58±3.07) were recorded with a thermo-hygrometer. After incubation intervals of 24, 48, and 72 hours cavity slides were observed under a light microscope. Pollen grains were considered to be germinated when pollen tube length was found to be greater or equal to the diameter of pollen grains (Tuinstra and Wedel, 2000). The pollen grains in each germination cavity slide were assessed in 10 micro-

scopic views; a total number of germinated and non-germinated pollen grains were counted in each view and expressed as a percentage of *in vitro* pollen germination. Statistical technique ANOVA was used to analyze the effect of hormones and their concentrations, sucrose concentrations, time, and plant species were assessed for *in vitro* pollen germination with the help of Excel 2016.

Fresh pollen grains were stored under an air-tight vial at three different temperatures, i.e., 6°C, -4°C, and -20° C. The viability of stored pollen grains was tested regularly with 0.5% TTC at an interval of 24 hours for seven days after that; viability was checked at an interval of a week, i.e., 14, 21, and 28 days, respectively, under a light microscope until pollen grains were found to be viable.

RESULTS

Pollen grains viability percentage in pre-anthesis stage (un-opened flower) was 28.57% ± 2.61% and in freshly opened flower (at anthesis stage) was 71.97% ± 4.30 % in case of *C. colebrookianum* while in case of *C. infortunatum* it was 19.37% ± 1.73% in pre-anthesis stage and 81.63%±3.23 during anthesis stage. Hence anthesis stage pollen grains were found to be more viable and suitable for pollination in both plant species (Table 3).

In vitro pollen grain germination showed a differential response with varied growth regulators (IAA, IBA, GA_3, and Kinetin), sucrose concentrations, and time in both the *Clerodendrum* species. In control (distilled water), exceptionally very low percentage of pollen germina-

Table 1. Effect of growth hormones on *in vitro* pollen germination in two *Clerodendrum* species

Hormone	Concentrations	Pollen germination %					
		C. colebrookianum			*C. infortunatum*		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
IAA (10% Sucrose)	100 mg L^1	39.88±3.70	7.12±0.81	1.16±0.25	25.45±2.89	6.00±1.01	0.75±0.16
	200 mg L^1	31.80±1.97	4.73±0.61	0.42±0.15	37.02±3.21	8.25±1.39	1.46±0.24
	300 mg L^1	26.86±3.08	4.64±0.70	0.67±0.18	26.16±3.13	3.77±0.55	0.38±0.08
IBA (10% Sucrose)	100 mg L^1	46.56±4.59	9.20±0.90	2.15±0.28	34.32±3.30	6.83±0.91	1.34±0.22
	200 mg L^1	35.87±2.07	6.37±0.58	1.01±0.20	48.61±1.79	10.47±0.91	2.22±0.30
	300 mg L^1	28.99±3.75	5.73±0.63	1.40±0.23	38.27±4.83	9.21±1.40	1.80±0.24
GA_3 (10% Sucrose)	100 mg L^1	34.02±1.71	5.63±0.66	1.25±0.19	39.56±3.70	7.00±0.88	1.63±0.24
	200 mg L^1	52.10±5.30	10.56±0.74	2.03±0.20	61.91±1.76	10.44±1.12	1.92±0.33
	300 mg L^1	44.48±3.26	6.52±1.16	0.88±0.15	42.88±5.25	7.19±1.01	1.69±0.29
Kinetin (10% Sucrose)	100 mg L^1	36.06±4.04	6.44±0.69	1.50±0.21	43.42±2.37	8.87±1.45	2.02±0.35
	200 mg L^1	23.37±1.67	4.85±0.55	1.22±0.18	31.68±2.56	5.94±0.73	0.44±0.12
	300 mg L^1	28.96±3.08	5.90±0.56	1.08±0.29	37.31±4.08	6.63±0.73	1.47±0.22

Results are shown as Mean ± SEM

Table 2. ANOVA for the effect of hormones, concentrations, time and species

Plant species	Response variable	df	MS	F	p
Clerodendrum colebrookianum	hormones	3	1548.28	7.86	<0.0001
	concentrations	2	759.69	3.42	0.0359
	time	2	2059.96	9.88	<0.0001
Clerodendrum infortunatum	hormone	3	2492.38	11.77	<0.0001
	concentrations	2	1569.22	6.35	0.0023
	time	2	2798.12	11.67	<0.0001
Between Species		1	0.04	0.07	0.7825*

*Non-significant

Table 3. Pollen viability tested with TTC for two *Clerodendrum* species

Species	*C. colebrookianum*	*C. infortunatum*
	Viable %	Viable %
Pre anthesis (un-open flower)	28.57%±2.61%	19.37%±1.73%
Anthesis (open flower)	71.97%±4.30 %,	81.63%±3.23

tion (0.85±0.23 and 0.78±0.78, respectively for *C. colebrookianum* and *C. infortunatum*) in the first 24 hours and later 48 and 72 hours no germination was recorded in both species. Sucrose concentrations (5% and 10%) were found to induce *in vitro* pollen germination and acted as a fundamental substrate compared to control. In 5% sucrose concentration, a low percentage of pollen germination was recorded with 5.19±0.60% in *C. colebrookianum* and 10.65±1.14% in *C. infortunatum* while at 10% sucrose concentration; still low germination of 7.54±1.21 was recorded for *C. colebrookianum* and fair germination (17.92±4.93%) in *C. infortunatum* in the initial 24 hours. Further, the germination percentage relatively declined as time passed, i.e., at 48 hours and 72 hours (Fig. 1). Significant differences ($p<0.0001$) were observed between distilled water and sucrose concentrations at 5% & 10% and time response (Table 4).

Maximum pollen germination was recorded in the first 24 hours, which decreased with time, i.e., 48 and 72 hours. The lowest germination percentage was observed after 72 hours in 100, 200, and 300 mg L^{-1} concentrations in all the selected growth hormones, i.e., IAA, IBA, GA_3, and Kinetin in both plant species (Table 1).

In *C. colebrookianum*, the highest *in vitro* pollen germination of 52.10±5.30% was recorded in GA_3 (200 mg

Table 4. ANOVA of the effect of distil water and sucrose 5% and 10% concentrations and time.

Response variable		df	MS	F	p
Clerodendrum colebrookianum	Control and sucrose 5% & 10%	2	100.94	16.69	<0.0001
	Time	2	648.13	76.54	<0.0001
Clerodendrum infortunatum	Control and sucrose 5% & 10%	2	511.80	17.57	<0.0001
	Time	2	3640.49	129.81	<0.0001

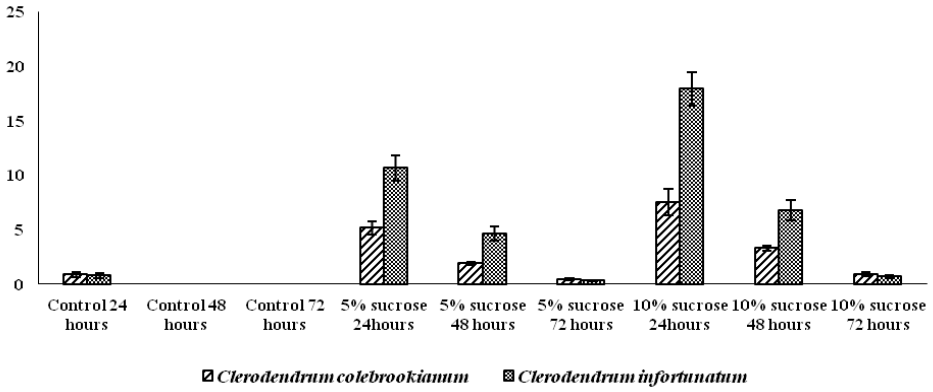

Fig. 1. *Impact of sucrose concentration on in-vitro pollen germination in C. colebrookianum and C. infortunatum*

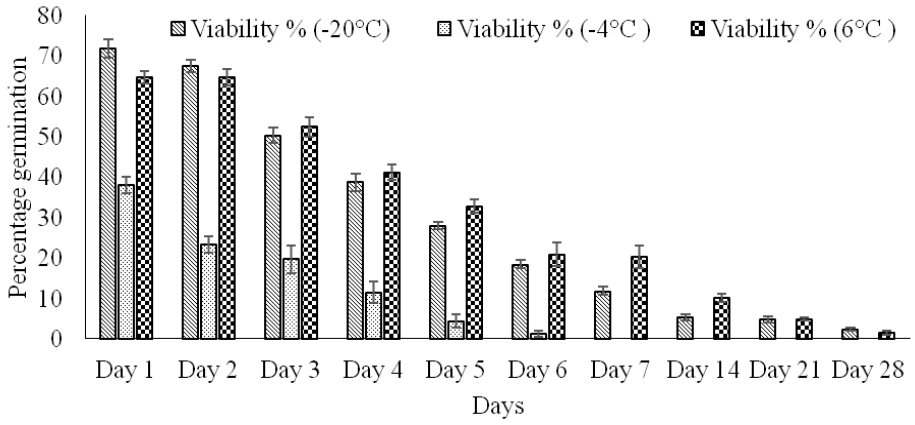

Fig. 2. *Pollen storage of C. colebrookianum under different storage temperatures (-20°C, -4°C and 6°C)*

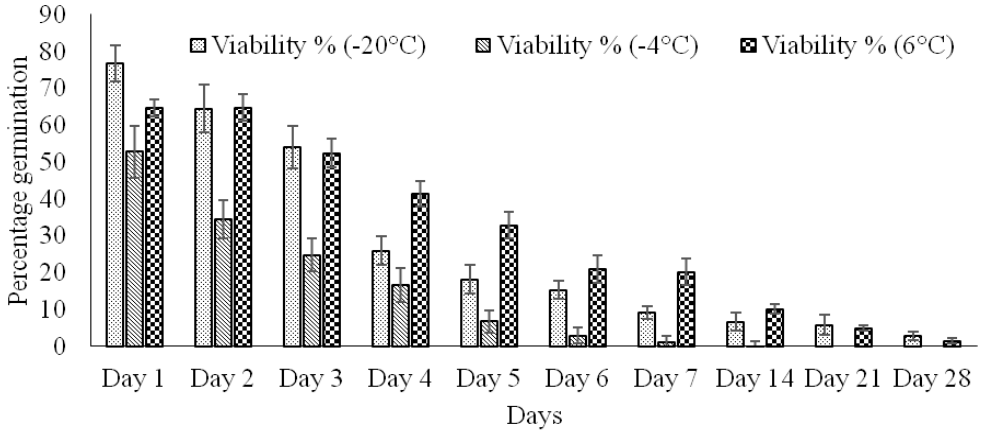

Fig. 3. *Pollen storage of Clerodendrum infortunatum under different storage temperatures (-20°C, -4°C and 6°C)*

L^{-1}), followed by 46.56±4.59 % in IBA (100 mg L^{-1}), 44.48±3.26% in GA_3 (300 mg L^{-1}) and least 23.37±1.67% in Kinetin (200 mg L^{-1}). In the case of *C. infortunatum*, the highest *in vitro* pollen germination of 61.91±1.76% was also recorded in GA_3 (200 mg L^{-1}), followed by 48.61±1.79 % in IBA (200 mg L^{-1}), 43.42±2.37% in Kinetin (100 mg L^{-1}) and least 25.45±2.89% in IAA (100 mg L^{-1}). Hence GA_3 (200 mg L^{-1}) was found to be the most suitable growth hormone concentration, followed by IBA (200 mg L^{-1} and 100 mg L^{-1}) for inducing *in vitro* pollen germination in both *Clerodendrum* species (Table 1). Statistically, the response of all the treatments viz. hormones, their concentrations and time, on *in vitro* pollen germination of *C. colebrookianum* and *C. infortunatum* was found to be significantly different ($p<0.05$) (Table 2). It was observed that among the treatments, different hormones and times gave higher significant responses ($p<0.0001$), followed by the hormone concentrations application ($p<0.05$). A non-significant difference was recorded between the study plant species for *in vitro* pollen germination (Table 3).

Under varied temperatures storage conditions such as -20°C, -4°C, and 6°C, pollen viability of both selected *Clerodendrum* species decreased with increased storage durations. It was observed that the pollen grains of both *Clerodendrum* species' stored at -20°C and 6°C showed relatively longer pollen viability duration; pollen grains lost their viability after 28 days. It was also observed that the pollens which were stored under -4°C lost their viability within 14 days of storage in two selected *Clerodendrum* species. Hence, storage conditions of both *C. colebrookianum* and *C. infortunatum* followed a similar trend (Figures 2 and 3). There is significant difference between storage days for both studied plant species ($p<0.0001$).

DISCUSSION

TTC test was found to be a dependable test for assessing pollen viability in different flowering stages, such as pre-anthesis and anthesis, to distinguish between alive and dead pollen in both *C. colebrookianum* and *C. infortunatum*. A color distinction in viable pollen stained with red while non-viable pollen with no stain was observed for both *Clerodendrum* species (Figs. 4 C and D). The color differentiation between alive and dead pollen using TTC was observed for *Jatropha curcas* (Abdelgadir *et al.*, 2012) *Prunus armeniaca* (Yaman and Turan, 2021), *Bursera* hybrids (Rico and Reyes, 2019), and *Fraxinus excelsior* (Buchner *et al*., 2022). Yang et al. (2021) found that the TTC test for pollen viability is reliable for *Amomum villosum* and *Amomum longiligulare*. The finding of a high viable percentage after anthesis in both the *Clerodendrum* species (Table 1), is similar to the findings observed for *Jatropha curcas* (Abdelgadir *et al*., 2012), *Passiflora cincinnata, Passiflora edulis, Passiflora edmundoi, Passiflora galbana, Passiflora gibertii, and Passiflora suberosa* (Soares *et al*., 2013). Shekari *et al*., (2016b) observed low viable and low germination percentage of pollen for *Leonurus cardiaca* before anthesis. Pollen viability is often correlated with pollen quality to be used in artificial pollination and breeding (Dafni and Firmage, 2000). Selection of appropriate anthesis stage for notably viable pollen grains is paramount in pollination, fertilization, and breeding of *Passiflora* sp. (Soares et al., 2013); improved cultivars of banana (Soares et al., 2015) and *Leonurus cardiaca* (Shekari et al., 2016b).

Pollen germination under *in vitro* experiments help to recreate the *in vivo* environment of pollen tube germination on the pistil. Sucrose helps to increase pollen germination and tube growth, thereby providing nutrients to the culture media (Lin *et al*., 2017). Appropriate sucrose concentration is a source of nutrition, osmotic balance, and vital carbon energy to induce pollen germination (Dong and Beckles, 2019). A high sucrose concentration may inhibit pollen grain germination (Lin *et al*., 2017). Within the same culture and media

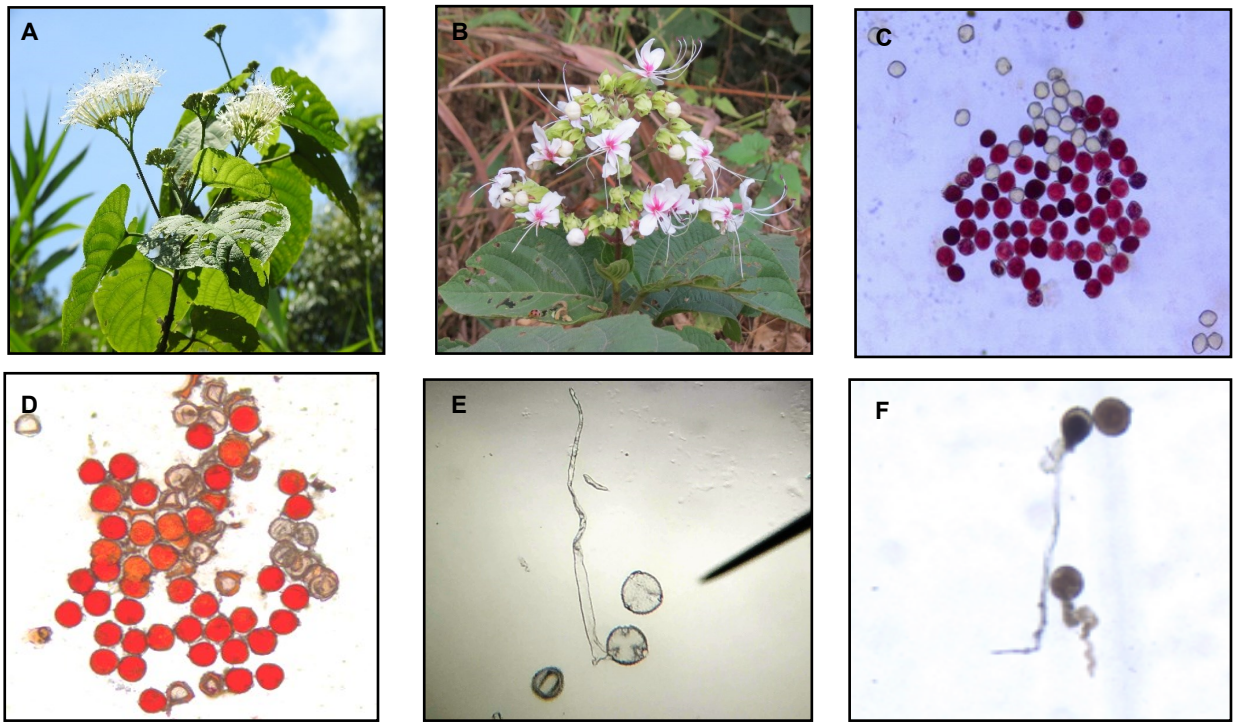

Fig. 4. (A & B): Habit of *C. colebrookianum* and *C. infortunatum*, (C& D): pollen viability in *C. colebrookianum* and *C. infortunatum*, pollen stained red is counted as viable, (E & F): *in vitro* germainating pollens of *C. colebrookianum* and *C. infortunatum*

variations, pollen germination might occur due to unbalanced osmotic pressure (Youmbi *et al*., 2015). Significant variation in pollen germination and tube growth was seen between the sucrose concentrations in both of the *Clerodendrum* species. Increased in sucrose concentration from 5 % to 10 % enhanced the pollen germination in both the *Clerodendrum* species (Fig.1.). Similar result in *Cunnighamia lanceolata* (Fragallah *et al*., 2019), *Psidium guajava* (Sarkar *et al*., 2018), *Impatiens balsamina* (Patel and Mankad, 2015) and *Leonurus cardiaca* (Shekari *et al*., 2016 a) were recorded for the effect of sucrose concentration on percentage of pollen germination. A low concentration of sucrose gives low pollen germination, while above >10% sucrose concentration gives high pollen germination in *Momordica subangulata* (Naik *et al*., 2016). The pollen germination rates varied significantly between incubation times and between treatments. Pollen germination rates were higher at 24 hours and less at 48 hours (Table 4). The results revealed that the pollen grains of both species of *Clerodendrum* need just 24 hours to start growing for germination (Fig. 1). A similar effect was observed for *Prunus laurocerasus* (Sulusoglu and Cavusoglu, 2014); *Cunnighamia lanceolata* (Fragallah *et al*., 2019) and S*pathodea campanulata, Bauhinia purperea* and *B. racemosa* (Sanjay *et al*., 2016). The rate of pollen germination determines the effectiveness of pollen germination media.

All four-growth hormones influenced pollen germination, but their rate differed (Table 2 and Figs. 4. E & F). Different concentrations of hormones give different germination percentages (Table 2). An increase in the concentration of GA_3 enhanced the pollen germination in the two *Clerodendrum* species; thus, a high concentration of GA_3 plays a vital function in pollen germination. A similar result was observed for *Acca sellowiana* (Xiong *et al*., 2016), *Prunus dulcis (*Maita and Sotomayor, 2015), and functional male flower of pomegranate (Engin and Gokbayrak, 2016). GA_3 promotes amylase activity, acid phosphatase, and β-glucosidase. It enhances the leaching of amylase and acid phosphatase enzymes to stimulate pollen germination (Sanjay *et al*., 2016). GA_3 significantly promoted *in vitro* pollen germination of *Vitis vinifera*, *Spathodea campanualata,* and *Momordica charantia* (Gokbayrak and Engin, 2015; Sanjay *et al*., 2016).

Kinetin regulates pollen germination and tube growth at different concentrations (Manonmani and Mekala, 2016; Marchioretto *et al*., 2019). A high concentration of Kinetin reduced pollen germination (Table 2) in both the *Clerodendrum* species, while a lower concentration of Kinetin was suitable for pollen germination and tube growth. Dziurka *et al*., (2019); Usman *et al*., (2022) reported low content of Kinetin in the plant improves regeneration, thereby increasing the efficiency of doubled haploid production; this supports our finding. Kinetin is reported to influence the germination of pollen and tube growth of *Prunus dulcis* (Maita and Sotomayor, 2015).

In *C. colebrookianum*, IBA and IAA at higher concentra-

tions reduced pollen germination, but in *C. infortunatum*, IBA and IAA increased pollen germination at higher concentrations (Table 2). Thus, suitable concentrations of IAA and IBA are needed for both the *Clerodendrum* species. IBA promoted *in vitro* pollen germination in four *Hibiscus* species (Li *et al*., 2015), *B. purpurea* and *B. racemosa (*Sanjay *et al*., 2016), *Litchi chinensis* (Zeng *et al*., 2018), and *Actinidia deliciosa* pollen (Marques, 2018). Abdelgadir *et al*. (2012) reported that suitable IAA concentration is needed for proper pollen germination and tube growth; this supports our finding too for the two *Clerodendrum* species. In addition, Kovaleva *et al*. (2005) reported that low concentrations of IAA promoted pollen germination while higher concentrations inhibited it. IAA stimulated pollen germination *in vitro* of male *Petunia hybrida* through osmoregulation by activating K^+ channels (Kovaleva *et al*., 2016).

IAA, GA3, IBA, and Kinetin influenced and regulated *in vitro* pollen germination for both *Clerodendrum* species. But their response in two selected *Clerodendrum* species differed with growth hormones and their concentrations, suggesting that the pollen grain of the two *Clerodendrum* species react differentially with different growth hormones.

Long-term pollen storage is essential for plant breeding, especially in asynchronous flowering species and germplasm exchange. The longevity of pollen differs from plant species to species and from minutes to months. Thus, there is a practical need to evaluate and standardize storage conditions of pollen grains to maintain their vitality for an extended period for making crosses between two varieties/ species which flower at different times. From the result of pollen storage, pollen grains of two *Clerodendrum* species which were stored (Fig. 2 and 3) at various temperatures (-20°C, -4°C and 6°C) for varying lengths of time (0-28days) revealed that the pollen viability decreased after storage at different temperatures with an increase in the interval of time. This would be due to the fact that the metabolic activity of pollen depends on temperature and time (Du *et al*., 2019). Among the stored temperatures (-20° C, -4°C and 6°C), viability percentage of pollen varies significantly in both *Clerodendrum* species. Temperature and humidity influence the viability of pollen (Sidhu, 2019; Du *et al*., 2019). Therefore, appropriate temperature is needed to be screened through more experimentation to extend the longevity of pollen viability of *C. colebrookianum* up to 70 days (as flowering occurred between July to December) so that species hybridization could be done with *C. infortunatum* (flowering occurred between February to April 2019) to develop new species with novel importance.

Pollen grains of *Fraxinus excelsior* lose their viability in warmer conditions, and they can be stored for a longer duration at temperatures of -20°C and -80°C than at 4° C; and the viability of stored pollen grains could be used to overcome crucial problem concerning ash dieback disease through future breeding program (Buchner *et al*., 2022). Pollen grains of *Juniperus communis* (a tree with meager seed sets due to pollen limitation under natural conditions) can be stored suitably with significant pollen viability at − 20 °C as compared to -4°C. The stored pollens were valuable for pollen supplementation experiments to enhance seed sets in the tree species (Kormutak *et al*., 2021). Hence pollen storage is crucial in pollen handling that can act as a valuable tool for breeders to overcome problems associated with differences in flowering time, pollen shedding, stigma receptivity, and pollen limitation in controlled pollination experiments. Further, the studies of long and short-term pollen viability and storage could help to make crosses among individuals of subpopulations growing geographically separated and adapted to biotic and abiotic gradients in racial hybridization to improve traits of interest.

Conclusion

It was concluded that TTC staining test is a dependable test to evaluate the viability of pollen grains for *C. colebrookianum* and *C. infortunatum*. Sucrose (5% and10%) induces low to fair pollen germination of the pollen grains of these species. GA_3 (200 mg L^{-1}) was found to be the most suitable growth hormone concentration, followed by IBA (200 mg L^{-1} and 100 mg L^{-1}) for inducing *in vitro* pollen germination in *C. colebrookianum* and *C. infortunatum* during the first 24 hours of incubation. Among the treatments, different hormones and times gave a higher significant response ($p<0.0001$), followed by the hormone concentrations application ($p<0.05$). There was a non-significant difference between the plant species for *in vitro* pollen germination. Pollen grains of both *Clerodendrum* species remained viable up to 28 days at -20°C and 6°C. Thus, pollen grains of both *Clerodendrum* species should be collected at anthesis stage for short-term storage of pollen grains which shall be valuable for future application in pollination, supplementation, hybridization, and breeding experiments in both *Clerodendrum* species.

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Flower-Pollinator Interactions in Liana (*Caesalpinia cucullata* Roxb.) in a Tropical Rain Forest of Mizoram

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***Abstract*:** Lianas (woody climbers) are relatively underexplored life forms of many forests, which predominantly forms tropical forests and provide food and shelter to a variety of animals. A study on flower-pollinator interactions in *Caesalpinia cucullata* Roxb. was conducted in the tropical rain forest of Indo-Burma hot spot at Tanhril area of Aizawl district of Mizoram. Floral visitors of *C. cucullata* were monitored and recorded during 24 field days, four to six hour per day with a total of about one hundred hours during the flowering season of November–December, 2016. The flowers of *C. cucullata* were found to be visited by three insect species belonging three families; nine passeriformes birds belonging eight families and one Hoary-bellied Himalayan squirrel (Irrawaddy squirrel) belonging family Scuiridae. Birds and squirrel mainly visited the flowers in morning hours while butterflies and bees exhibited diurnal pattern of foraging. The results revealed that the bird pollination is supported by butterflies in *C. cucullata*. The flowers of *C. cucullata* would be excellent food resource to the dependent animal species during dry cold period in the extreme tropical sloppy mountain forest site when availability of floral resource is very scarce. Therefore, *C. cucullata* could be a valuable liana species for the conservation of valuable species of insects, birds and squirrels.

***Keywords*:** Liana, Birds, Insects, Pollination, Tropical forest and conservation

Tropical forest community contains enormous diversity of flora and fauna with their ecological interactions. One of the peculiar and mesmerizing life forms found in tropical rain forest is woody climbers, i.e., liana, which remained a relatively underexplored plant life forms compared to that of tree species (Rice et al 2004). In the last two decades there is a pulsating trend in liana research owing to their growing significance in tropical forest dynamics due to global change (Ledo and Schnitzer 2014). Liana competes with trees for above and belowground resources, leading to decrease in recruitment, regeneration, growth, fecundity and survival of trees especially in disturbed forest sites (Phillips et al 2002, Schnitzer and Bongers 2011). Conversely, lianas act as an important resource to the forest functioning like stabilizing microclimatic conditions of under canopy, trail for arboreal animals to crossways the tree tops, acts as foliar, floral, fruit and nesting resources to a diverse group of vertebrate and invertebrate fauna (Emmons and Gentry 1983, Yanoviak and Schnitzer 2013). Birds are reported to depend on lianas for a variety of direct resources such as fruit (many lianas produce fleshy fruits which are frequently consumed by birds) and nectar. -Indirect resources like sheltering, nesting sites, perching space, insects and their larvae for feeding (Kominami et al 2003, Sankamethawee et al 2011). Nectar of lianas act as floral rewards to the diverse array of birds such as humming birds, honeyeaters, warblers, parrots, blackbirds, cardinals and orioles (Stein 1992, Peres 2000, Fleming et al 2005). Many liana-harboring insects (mainly Hymenopterans, Dipterans and Hemipterans) extract floral nectar as their feed, such insects turn act as a food resource to insectivorous birds (Gryj et al 1990). Lianas are also reported to be harbouring large number of endophytic fungi (Biplab 2018). Lianas and their tangles offer either obligate and facultative nesting and/or roosting niche for a spectrum of bird species (Mack and Wright 1996, Michel et al 2015). Intense liana entangles offer excellent habitat for birds to hide and protect from predators (Boinski et al 2003), song and display perches (Durães 2009). In turn, lianas get benefited from a range of services by birds in pollination, seed dispersal and protection from insects in herbivory (Gryj et al 1990, Stein 1992, Lenz et al 2011, Michel et al 2015), while some birds used to rob nectar and predate seeds of liana, thus affecting it negatively (Lara and Ornelas 2001).

The trend of bird population and their species diversity are declining globally (Sekercio˘glu et al 2004) and presently, 21% of bird species are measured to be extinction-prone and 13-39% of bird species are speculated to be extinct by 2100 (Sekercio˘glu et al 2004). Specialized fruit and nectar eating

bird species are more vulnerable to extinction than other functional groups. Therefore, the decline in population of specialist bird species involved in pollination and seed dispersal of liana are going to impact the liana and other key plant groups (Ansell et al 2011). Contrary to this, the generalist bird species are reported to increase with increasing liana abundance and diversity in logged rain forest site (Biamonte et al 2011). Therefore, it is utmost important to understand the nature and degree of liana-animal interaction for the sustainable conservation of lianas, mammals, birds, insects and tropical forest communities.

Caesalpinia cucullata Roxb. (Syn. *Mezoneuron cucullatum* (Roxb.) Wight and Arn.) commonly known as hooded-flowered brasiletto, Sahyadri thorn, (Locally known as Hling-Khang in Mizo) is a large climbing shrub with thorns and is reported to be an armed straggler (Muthumperumal and Parthasarathy 2009). It bears fragrant flowers in terminal and axillary racemes of 20-40cm in length. Yellow flowers appeared like hoods with long stamen filament and protruding red anthers. *C. cucullata* is distributed throughout the north eastern hilly states of India (Barik et al 2015), and sparsely found in evergreen forests of Sahyadri hills and its presence is also recorded from North Andaman in Semi evergreen and littoral forests (Ghosh 2013). It is closely related to *Caesalpinia decapetala* (Deshmukh et al 2013). Its beans are locally consumed by the tribals of Koraput, Orissa (Mishra and Padhan 2011) and roots are used in curing sprains (Bandopadhya and Mukherjee 2010). In Chinese traditional medicines it is reported to be an effective anti-abortion agent (Xiaoping and Shaanxi, 2003). A variety of active phytochemicals was isolated and characterized from the different parts of *C. cucullata* (Cheng-yu et al 2013). There is no scientific report on flower-animal interaction (floral visitors) of *C. cucullata* so far and this study is the first report on the flower visitors and their role in pollination and resource utilization for sustainability and conservation of dependent pollinators as well as the liana species.

MATERIAL AND METHODS

Five individuals of *C. cucullata* were identified along deep mountain slopes inside the Mizoram University campus, Tanhril, Aizawl (latitude 23°.43'53. 19" N to 24°.35' N and longitude 92°.39'44.21" E to 93°.29' E and altitude 832 m). A reconnaissance survey was also made all across the campus to locate other individuals of *C. cucullata* but other individuals were not found which might be due to highly dense and close forest canopy cover, inaccessible steep forest mountain slopes in the study area. Hence, recording of phenological events (for two seasonal calendars i.e., 2016-17 and 2017-18) and pollinator floral visitors (in 2016) were done on five individuals located nearby to each other with the help of binocular and camera. Floral visitors of *C. cucullata* were monitored and recorded during 24 field days, four to six hours per day with a total of about one hundred hours during the flowering season of November-December 2016. Five branches per individuals were chosen randomly and the observations were recorded over the course of whole day length between 0600 h morning to the 1700 h dusk in five blocks (0601-0800; 0801-1000; 1001-1200; 1201-1400 and 1401-1700). The visitation rate of the floral visitors was assessed in terms of visits per branch per day (visits/branch/day) and the pollinators were classified as regular and occasional visitors on the basis of their frequency. Floral visitors included insects, birds and squirrels. Floral visitors were monitored with the help of binocular, camera, and also directly when they visited the flower. Bird's mode of approach, landing, probing behaviour with bill while perched, floral resource used by the flower visitors, contact with reproductive organs which can potentially promote pollination were recorded. The allocation of each monitoring time block was done in such a way that all the selected individuals of lianas in a group was monitored in each observation block during field visit. Floral visitors were identified with the help of standard handbooks and manuals (Ali 1943, Richard et al 2011). The structure and brief tree floristic diversity of the forest and climatic features of the study site were described by Kumar and Khanduri (2016).

RESULTS AND DISCUSSION

Caesalpinia cucullata flowered during cool dry period from mid of November to first week of January with peak flowering (i.e. blooming) recorded during second to third week of December in 2016 (Fig. 1A-B). Early fruiting started in first week of January which coincided with late flowering phase. Both fruits and flowers can be observed simultaneously in the same branch of *C. Cucullata* (Fig. 1C). Fruiting phase was extended from first week of January-2017 to last week of April-2017. Fruit maturity took place in February-2017 and dispersal was recorded in March and April-2017 (Fig. 1D and Fig. 4). In 2017, floral budding was initiated in the third week of November-2017 and just before the anthesis and blooming phase a brief period of atypical intense rainfall occurred during 9-11 December-2017 (Fig. 3) which disrupted the whole flowering phase, pollination and fertilization and all unopened floral buds from the branches fell down within a week (Fig. 1E). Consequently, no animal foraging and fruit set were recorded during the season 2017-2018 (Fig. 4).

Three insect's species belonging to three families; nine

passeriformes birds belonging to eight families and one Hoary-bellied Himalayan squirrel (Irrawaddy squirrel) belonging to family Scuiridae were found visiting the flowers of *C. cucullata* during the flowering phase of December-2016. *Vindula erota erota* (butterfly) is one of most prolific floral visitor recorded exhibiting peculiar sexual dimorphism in morphology (colour) between male and female individuals (Fig. 1 H-I). Diurnal foraging activity for *V. erota erota* was

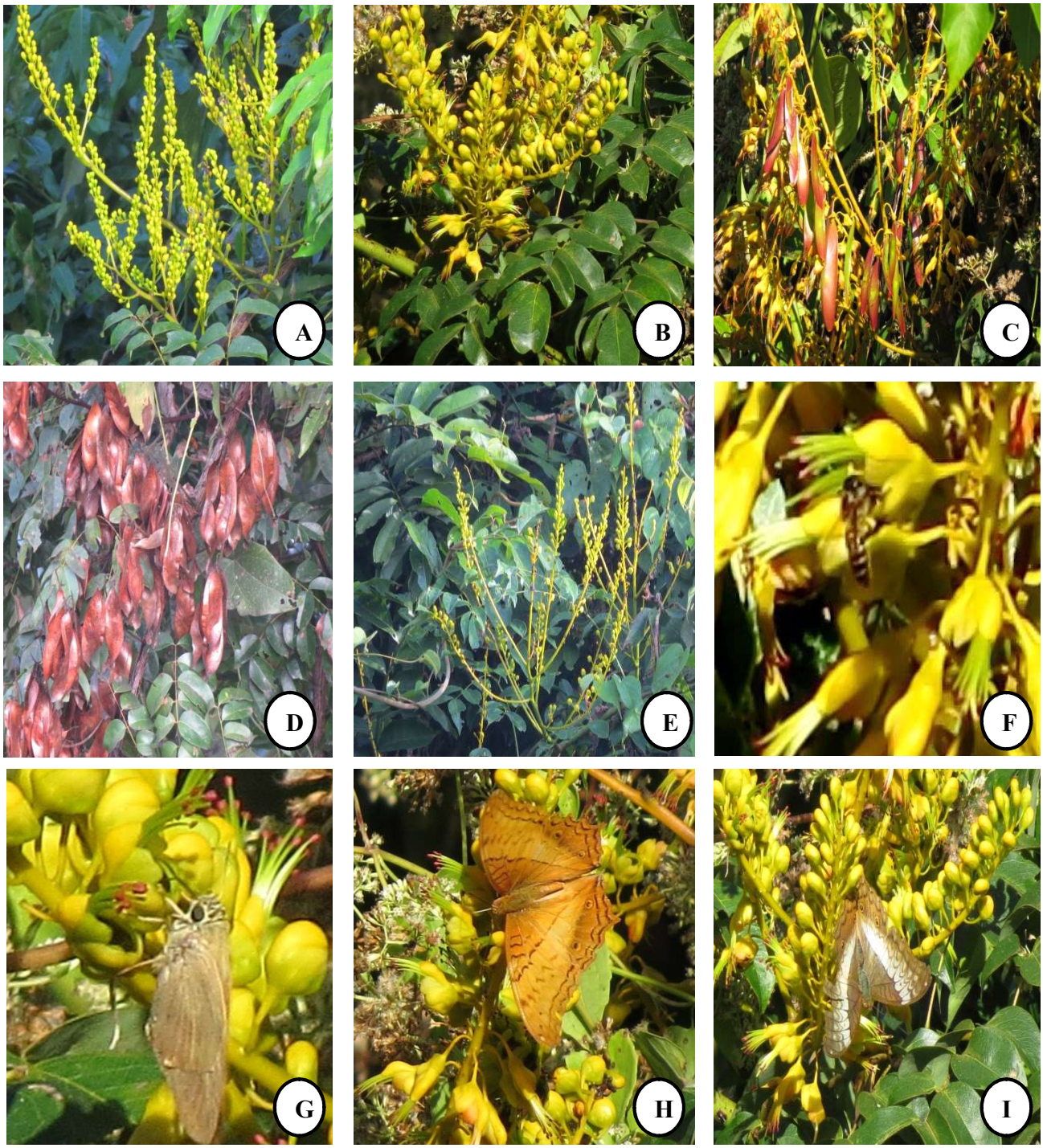

Fig. 1. Phenological phases and insects floral visitors of *C. cucullata* : (A) Early unopened floral bud-2016 (B) Flower in blooming phase-2016 (C) Early fruiting phase coincide late flowering phase-2016 (D) Mature fruiting stage-2016 (E) Detrimental impact of unusual out of season rainfall on flowering phase (all floral buds before anthesis fell down)-December 2017 (F) *Apis cerena* (G) *Badamia exclamationis* (H) *Vindula erota erota* (male) (I) *Vindula erota erota* (female)

observed with the peak foraging activity during morning and afternoon hours, however, inter flower movement was slow (Table 1). *Badamia exclamationis* (butterfly) occasionally visited the flower with peak visitation during 0801-1000 hours. Both species of butterfly exhibited hovering and sitting activity on flowers. During foraging, they make definite contact with stamens and stigma of *C. cucullata* and the nectar and pollen were harvested as their food resource (Fig. 1G-I). *Apis cerena* (bee) was recorded foraging the flowers of *C. cucullata* diurnally with peak visits during morning and afternoon. *A. cerena* makes inter flower movement and in one bout it visited 2-6 flowers in a branch. *A. cerena* extracts floral nectar without making regular contacts with protruding red-coloured stamens (Fig. 1F). While foraging, *A. cerena* very occasionally contacted the stamen of the flower for pollen resource and therefore they mainly visited for nectar of the flower.

C. cucullata offered some of distinguishing features for bird pollination (ornithophily) such as; (i) upright branches facilitating bird perching, (ii) large number of red colour protruding stamens in flowers can be easily sighted from distance, (iii) production of nectar in protected cup like yellow flower, (iv) corolla colors range from yellow to yellowish orange and (v) prolonged anthers seems to be important feature for pollen transfer. As bird forages for deep seated nectar, a definite contact with anthers to beak, head and neck region of birds was observed (Fig. 2 A-H).

Table 1. Butterflies, bee, passerine birds and squirrel visitors to the flowers of *C. cucullata*

Animal species	Common name	No. of visits/ branch/day (n=10 d) (Mean ±SD)	Frequency	Peak time of visitation	Floral resource sought	IUCN Status
Insects (Lepidoptera & Hymenoptera) Family : Nymphalidae *Vindula erota erota* Fabricius	Common cruiser	30.9±10.72	Regular	0801-1200; 1401-1700	Nectar, Pollen	NA
Family: Hesperiidae *Badamia exclamationis* Fabricius	Brown awl	3.5±1.77	Occasional	0801-1000	Nectar, Pollen	NA
Family: Apidae *Apis cerena* Fabricius	Asiatic honey bee	55.2±12.7	Regular	0801-1200;1401-1700	Nectar Pollen	NA
Birds (Passeriformes) Family: Chloropsidae *Chloropsis aurifrons* Temminck	Golden-fronted leaf bird	8.3±2.86	Regular	0601-1000	Nectar	LC,⇌
Chloropsis cochinchinensis Gmelin	Blue winged leaf bird	3.3±2.62	Occasional	0601-0800	Nectar	NT, ↓
Family: Pycnonotidae *Pycnonotus cafer* Linnaeus	Red vented bulbul	5.6±2.59	Regular	0801-1000	Nectar	LC, ↑
Family: Nectariniidae *Arachnothera longirostra* Latham	Little spider hunter	2.6±1.77	Occasional	0801-1000	Nectar	LC,⇌
Family: Cisticolidae *Orthotomus sutorius* Pennant	Common tailor bird	3.1±2.18	Occasional	0801-1000	Nectar	LC,⇌
Family: Zosteropidae *Zosterops palpebrosus* Temminck	Oriental white eye	5.3±2.35	Regular	0601-1000	Nectar	LC, ↓
Family: Tamaliidae *Mixornis gularis* Horsfield	Pin-striped tit-babbler	1.8±1.75	Occasional	0801-1000	Nectar	LC,⇌
Family: Phylloscopidae *Phylloscopus* sp.	Leaf warbler	1.4±1.5	Occasional	0801-1100	Nectar	
Family: Dicruridae *Dicrurus macrocercus* Vieillot	Black drongo	0.9±0.73	Occasional	0801-1000	Nectar	LC,?
Squirrel (Rodentia) Family: Sciuridae *Callosciurus pygerthus* I. Geoffroy Saint Hilaire	Hoary-bellied Himalayan squirrel	2.1±1.28	Occasional	0801-1000	Flower, Nectar	LC,⇌

NA (Not Available); LC (Least Concern); NT (Near Threatened); ⇌ (Stable); ↑ (Increasing); ↓ (Decreasing) ;?(Unknown)

Chloropsis aurifrons was recorded to be the regular visitor to the flower of *C. cucullata* during early morning hours (0601-1000; Table 1); it first perched on the branch of liana then try extracting nectar from more than one flowers in one sitting and spared about 15-35 seconds in a branch and 4-6 seconds per flower. On an average it pokes and extracts nectar from 5-15 flowers in one bout and makes definite contact with stamens through its neck, head and beak (Fig. 2D). *Chloropsis cochinchinensis* was foraging mostly during early morning hours and its behaviour was almost similar with

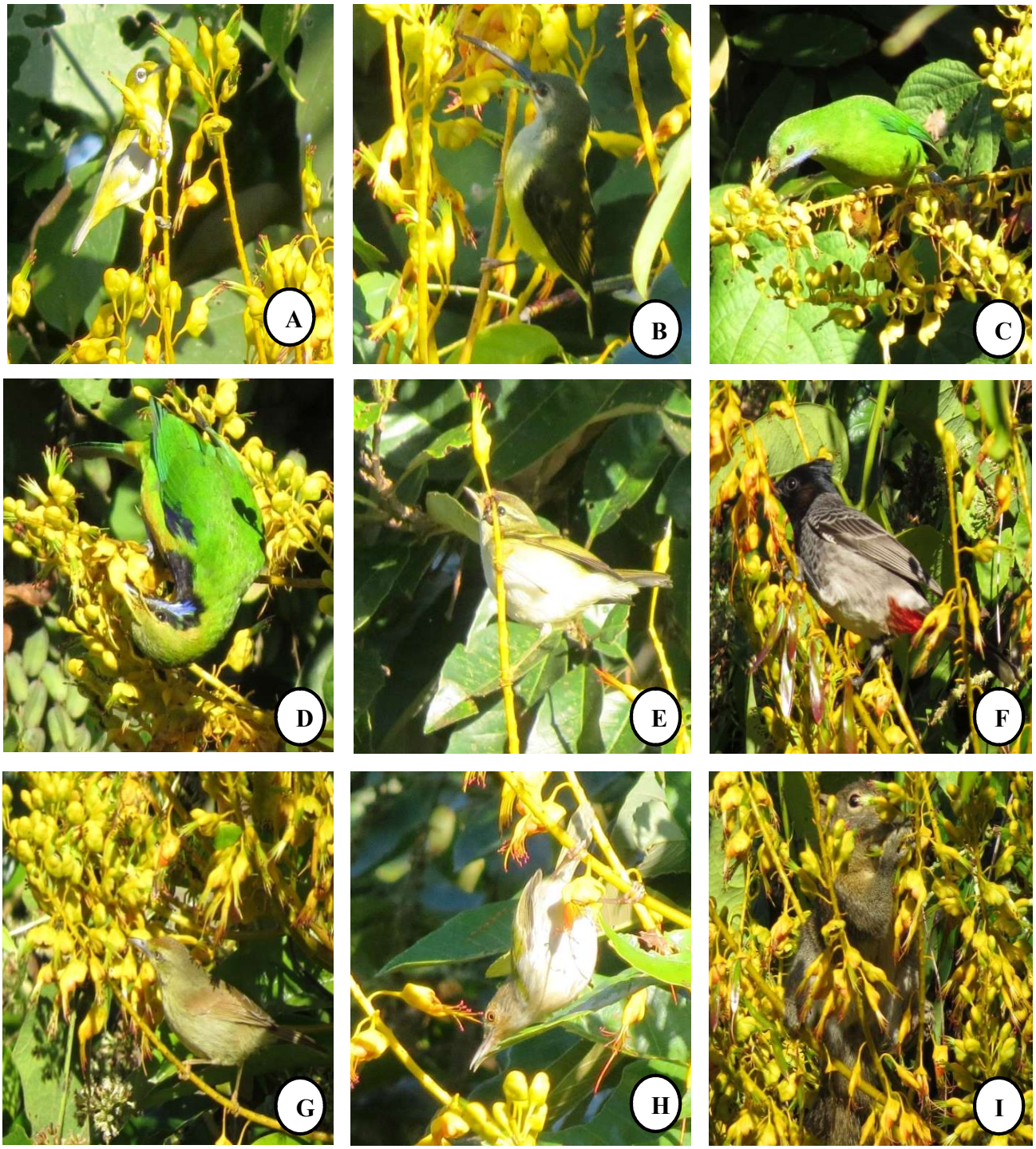

Fig. 2. Floral visitors (birds & squirrel) showing interactions with reproductive floral parts of *C. cucullata* (A) *Zosterops palpebrosus* (B) *Arachnothora longirostra* (C) *Chloropsis cochinchinensis* (D) *Chloropsis aurifrons* (E) *Phylloscopus* sp. (F) *Pycnonotus cafer* (G) *Mixornis gularis* (H) *Orthotomus sutorius* (I) *Callosciurus pygerthus*

C. aurifrons but it occasionally visited the flower for nectars (Fig. 2C, Table 1).

Pycnonotus cafer was observed as a regular visitor to flower with the average visit of 1-3 flowers per visit and was found to be very alert while foraging. It sensed small movement and fly away from the source. It extracted nectar during foraging and spared about 10-15 seconds per flower and simultaneously made precise contact with reproductive parts of flower (Fig. 2F). Oriental white eye (*Zosterops palpebrosus*) visited the flower both singly and in small flocks (Fig. 2A). The peak visitation was recorded during 0600-1000 h. The flower handling time was very less (~3-5 seconds). After harvesting nectar, it rubbed its beak on branches after nectar drinking. Little spider hunter (*Arachnothera longirostra*) occasionally visited the flower. It produced typical noise before and after foraging the flower and rubbed its beak on branches after nectar harvest. It legitimately foraged the flower with long curved beak. The beak and head of *A. longirostra* make precise contact with reproductive parts of flower (Fig 2B) . It spent around 3-5 seconds per flower. *Orthotomus sutorius*, *Mixornis gularis* and *Phylloscopus* sp. were found occasional visitors to flowers of *C. cucullata* and were observed visiting during morning hours (0800-1000) (Fig. 2F, G and H). They make firm perched on the branch and then precisely foraged flower for nectar. At one perch they poked 1-3 flowers. *Dicrurus macrocercus* was found to be very occasional visitor to flower, it perched on branch and try to harvest nectar from flower; while foraging it damages the flowers too. *Callosciurus pygerthus* (Irrawaddy squirrel) to the visited singly flowers of *C. cucullata*, during visits it makes extensive noise, while harvesting nectar, damaged the flower and also did florivory. The prehensile tail of *C. pygerthus* acts as balancer while moving from flower to flower, it spent good amount of time per visit.

Insects mostly exhibited bimodal pattern of regular foraging visit while bird visited mostly in the morning hours with unimodal pattern. *C. aurifrons, P. cafer* and *Z. palpebrosus* were recorded most regular visitors to the flowers of *C. cucullata* while others foraged occasionally for nectar. After web search on IUCN red list to assess the conservation status of floral visitor species, except blue winged leaf bird (*C. cochinchinensis*) which is found to be near threatened (NT), all other birds were found to be in least concern (LC) category. Population trend found be variable with stable population trend for *C. aurifrons, A. longirostra, O. sutorius, Mixornis gularis,* increasing trend for *P. cafer* while decreasing trend for *C. cochinchinensis* and *Z. palpebrosus. Callosciurus pygerthus* was enlisted in LC with stable population trend.

The majority of perennial plant species found in tropics exhibit some degree of seasonality in growth and reproduction to climatic factors (van Schaik et al 1993, Aide 1993). Such a periodicity in tropical long-lived plants is tightly coupled with activities of dependant animal species for plant resources such as emerging leaves, nectar, pollen, fruits and seeds. In turn, animals render their valuable services as pollinators, seed dispersers and protecting from herbivory from other animals. Thus, uneven patterns of climatic factors may have profound impact on such plant-animal interactions which may lead loss of biodiversity and associated ecological functions (Butt et al 2015). In present study erratic intense brief rainfall during 9-11 December, 2017 (Fig. 3) leads reproductive failure of *C. cucullata* in the season. The month of December in general considered as a dry month in present study site, as there were only two episodes of rainfall i.e. 56 mm and 37.6 mm in December-2010 and December-2017, respectively has been recorded during the past decade since 2005. Such atypical climatic events not only affected the liana flowering and reproduction but also influenced the dependent animal species (recorded three species of insects; nine species of birds and one squirrel in the study) for their food resources. Changes in temperature and intense erratic rainfall are reported to be most important factors affecting phenology (flowering and fruit drop) in tropics (Wright and Calderón 2006, Gunarathne and Perera 2014) and in turn has cascading effects on dependent vertebrate fauna.

C. cucullata might be a critical food resource during dry cold period, when moisture availability for plant growth and development is limited for deciduous trees and annual herbaceous community in the present tropical sloppy

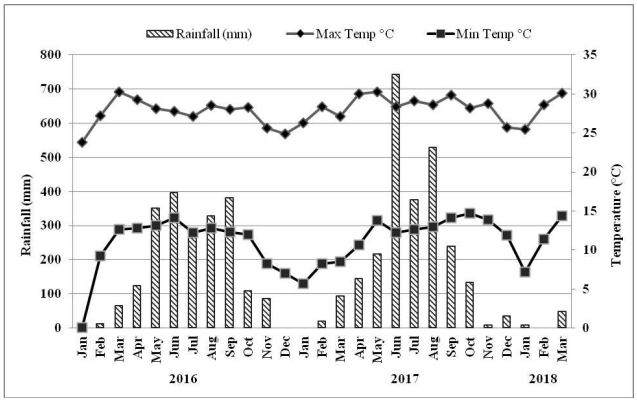

Fig. 3. Mean monthly rainfall, minimum and maximum temperature distribution in study area during study period 2016-18 (Source: ENVIS Centre, Mizoram). Atypical rainfall (35.6 mm) in the month of December-2017 on dated 9, December (5mm), 10 December (19mm) & 11 December (11.6 mm)

	2016						2017												2018					
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr		Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Flowering phase					**	****	* *										*	**						
Fruiting phase							ψψψ ψ	**ψψψ ψ**	**ψψψ ψ**	**ψψψ ψ**														

Fig. 4. Phenogram of *C. cucullata* for reproductive stages (*-Flowering; **ψ**-early fruiting; ψ-mature fruits) in two annual seasons (i.e. 2016-17 & 2017-2018); atypical rainfall during second week of December, 2017 negatively affected flowering phase, pollination and fruiting, hence no fruit set was recorded in 2017-18

mountain forest site. The floral resource availability to the dependent animals is very scarce in the months of November and December as compared to spring and rainy seasons. Other tree species which were overlapping with *C. cucullata* flowerings in December in the study site were *Bombax insigne*, *Parkia roxburghii*, *Neolamarckia cadamba* and *Prunus cerasoides* (Khanduri and Kumar 2017). Floral nectars are rich source of sugars, amino and organic acids which are a suitable source of food to a broad spectrum of animals (Koptur 1992). Floral nectars of liana were reported to serve as an important food resource to birds and around 94 bird species belonging 22 families mainly humming birds, honey eaters and warblers were reported so far (Michel et al 2015). Passerine birds have been recorded as major floral nectar feeders in tropical dry deciduous forest during low food availability in other species of liana *e.g., Combretum fruticosum* (Gryj et al 1990). The bird's acoustic activity and patterns were more complex in liana rich forest site as compared to low liana abundance site in tropical deciduous forests of Costa Rica, thus, indicating importance of liana as direct and indirect resource to floral bird communities (Hilje et al 2017).

CONCLUSIONS

On the basis of visitation frequency, time, and behaviour of observed animal visitors in *C. cucullata*, it is ample clear that the birds are main potential pollinators in *C. cucullata* duly assisted by butterflies (*Vindula erota erota* and *Badamia exclamationis*). However, diversity of floral visitors reveals that *C. cucullata* is a valuable liana species in the moist tropical forest for conservation of animal visitors, as it flowers during low resource availability. Moreover, the impact of atypical climatic rain exposed its vulnerability to reproductive success that also may influence the dependent animal species for food resources. *C. cucullata* could be a valuable liana species in green urban landscaping for their aesthetic as well conservation value for the dependent animal community.

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Chapter 1:
Introduction

The forests accomplish the needs of the rural communities for their livelihood and subsistence. Indigenous peoples are very familiar with their surroundings' floral and faunal diversity and have developed a deep relationship with their nature and natural resources. Non-timber forest products (NTFPs) are any forest items obtained from forests, aside from timbers. Thus, the term 'Non-Timber Forest Products (NTFPs) encompasses all biological materials other than timber, which are extracted from forests for human use (Soe & Yeo-Chang, 2019). NTFPs play a vital role in the rural economy, creating employment generation, revenue earning potential and socio-cultural and life support opportunities. Rural people in developing countries earn 20-25% of their income from NTFPs (Uprety et al., 2016). NTFPs are regarded as secondary forest products and have been historically and culturally used for hundreds of thousands of years by indigenous societies for foods, medicines, fuel woods, fodders, fibres, building materials, and other uses (Shackleton, 2015). The traditional health care system depends mainly on NTFPs in the form of medicinal plants with ethnic and cultural significance. To treat common ailments and other diseases, local people used to prepare traditional medicines from the plant species found in their natural landscape.

Among the different categories of NTFPs, medicinal plants are more important than other categories. Many species of NTFPs have medicinal benefits for treating various ailments such as stomach aches, cuts and wounds, diarrhoea, ulcers, infertility, malaria, fever, and blood purification (Popoola & Obembe, 2013). The demand for medicinal plants is rising globally and in the domestic market. Ayurveda, Chinese, Unani, Siddha, Tibetan, and other traditional medical systems worldwide rely on medicinal and aromatic plants (Gewali & Awale, 2008). Numerous drugs or medicines are extracted/synthesized from wild plants or their extracts. The World Health Organization (WHO) estimated that 80% of the population relies on plant-based medicine traditionally for their primary health care due to its ease of asses and availability to the people (Uritu et al., 2018).

According to the International Union for Conservation of Nature and the World Wildlife Fund (IUCN), between 50,000-80,000 plant species are used in traditional and modern medicinal systems (Romanciuc, 2019). About 20,000 species of higher plants are contributed by India, with 500 plant species classified as having medicinal potential and one-third of them being endemic (Krishnan et al., 2011). Almost all societies used medicinal plants as a source of healing purposes. Medicinal plant resources are one of the most important Non-timber forest products (NTFPs) in tropical regions. 2/3rd of the rural population in the tropics still depends on traditional medicinal plants for their primary healthcare (Muthu et al., 2006).

Medicinal plants contain physiologically active biochemicals that has therapeutic effect, such as saponins, tannins, essential oil, terpenoids, flavonoids and alkaloids (Okigbo et al., 2009). This various chemical composition is present in the form of secondary plant metabolites. Plant- based medicine, drugs, and extracts are used to treat various illnesses like HIV/AIDS, malaria, diabetes, sickle cell anaemia, mental problems, and microbial infection (Okigbo et al., 2009). Pharmaceuticals, nutritional supplements, natural health products, beauty aids, cosmetics, personal care products, aromatic and essential oils, and various items are all examples of plant- based medicines, nutraceutical and aromatic products.

North East region of India is rich in biodiversity and ethnicity. The diversified landscape of North East India is a vast reservoir of medicinal plants used by local people in primary health care management. Medicinal plants, which are relatively common in their natural habitat in the region are now quickly diminishing due to forest fragmentation, forest fire, jhum cultivation, logging, and landslide, thus threatening to the valuable plant biodiversity of the forest (Deb et al., 2015). Other factors include climate change, urban development, industrialization, pollution, destructive harvesting practices, and indiscriminate use have decreased the wild population of medicinal plants. Consequently, the ICUN has listed medicinal plants as "vulnerable" and "threatened" depending on their population status (Smitha & Thondaiman, 2016). Thus, there is a need to educate the local population for conservation and sustainable use of natural recourses particularly the medicinal plants found in the wild (Kennish, 2002).

The genus Clerodendrum (Lamiaceae) is one of the important medicinal plant genera. They possess various secondary metabolites and are used in various ethnic and folk medicine, indigenous systems such as Ayurveda, Unani, and Homeopathy, and as a source of drugs in various pharmaceutical industries (Poonam and Singh, 2009). Members of the genus are used as a remedy for high blood pressure, cardioprotective, diarrhoea, dysentery, anti-colics, abdominal pain, dizziness, gastric disorders, cough, and skin diseases. Also reported for anthelmintic, anticonvulsant, analgesic, wound healing, antioxidant, anticancer, antimalaria, antifungal activities, asthma, inflammation and wounds etc. (Kalita et al., 2012). The genus Clerodendrum includes 580 species (Shendge et al., 2018) which are widely distributed in tropical and subtropical regions of the world. Maximum species in the genus are found in tropical Africa and southern Asia, but in tropical America and northern Australasia, they are scarcely found, and some are scattered in the north, continuing into the temperate zone in eastern Asia (Mabberley, 2008). Among 580 species, India has 23 species (Kar et al., 2014), primarily distributed in the north-eastern region of India. The present study was concentrated on three Clerodendrum species, namely Clerodendrum colebrookianum Walp., Clerodendrum infortunatum L. and Cleroldendrum serratum (L.) Moon.

C. colebrookianum is an important edible plant for its medicinal value. It is used as a home remedy in the treatment of hypertension by different tribes of northeast India; the young leaves of the plant are mostly cooked and boiled for eating (Yadav, 2012). In Mizoram local traditional medicinal practitioners use the plant for anticolics pain in infants (Sharma et al., 2001). In Nagaland the plant is used for the treatment of helminthic infection, dizziness, and greenish (Jamir et al., 1999); in Arunachal Pradesh it is used for stomach disorders (Namsa et al., 2011) and in Manipur it is used for skin diseases, cough and dysentery treatment (Singh & Singh, 2009). The ethnic tribe in Dibrugarh district, Assam, has reported C. colebrookianum as vulnerable shrub (Gogoi & Nath, 2021). A study by Choi et al., (2004) reported that C. colebrookianum has antidiabetic, antihypertensive, and sedative properties. Leaves were reported to possess clerosterol, colebrin, sitosterol, octacosanol, daucosterol, and fatty acids (Yang et al., 2000).

C. infortunatum is a shrub medicinally useful in relieving thirst and burning sensation, foul orders, and blood diseases (Rej et al., 2014). Leaf extracts are effective against scorpion sting, pain reliever, and act as expectorant and vermifuge, while bark juice relieves abdominal pain and indigestion (Nandi & Lyndem, 2016). In homeopathy, it is used as remedial medicine for diarrhoea and fresh wounds (Helen et al., 2017). C. infortunatum is reported to have several pharmacological properties, viz. anthelmintic, anticonvulsant, analgesic, wound healing, antioxidant, anticancer, antimalaria, and antifungal activities (Bhattacharjee et al., 2011 and Saha et al., 2018). C. infortunatum also contains saponin, diterpene (Clerodin), triterpene (lupeol), steroid (β-sitosterol), flavonoids, glycerides of stearic acid, linoleic acid, and lignoceric acid (Bhattacharjee et al., 2011).

C. serratum has been one of the most important medicinal plants since ancient times. The plant species are used to treat a variety of human ailments. Different part of the plant is used for various medicinal treatments; the root and leaves are more commonly used. The roots of the plant are bitter in test, and they are used for curing diseases like asthma, body ache, bronchitis, cholera, drops, eye diseases, fever, inflammations, malaria, ophthalmic, rheumatism, snakebite, tuberculosis, ulcers and wounds (Patel et al., 2014). The leaves of the plant are used for the treatment of cephalalgia and ophthalmia by external applications.

In Assam, the tender leaf extract and leaf juice of C. serratum are used for the treatment of helminthic diseases, dysentery, seasonal fever, and fruit for dietary purposes (Yadav et al., 2018). The Jaintia tribes of the North Cachar hill district of Assam used C. serratum for curing different ailments (Sajem & Gosai, 2006). The leaves and roots of C. serratum are used as medicine for treating malaria, febrile and catarhitic infections, fever, cephalalgia, and snake bites in Western Ghats of India (Raviraja, 2005). The root of C. serratum is used to treat asthma in Andhra Pradesh (Savithramma et al., 2007). The ethnomedicinal uses of C. serratum have been reported from different region of India and also from many countries of the world. China, Japan, Korea and Thailand also reported the use of C. serratum for various medicinal treatments such as syphilis, typhoid, cancer, jaundice, and hypertension (Yadav et al., 2018). The roots of C. serratum contains sapogenins, D-mannitol, and stigmasterol, and the leaves contain flavonoids and phenolic acids (Apana et al., 2021). Deforestation, high exploitation and low seed germination of C. serratum are some of the result for causing reduction in the natural plant population (Sharma et al., 2009). The Chhattisgarh Medicinal Plant Board has reported C. serratum as a threatened species (Upadhyay & Koche, 2015). C. serratum is also enlisted as “vulnerable” and “endangered” species in India (Apana et al., 2021).

Understanding of the pollination biology of wild and domesticated plant genetic resources is essential for management of the rapidly diminishing tropical biodiversity and increasing plant species diversity, evenness, and productivity (Krishnan et al., 2020). Medicinal plants have received much less attention in reproductive and genetic studies and species improvement programs than agricultural and forestry crops. There is an urgent need to develop a database that is essential for the conservation and management of medicinal plant resources since there is a severe lack of baseline data on pollination biology for tropical medicinal plant species, especially from North East India. The present study was done to discern detail information about the anthesis, anther dehiscence, female receptivity, pollen production interactions, pollen production, in-vitro pollen germination and pollen storage of three important Clerodendrum species in the tropical forest of Mizoram.

1.1. Floral and pollination biology

Despite of enormous importance for medicinal purposes and other uses, the raw material for Clerodendrum species is still primarily taken from the wild; hence proper cultivation methods need to be established. The cultivation of this medicinal plant is a challenging task since the floral and pollination biology of Clerodendrum species is less known. Understanding floral biology enables us to understand the various phenophases of a species as well as the processes that take place from the creation of gametes to the germination of seeds as well as the limitations on the natural reproduction of the plant species. Studies on the floral and pollination biology of vulnerable medicinal plants species will offer useful knowledge information’s, such as the timing of flowering, fruiting, and plant-pollinator interactions. This will help in the hybridization, cultivation, sustainable use, and conservation of that particular plant species.

The length of time and frequency play a key role in defining flowering and fruiting patterns. There is a need to identify the limiting factors influencing the floral and pollination biology of medicinal plants, such as time of onset, synchronization, duration of flowering, amplitude and variation in flowering quantity. The onset of flowering may be regulated by a variety of weather conditions, such as temperature, humidity, rainfall, and solar radiation (Requile et al., 2021). The stability of the fruit and seed production of various commercially significant crops is severely hampered by ineffectual pollination. Limitation of pollen on flowers may occur when the flower depends on pollinators for pollination. A reduced pollinator activity might affect reproductive success in producing fruit and seed sets. Major biotic elements influencing pollination success in animal pollinated plant species include the population density of pollinators, their diversity, frequency of visits, and the quantity and quality of pollen that reaches the stigma (Kremen et al., 2007).

Pollination and fertilization of a flower occur in different stages (a) time of opening flower- anthesis, (b) stomium split at base and apex of the theca – anther dehiscence, (c) released of pollen from the dehisced thecae, (d) released pollen from anther are deposited and germinated on stigma and (f) pollen tube growth. Reproductive success in the plant is related to floral arrangement, the number of flowers that bloom per day, and the frequency of legitimate pollinator visits for pollination and fertilization (Thakur et al., 2018). The degree of outcrossing and selfing within a plant population depends on synchronous and asynchronous flowering, which affects plant reproduction (Fuchs et al., 2003). The majority of the angiosperm produced hermaphroditic flowers and developed a range of strategies to encourage outbreeding (Lora et al., 2011). Dichogamy, i.e., time separation in the maturity of male and female reproductive organ, is observed in angiosperm species (Endress & Lorence, 2004). Timing and duration of stigma receptivity are critical factors in regulating the separation of the male and female reproductive phase, which controls the pollen's adherence, hydration, and germination. Stigma receptivity directly influences the duration of adequate pollination time and, subsequently on fruit yield. Different species have different lengths of stigma receptivity which can be influenced by the environmental factor (Hedhly et al., 2003).

Scarce information about the mating behaviour and incompatibility in medicinal plants was available in spite of their medicinal uses. The improvement, conservation, and management of plant species are impossible without the proper knowledge of their type of mating system. Scientific studies on floral features and pollinators responsible for the mating system will help to formulate conservation measures for rare, vulnerable, and endangered plants of ecological and economic importance.

1.2. Pollen production in mating success of plants

Pollen is a male gametophyte of seed plants that creates male gametes. Compared to other plant organs, pollens are anatomically simple (Crang et al., 2018). The germination of pollen and pollen tube growth is essential for fertilization and seed development. The biotic and abiotic factors help in the success of sexual reproduction, which play an important part in the successful mating and fruit set (Kremen et al., 2007).

The study of pollen grains production is essential because it determines the pollen availability in and across the populations by gene flow which impacts mating success (Allison, 1990). Flowering plant species produce and release enormous amount of pollen into the atmosphere every year. The number of the stamen and flower in the individual plant is reported to be positively correlated with the number of pollen grains produced (Mazzeo et al., 2014). Pollen production is influenced by the number of flowers, the inflorescence number of a plant, and the environment in which it develops. In addition,

anthers are the main determinants of pollen production (Pletsers et al., 2015). The flower density in the plant is influenced by climate factors (Khanduri et al., 2013). Anemophilous plants produce enormous volumes of pollen to fulfil their fundamental role (Piotrowska, 2008). Information about the pollen production of a particular species will help to aero biologists to predict the pollen season and amount of pollen emission to the air (the amount of pollen in the air is influenced by plant density, flowering season length, and pollen production), tree breeders for making timely crosses, foresters and silviculturists to improve the quality of the wood through provenance testing and mating design, and ecologists to study natural selection and evolution of plant species.

Between species and populations, the production of pollen per anther and the quality of pollen vary considerably for the same plant species. Dactylis glomerata provides an excellent example of pollen production estimation under various climatic and geographic situations (Severova et al., 2022). Pollen production varies greatly depending on the location and altitude of the same species. The differences in pollen production among the population define the species' reproductive costs, which significantly impact an individual's fitness (Kyogoku, 2015). Within a species and across sites and years, the amount of pollen production in woody species varies greatly and is regulated by environmental conditions (Barwise & Kumar, 2020). In animal pollinated plants, the quality of pollen supplied during pollination offers reproductive assurance when pollinator numbers are limited (Timerman, & Barrett, 2021). The production of seeds and fruits in various plant species have been linked to pollen limitation which results because the pistil gets insufficient pollen grain to fertilise all the ovules (Harder, & Johnson, 2008). Although the quantity of pollen grain that land on the stigma is significantly higher than the number of accessible ovules but fertilization and fruit set does not occur. A minimum number of pollen grains on the stigma are required to enable pollen germination and pollen tube growth which is associated with pollen production effect and is linked to a phenomenon i.e. pollen density dependent. Pollen production has an important role in natural regeneration of the plant communities in tropical forests and had a substantial impact on a species reproduction (Schwartz et al., 2017). Production of a high number of pollens enables stigma to receive sufficient pollen, which helps in high fruit/seed set, ultimately affecting plant abundance and population viability and raising the possibility of a successful plant mating system (Khanduri et al., 2015b).

1.3. Pollen viability

Pollen sterility is a crucial obstacle to sexual recombination. The viability and vigour of the pollen determine the rate of pollen quality. Pollen viability, artificial pollination, and inbreeding experiment are essential for understanding the sterility issues and hybridization programs, fruiting, breeding programs, and evolutionary ecology.

The pollen grains developed inside the anther and they start dehisce in a dehydrated and metabolically inactive state at the time of anther maturity. Once the pollen is released from the anther they act as autonomous functional unit and are exposed to ambient environment and the part of pollen gets transferred on to the receptive stigmas through different pollinating vectors at a limited period of time, within the limit of pollen grain viability, which led to pollination. After deposition on the stigma, pollen germinates within a few hours to several days and pollen tube grows within the style and reaches to the embryo sac. It has been reported that in most angiosperms, pollen grains germinated by the time when the ovule structure and embryo sac have fully developed. Hence, fertilization normally occurs with days and month after pollination (Chen & Fang, 2016).

The time period between the anthesis and receptivity of stigma is influenced by numerous distinctive environmental factors which might reduce the viability rate of pollen and there are chances for dryness of stigma when exposed to high temperature. During reproduction process plant might face many environmental factors. To achieve successful reproduction, plant modifies their reproductive system to accommodate pollination success and fertilization. Encountering the above view in mind, artificial pollination and breeding experiments are done for estimation of pollen viability to understand the sterility problems and hybridisation programs, fruiting breeding programs and evolutionary ecology (Bandeira et al., 2021).

Examination of pollen germination and pollen tube growth in-vivo are quite troublesome due to the presence of pistillate tissue in nature. Therefore, in-vitro pollen germination has been used significantly on a variety of pollen framework. The study on in-vitro pollen germination is vital as it can provide abundant knowledge

There is linear relationship between pollen viability and in-vitro pollen germination, and in many species, it has come up with direct correlation with fruit and seed set.

It is critical to know an appropriate method for measuring pollen viability while breeding of plant species (Dafni & Firmage, 2000). A variety of dyes have been used for testing of the viability of pollen. The dye which is used for testing the viability of pollen might also have the chances of staining the death pollen. TTC, MTT, X-Gal, aniline blue with lactophenol, acetocarmine and Alexander's produce are few of the common dyes for testing the viability of pollen, but all the above dye has recently been criticised strongly that they also stain the death pollen (Rathod et al., 2018). Therefore, the viability test conducted through the above dyes give us a confused idea that weather the dyes are able to differentiate between live/viable pollen and death pollen and which pollen can germinate and which cannot germinate. So, using above mentioned dyes for pollen viability test cannot tell whether the pollen is alive or not. To support the pollen viability test further in-vitro pollen germination test is necessary.

1.4. Influence of growth hormones on pollen germination in medicinal plants

Compared to the medicinal importance of C. colebrookianum, C. infortunatum and C. serratum there are minimal studies on its propagation (Lalramnghinglova, 2016) and reproductive biology. Pollen viability, germination, and storage are essential aspects of reproductive biology and breeding of a plant species, as viable and fertile pollen is critical for efficient sexual plant reproduction. The pollen's viability and vigor determine the pollen quality rate, which is crucial in artificial pollination and inbreeding experiments for understanding sterility and hybridization (Shivanna, 2019). Proper germination and growth of pollen grains are essential for fertilization, fruit, and seed development (Shivanna & Rangswamy, 2012). In vitro pollen germination is used significantly on a variety of pollen viability tests (Hao et al., 2022). The study of pollen germination is vital in plant developmental biology. It can provide abundant knowledge on the nutritional and physiological requirements of pollen germination and its growth (Shivanna & Rangswamy, 2012).
Plant hormones influence the growth of pollen tube, in addition to pollen germination. Water, amino acids, sugars, boron, calcium, growth stimulating substance such as gibberellins, auxins, kinetin, IBA and Indole-3-acetic acid (IAA) are some of the main components for pollen germination culture media.

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Sucrose, boric acid, calcium nitrate, potassium nitrate and magnesium sulphate

are also some of the organic and inorganic substances which had an effect on in-vitro pollen germination. It has been reported that gibberellins are endogenous plant growth hormone which involved in many aspects of plant development, seed germination, trichome development, stem and leaf elongation, flower induction, anther development and fruit and seed development. Study from different author reported that the gibberellins help in the enhancement of pollen tube growth in-vitro (Binenbaum et al., 2018). Auxins help in promoting pollen tube growth via an effect on Ca2+ channel activity in apical pollen tubes, with the influenced of ATPase activity regulation (Gao et al., 2019). The application of kinetin at low concentrations in in-vitro culture improves pollen germination and at high concentration of kinetin it can inhibit pollen tube growth (Soni & Bohra, 2021). Exogenous indole-3-acetic acid (IAA) facilitate in-vitro pollen tube growth (Zhang et al., 2018) and they involved in the pollen-pistil interaction directly or indirectly. During plant sexual reproduction, IAA plays an important role for controlling the development of stamens and ovaries, promoting the maturation of egg cells and inducing the axial polarity and polar development of embryo (Aloni, 2021).
1.5. Pollen storage and its practical applications in medicinal plants
Long-term pollen storage is essential for plant breeding, especially in asynchronous flowering species and for germplasm exchange. The longevity of pollen differs from plant species to species and from minutes to months. Thus, there is a practical need to evaluate and standardize storage conditions of pollen grains to maintain their vitality for an extended period for making crosses between two varieties/ species which flower at different times. Appropriate temperature is needed to be screened through more experimentation to extend the longevity of pollen viability so that species hybridization could be done to develop new species with novel importance.
Pollen storage is valuable for breeding programs, hereditary preservation, artificial fertilization and self-incompatibility. The life span of pollen changes significantly with plant species and storage conditions (Mesnoua et al., 2018). Numerous techniques are taken after these days to preserve the viability of pollen beneath storage conditions. In addition, pollen storage is vital for artificial hybridisation of fruits grown under controlled conditions. For tissue culture pollen are used as an explant source to produce haploid plants (Lone et al., 2020). Natural solvents, refrigeration, freeze drying and cryopreservation are distinctive strategies of pollen storage (Sidhu, 2019). The duration of pollen storage can be expanded by controlling temperature, relative humidity and storage atmosphere (Yang et al., 2010; Mesnoua et al., 2018; Jaskani & Naqvi, 2017).
Looking into the economic medicinal importance of C. colebrookianum, C. infortunatum and C. serratum and to increase the frequency of occurrence of plant species, proper information about pollen biology, in-vitro pollen germination, pollen tube growth rate and pollination mechanism is needed. The knowledge about the functional quality of pollen will help in establishing a relative method to monitor

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pollen vigour during storage, genetic and pollen-stigma interaction studies, crop improvement and breeding, incompatibility and fertility studies. Short-term storage of pollen

grains shall be valuable for future application in pollination, supplementation, hybridization, and breeding experiments. Keeping in view the all above facts in consideration, this study was conducted in three valuable medicinal plants with the following broad objectives:
Objectives:

1. Anthesis, anther dehiscence, female receptivity and pollen- pollinator interaction in relation to the time of the day and associated weather conditions.
2. Assessment of pollen production, pollen/ovule ratio and its impact on reproductive success.
3. Effect of growth regulators on in vitro pollen germination with pollen longevity tests.

Chapter 2: Review of Literature

2.1. Medicinal utilities of Clerodendrum species:

Research interest in medicinal plants and their traditional uses during the past few decades has raised worldwide (Rossato et al., 1999). According to the World Health Organization (WHO) survey, between 70-80 percent of the world's population uses herbal plant medicine for their primary healthcare (Al-Snafi, 2016). Medicinal plants were used to extract important medicines and biologically active substances (Al-Snafi, 2015). The commercial demand for natural health products (nutraceuticals), herbal medicines, and secondary metabolites of medicinal plants is increased throughout the world (Nalawade et al., 2003). According to a conservative estimate, the earth is losing at least one key drug candidate every two years, and the rate of plant extinction is currently between 100-1000 times higher than what is expected from natural extinction (Pimm et al., 1995). Many medicinal plants are lost due to overharvesting and habitat damage (Sharma et al., 2010). According to the IUCN Red List, 91% of plant species are threatened due to habitat loss and degradation (Hoffmann et al., 2008). Understanding the ecological requirements of endemic and threatened plants is required for using plant species wisely (Kala et al., 2006). The perennial shrub Clerodendrum, family Lamiaceae, is common in the tropical parts of Asia, such as India, Myanmar, Bangladesh, Malaysia, Indonesia, Thailand, Bhutan, and Nepal, as well as in temperate Tibet (Chathuranga et al., 2019). The Clerodendrum genus has a number of species that have been reported to have significant ethnomedical use in several indigenous medical systems and as folk remedies (Chakraborthy & Verma, 2013). For the treatment of numerous life-threatening disorders like syphilis, typhoid, cancer, jaundice, and hypertension, the genus is specifically utilized as medicines in the Indian, Chinese, Thai, Korean, and Japanese systems of medicine (Chakraborthy & Verma, 2013). Clerodendrum spp. is frequently used to treat respiratory conditions broadly (Chakraborthy & Verma, 2013). Clerodendrum genus reported for its anti-inflammatory, anti-nociceptive, antioxidant, anti-hypertensive, anti-cancer, hepatoprotective, memory-enhancing, and neuroprotective properties (Wang et al., 2018). Natural compounds of twelve Clerodendrum species were reported to be used as possible therapeutic candidates against SARS-CoV-2, considering the fact that Clerodendrum species are often used in respiratory ailments (Kar et al., 2021). The root of C. colebrookianum is recorded to possess pharmacological properties such as anthelmintic, anti-bacterial, and anti-fungal, used in the treatment of bronchial asthma, gastrointestinal tract diseases, syphilis, gonorrhea, and hematological disorders. Local peoples in the North-Eastern region of India utilize this plant's leaves and leaf twigs as a home treatment for high blood pressure (Rajbongshi, 2014). The Mizo inhabitants of this region claim that the prevalence of hypertension in their society is extremely low, and this is because they regularly consume the tender leaves and shoots of C. colebrookianum, locally known as "Phuihnam" (Devi & Sharma, 2004). It is grown by rural and urban residents in kitchen gardens, and private nurseries in urban areas sell the saplings for Rs. 10.00 each (Bordoloi & Borthakur, 1997). Traditional healers have used C. colebrookianum to treat intestinal tapeworm infections because of its potent anthelminthic qualities (Yadav, 2012). Leaves and roots were reported to possess sterol glycoside clerosterol, colebrin A-E, clerodolone, octacosanol, daucosterol,triacontane,(24s) ethylcholesta−5,22,25−triene−3β−ol, α−amyrin, β−sitosterol and fatty acids (Yang et al., 2000 and Rajbongshi, 2014).

C. colebrookianum leaves are being consumed for better health in the North East region of India since they have definite cardioprotective potential (Devi & Sharma, 2004). (Rajlakshmi et al., 2003) reported that the leaf extract of C. colebrookianum increased the blood's antioxidant capacity and had an inhibitory effect on the liver's and kidney's basal levels of lipid peroxidation. Phytoconstituents with antioxidants potential, prevent the formation of uric acid, anti-tumour, enhancing zinc's bioavailability, against high blood pressure were identified in the C. colebrookianum through GCMS analysis (Payum, 2020). C. infortunatum extracts were reported to be used in Ayurveda to treat leprosy, worm dyspepsia, itching, cough, and colds; its leaf was used in treating scorpion stings, and bark juice was effective at treating indigestion and abdominal pain (Nandi & Mawkhlieng, 2016). It is reported to use in Indian homeopathy for treating fresh wounds, post-natal problems, and diarrhoea (Nadkarni & Nadkarni, 2002). Various parts of plants such as roots, stems, and leaves are reported to be utilized as a medicine in India by the tribal people of the Chotanagpur plateau in eastern India to treat conditions like asthma, cataracts, malaria, and blood, skin, and lung problems (Singh, 2007). Similar uses have been documented in Thailand; the leaves and roots were used to treat kidney failure and intestinal infections (Islam et al., 2013). The leaf juice is applied externally to treat tumours, skin conditions, snake bites, and scorpion stings in Bangladesh, and it is also used as an anthelmintic, emetic, moderate laxative and cholagogue (Ghani, 2003). The plant extracts of C. infortunatum were found to contain high antioxidant and pharmacological properties that support the traditional therapeutic claim for wound healing activity (Gouthamchandra et al., 2010). Root, leaf, and stem extracts of C. infortunatum are used in traditional medicine to cure common ailments such as intestinal disorders, diarrhoea, tuberculosis, and respiratory problems (Waliullah et al., 2014). The essential oils (fatty acids and their esters, aromatic monoterpenes like limonene, a-pinene, ß-pinene, p-cymene, and myrcene and sesquiterpenes), are extracted from the leaves and root bark of the plant C. infortunatum which is highly used in Ayurveda (Jirovetz et al., 1999).

C. serratum plant has been reported to have significant ethnomedical value in several traditional medicinal systems including Ayurveda, Siddha, and Unani, for the treatment of fatal illnesses, including syphilis, typhoid, cancer, jaundice, and hypertension (Singh et al., 2012). Additionally, it is said to have been traditionally used as an anti-rheumatic, anti-asthmatic, febrifuge, cephalalgic, and ophthalmic remedy. Roots are recorded for their anti-oxidant, anti-bacterial, anti-fungal, anti- tumours, and anti-inflammatory (Singh et al., 2012 and Dongare et al., 2020). Icosahydropicenic acid (IHPA), a new pentacyclic triterpenoid saponin isolated from the roots of C. Serratum can be used for the treatment of asthma (Bhujbal et al., 2010). The aerial portions and roots of C. serratum have been traditionally used to treat a wide range of inflammatory illnesses because they have anti-rheumatic effects (Shareef et al., 2013). It is reported that the plant drug Sirutekku is extracted from the root of C. serratum, which is used in Siddha medicine (Narayanan et al., 2014).

2.2. Plant-pollinator interactions in plants

Pollination biology is a mutualistic relationship between plants and their animal pollen carriers, with energy rewards as the foundation for co-evolution (McCallum et al., 2013). Animal pollinators and pollination have long been thought to have been crucial to the diversity of the angiosperms (Coyne & Orr, 2004). There has been a surge in research on the pollination ecology of agricultural crops, forest trees, and ornamental plants in recent years, but comparatively less research on the reproductive biology of plants with medicinal importance from natural stands (Kulloli & Sreekala, 2009).

Insects play a vital role in transporting pollen, enabling a plant species to outcross with other conspecific members, thus helping in gene flow at a population level (van Ginkel & Flipphi, 2020). The length of the flower, colour, odour, nectar, pollen, and other flower rewards have a significant impact on pollinators (Faheem et al., 2004); thus, floral features influence pollination efficiency. Flowers that are pollinated by bees are bright in colour, reflect light in the blue to violet spectrum, and have nectar guides for nectar during the day (Faheem et al., 2004). Bright or light-coloured flowers with suitable odour and nectar guides that produce nectar at night are pollinated by moths and bats (Zariman et al., 2022). Pinkish and red flowers are well-known to attract butterflies (Abrol, 2012). The odour or fragrance of the flower attracts specific pollinators on them; flowers pollinated by insects mostly release order or fragrance, while flowers pollinated by birds are odourless (Johnson & Govender, 2022). For long-distance advertising, nocturnal flowering plants with a distinctively strong and pervasive floral odour are required.

Over 2, 50,000 species of angiosperms occur in the world, of which 70% of plants rely on insect pollinators (Pannure, 2016). Nectar is predominantly a sugar solution; it has been regarded as the most crucial floral food reward for animal pollinators (Nepi et al., 2012). Nectar is a vital source of nutrition and energy for pollinators, it consists of a complex mixture of carbohydrates, amino acids, proteins, lipids, vitamins, antioxidants, alkaloids, organic acids, and inorganic substances like minerals (Burkett,1998), and they play a significant role in pollination. Generally, a small amount of concentrated nectar is available in the insect-pollinated flowers, thus encouraging insect pollinators to visit more flowers to quench their thirst, which increases the floral visit and the level of outcrossing (McCallum et al., 2013). Bats and hawk moths pollinated flowers secrete a copious amount of nectar at night, while flowers that bees, butterflies, and birds pollinated one do so during the day (Lemaitre et al., 2014). Many insects, especially apidae, beetles, flies, thrips, and butterflies, depend on pollen as a food source and nutrition (Cane, 2016). According to flowers, pollinators' foraging range varies from 3 to 12 km, and their foraging rates also change (Anonymous, 2003). Bumble bee foraging rates are twice as fast as honeybee foraging rates, although solitary bee foraging rates vary greatly depending on size (Goulson et al., 2002). Honeybees, flies, bees, butterflies, and hawk moths have all been observed moving swiftly between flowers during foraging (Kulloli et al., 2011).

Attitude, temperature, light, wind, and rainfall influence the foraging behaviours, flower visits, and pollination efficiency (Akhtar et al., 2018). The foraging behaviour of pollinators is influenced by the altitudinal gradient, for example, hummingbirds are more effective pollinators at higher elevations than bees, and moths are at intermediate and lower altitudes (Klomberg et al., 2022).

Pollinators are significantly impacted by temperature (Conrad et al., 2017). Temperature changes have a significant impact on bee foraging, as no foraging occurs below 8°C, some activity occurs between 8°C and 16°C, optimum activity occurs between 16°C and 32°C, and foraging is reduced above 32°C, though in some cases foraging may continue up to a temperature of 42–48°C (Abrol, 2011). The interaction between plants and their pollinators is hampered by habitat fragmentation; for example, plantation of two shrub species, Acacia brachybotrya and Eremophila glabra growing in linear vegetation, received less pollen than conspecifics in adjoining reserves (Jabeen & Bhat, 2013).

The buzz-pollination syndrome is the term used for the removal of pollens from the anther by vibrations (De Luca et al., 2022). Bees and other insects harvest pollen from the anthers utilizing vibrations known as sonication or buzzes (De Luca et al., 2022). The vibrations of bees help to add pollen collection from various plant species with different morphologies, such as Cistus, Papaver, Pedicularis, Myrtaceae, and Solanum (Gottsberger, 2012).

2.3. Plant-pollinator interactions in Clerodendrum species

Rohitash (2018) reported anthesis between 0600-0630 h, anther dehiscence between 0730-0900 h and stigma receptive between 1100-1400 h for Clerodendrum splendens. Furthermore, he observed only black ants as floral visitors in C. splendens, and they are highly self-incompatible. Further, he also mentions that the reason for self-incompatible might be the absence of nectar reward and effective pollinator (Rohitash, 2018). Jai (2010) reported Xylocopa, Eumenes sp. and Componotus campestris (black ant) are the most effective pollinator visitors of C. splendens.

The anthesis of Clerodendrum inerme was recorded between 1500-1600 h in afternoon, and the anther dehiscence after an hour form anthesis with the longitudinal split of the anther (Aluri et al., 2016). Nectar was released during the post-anthesis phase, and the stigma with forked lobes becomes receptive between 2-3 days after anthesis (Aluri et al., 2016). C. inerme is reported to be pollinated by hawk moths (Primack et al., 1981), and pollination of flowers is mostly adapted towards entomophily (Aluri et al., 2016). Raju & Kumar (2016) observed bird and hawk moth pollination and nectar robbery in C. inerme. Bees and butterflies pollinate C. laevifolium, according to Keng, (1990). Rohitash, (2017) reported Macroglossum sp., Apis cerana, Apis dorsata, Bumbus lapidaries, Danaus genutia, Neptishylas papaja, and Eurema hecabe as floral foragers of C. inerme and he also noted that honey bees and bumble bees were more active than butterflies.

The anthesis of C. infortunatum was observed in the morning hours with the physical separation of male and female (distinct herkogamy), and stigma was receptive mostly during the female phase (Mukhopadhyay & Quader, 2022). C. infortunatum flowers exhibit temporal dioecy and are extremely protandrous, herkogamous, and dichogamous (Kumar et al., 2017). The papilinoid butterflies (Papilio polytes, P. polymnestor, and Atrophaneura hector) are observed to pollinate on C. infortunatum, through pterogotribic pollination by striking the anthers and stigma with their wings, according to Byragi & Subba (1995). Tapinoma melanocephalum and Trichomyrmex destructor harvest nectar for C. infortunatum without pollinating the flower (Mukhopadhyay & Quader, 2018).

Sakamoto et al., (2012a) observed the effect of three pollinators Papilio spp., Macroglossum pyrrhosticta and Xylocopa appendiculata on fruit and seed production of Clerodendrum trichotomum, and they found out that pollinator Macroglossum pyrrhosticta promote self-pollination among the flowers.

Clerodendrum molle is visited by nocturnal pollinators, which includes ants, spiders, hawk moths, and roaches, and they are also visited by diurnal pollinator such as carpenter bees and ants (McMullen, 2011). Pollinators play a significant role in a Clerodendrum species that reproduces primarily by xenogamy and geitonogamy (Mukhopadhyay, & Quader, 2020).

Clerodendrum trichotomum and Clerodendrum izuinsulare were reported to be visited by insects such as diurnal hawkmoths, bees, swallowtails, and nocturnal hawkmoths. They were pollinated nocturnally and diurnally, and shared pollinators are common to both species (Miyake & Inoue, 2003).

Numerous plants may be suited to various pollinator types based on differences in floral appearance. Lepidoptera, nectar-feeding bees, Hymenoptera, Coleoptera, Diptera, Japanese black swallowtail butterflies, nocturnal hawk moths, diurnal hawk moths are among the pollinators of C. trichotomum or C. izuinsulare (Mizusawa et al., 2014).

Long-tongued hawkmoth is pollinator for the tubular flower Clerodendrum viscosum; many black ant and butterflies were observed moving from flower to flower. Though the flower appears hermaphrodite during anthesis, C. viscosum stigma cannot accept self-pollens due to their arrangement (Liza et al., 2010).

Three floral visitors behaviours i.e., Macroglossum pyrrhosticta, Xylocopa appendiculata and Papilio dehaanii was studied in Clerodendrum trichotomum and it was concluded that pollination dynamics differ among pollinator species, rapid visitation behaviours of pollinators and flower visits and pollination rate are not equal (Sakamoto et al., 2012a).

2.4. Pollen production and reproductive success in plants

Pollen production varies widely among anemophilous and entomophilous plant species (Mondal & Mandal, 1998). Several studies have addressed pollen production per plant in various species (Khanduri et al., 2015b). During pollen grain development within the anther, microsporogenesis may be affected by both genetic and environmental stress (Garcia Mozo et al., 2005). Pollen is exposed to severe environmental factors during the dispersal phase that can affect pollen viability and germination capability (Bots & Mariani, 2005). Viability mainly depends on relative atmospheric humidity at shedding and during pollen transport (Fonseca & Westgate, 2005).

Pollen limitation, inadequate compatible pollen receipt by stigma and ovules, is common among animal-pollinated plants (Knight et al., 2006). Pollen limitation is often considered a negative consequence of small population sizes or fragmentation (Jump & Penuelas, 2006). Low reproductive success due to pollen limitation is widely reported in angiosperms (Larson & Barrett, 2000) which is predicted to be the most prominent in an environment where pollinator services are unreliable or low in abundance (Burd et al., 2009). Scientific studies have reported the effect of pollen production and yield of flower on seed settings in entomophilous (Khanduri & Sharma, 2001) and anemophilous plant species (Damialis et al., 2011).

Pollen production is crucial to plants' reproductive fitness and success in forest ecosystems. Anemophilous plants emit enormous volumes of pollen into the atmosphere each year. Most temperate tree species are pollinated by the wind, and successful fertilization requires a large amount of airborne pollen (LaDeau & Clark, 2006). Numerous temperate trees, including conifers, experienced interannual variation in pollen production that led to varied fruit sets and seed production (Shibata et al., 2002). Pollen is a rich reservoir of food resources since they contain essential amino acids, trace elements, enzymes, B-complex, and vitamins C and E naturally (Godswill et al., 2020). The length of the anthers and the size of the pollen grains have a significant impact on pollen production (Piotrowska, 2008). Environmental factors such as temperature and precipitation influence pollen production; increase in atmospheric carbon dioxide has an impact on pollen production in Ambrosia artemisiifolia (Ziska & Caulfield, 2000).
Among anemophilous plant species, pollen production varied widely (Quamar & Bera, 2014). Increased temperatures can hurt plant sexual reproduction, which can lower fertility. Male gametophytes are more susceptible to heat stress during all stages of development; it impacts pollen production and shape, cell wall structure, and most significantly, pollen metabolism (Hedhly, 2011). Different species have different sensitivity to heat stress levels when the high-temperature modes are applied (Parrotta et al., 2016). More than 12000 species in the Poaceae family are pollinated by wind. Pollen from the Poaceae family is now recognized as the world's most significant source of airborne biological pollution and the main factor contributing to pollen allergies (Garcia, 2017). Piotrowska, (2008) study the structural characteristics and pollen production of five allergenic anemophilous plants species such as Betula verrucosa, Secale cereale, Rumex acetosella, Plantago major and Artemisia vulgaris and observed pollen production per anther was highest in Secale cereale 22360, followed by Betula verrucosa 11160, Rumex acetosella 10850, Artemisia vulgaris 9580 and lowest in Plantago major 5870.
According to Jai, (2010) Clerodendrum splendens has 2160$\pm$380 pollens per flower, with 4 ovules per flower and a pollen ovule ratio of 540:1. He concludes that just 2% of the flowers in Clerodendrum splendens exhibit open pollination with significantly less seed set percentage. In Clerodendrum inerme, the pollen productivity per anther is 796$\pm$51.2, while the overall pollen productivity in individual flowers with four and five stamens is 3184 and 3980, respectively (Aluri et al., 2016). Gupta et al. (1982) observed pollen production in C. viscosum 1799 to 2646 grains/ stamen and 7979 to 9595 grains/flower. A total of 58 angiospermous plant species have been determined for pollen production, increase in pollen production from herbs>shrubs>trees was observed and analyzed (Mondal & Mandal, 1998). Pollen production varies among the anemophilous, entomophilous and amphiphilous plants. The average number of pollen production per anther in C. japonicum was observed as 9097, and pollen production per flower as 36388 with four anthers per flower. The average pollen production per anther for C. indicium was 11137, and pollen production per flower was 44548 with four anthers per flower (Mondal & Mandal, 1998).
Pollen/ovule (P/O) ratio and breeding system correlations have typically been explained by the sex allocation hypothesis or the idea that P/O represents pollination efficiency (Gallardo et al., 1994). Plant breeding systems have been roughly estimated using the pollen: ovule ratio (P/O). Plant breeding techniques have been proven to be connected to specific floral features. Correlation between P/O ratios and breeding methods within some plant groupings has been demonstrated (Alarcon et al., 2011). Autogamy is connected with low P/O levels, while outcrossing is correlated with higher P/O values (Cruden, 1977). P/O ratios are also known to be affected by pollination mode: Species with particular pollen packaging strategies (pollinia, polyads, or viscin threads) have much lower P/O ratios than species without such strategies because pollen is transferred more efficiently, and species offering pollen as a reward have much higher P/O ratios than species offering nectar because large amounts of pollen are consumed (Cruden, 2000). Other characteristics of plants, such as life form and life history, have an impact on P/O ratios as well (Alarcon et al., 2011).
According to Cruden, (1977) cross-pollinated plants generate more pollen grains than self-pollinated plants because they are less efficient at pollination and have larger P/O ratios. Higher P/O ratios in wild species compared to domestic species were explained by Shanker & Ganeshaiah, (1984) as a result of the higher pollination hazards that the wild species had to deal with. In a different study Shanker & Ganeshaiah, (1984) found a correlation between the decline in P/O ratio with increasing Croton age and the likelihood that pollen grains will successfully reach the stigma. Etcheverry et al., (2012) determined P/O in 21 Leguminosae species; he classified 15 species as obligate xenogamous, despite some of them having been recorded as facultative xenogamous, and have concluded that P/O variability is influenced by the taxonomic position and pollination strategy.
The angiosperms contain a wide range of pollen and ovule for each flower and considering how important these spores are to reproduction (Burd, 2011). One of the most common patterns is that the ratio of pollen to ovules per flower (P:O ratio) is frequently an approximative indicator of a breeding system (Cruden, 1977); local mate competition influences sex allocation. Self-pollination through geitonogamy or autogamy creates local rivalry between them, which favours selection favouring higher female investment in sex and hence a lower P:O ratio.
The reproductive capabilities of 32 species of legumes from the Mediterranean region by Galloni et al., (2007), noted that the species with high P/O values are suggestive of a poor pollen transfer efficiency, which is typical of xenogamous species. While species with the highest P/O had primary pollen presentation, those with the lowest P/O exhibited brush or explosive tripping mechanisms. Their study got to the conclusion that reducing autogamy rates requires the stigmatic cuticle. The importance of P/O ratio is a close indication of species breeding strategy was confirmed by productivity studies. The pollen/ovule ratio of Clerodendrum inerme is low, indicating highly effective hawkmoth pollination of this specie (Primack et al., 1981).

It has been postulated that in hermaphroditic plants, the ratio of pollen and ovule dictates the plant mating system with the rule that the higher the P: O ratio, the more will be the chances of outcrossing (Khanduri et al., 2015b).

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Flowering plants are hermaphrodites; the amount of seeds fertilized by self and outcross pollen varies greatly among species, from predominate self-fertilization to exclusively outcrossing (

Goodwillie et. al., 2005). The degree of outcrossing or selfing (the mating system) among individuals and populations can affect the genetic makeup of populations, the rate of gene flow, the size of an effective population, and the manifestation of inbreeding depression (Barrett & Harder, 2017). Research on the evolution of mating systems has concentrated on factors that encourage self-fertilization within populations, such as reproductive assurance when pollen transfer possibilities are scarce and the genetic transmission benefit of selfing in populations with minimal inbreeding depression (Butcher et al., 2011). The majority of facultative xenogamous species are found in the climax or other stable ecosystems; delayed autogamy, self-compatible, evolved cross-pollination, self-compatible, and flower pollinator activity may be limited or unreliable (Cruden & Lyon, 2019).

2.5. Pollen viability, in vitro pollen germination and pollen longevity

The rate of pollen tube growth and the speed at which pollen grains germinate significantly correlated to pollen vigour (Sulusoglu & Cavusoglu, 2014). In vitro pollen germination assays have been used to calculate the pollen germination percentage and can also be used to evaluate pollen vigour by monitoring the rate of germination over time or the length of pollen tubes (Shivanna & Ram, 1993). The study of pollen vitality is essential in the investigation

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of pollen biology. Such as monitoring pollen vigour during storage, genetics and pollen-stigma interaction, crop improvement and breeding programmes, maintaining gene banks, incompatibility and fertility studies, assessing pollen germinability after exposure to specific conditions, and assessing dispersal and gene flow

are some of the topics covered (Dafni & Firmage, 2000). Pollen quality is determined by pollen viability and vigor. In the hybridization process, pollen viability and fertility are of the utmost importance. Most plants need to be successfully pollinated in order to fertilize and produce seeds, and any sensible strategy to boost pollen production must take into account in pollen biology, including pollen viability, pollen germination, and pollen tube expansion (Rathod et al., 2018). High temperatures reduce pollen viability, resulting in fruit production decline (Paupiere et al., 2014). Numerous methods are used to evaluate pollen viability. Such as counting the number of seeds that are produced after pollination, tracking pollen germination and pollen tube growth in vivo, tetrazolium salts to detect dehydrogenase activity, aniline blue to identify callose in pollen walls and pollen tubes, acetocarmine or Alexander stain to identify cytoplasmic contents, and determining the plasma membrane after in vivo test and the choice of approach depends from species to species and on establishing a link between the test and fertility (Dafni & Firmage, 2000). The ability of pollen to accomplish fertilization and seed set is the most precise means of determining pollen viability (Impe et al., 2020). The staining approach for determining pollen viability is unreliable for many plant species; hence in-vitro pollen germination must be done (Sulusoglu & Cavusoglu, 2014). The spread of a species fitness, and the survival of plant generation depend on viable pollen. It is also necessary for plant breeding and crop improvement. Pollen performance, such as fertilization potential, germinability, and stainability, are crucial aspects of pollen viability (Dafni & Firmage, 2000). Rodriguez and Dafni, (2000) tested the viability of pollen using four different dyes to distinguish between fresh pollen and dead pollen. They discovered that the peroxidase test and 2,5-diphenyl monotetrazolium bromide (MTT) did not stain dead pollen; thus, techniques effectively differentiate pollen quality.

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High humidity (<95% RH) and temperature (38°C) or storage stress of Nicotiana tabacum, Agave sp., Tradescantia virginiana and Iris sp. affected pollen vigour before affecting pollen viability (

Shivanna et al., 1991). 83% viability and 58% in vitro pollen germination with 763.65±34mm long pollen tube were observed for Clerodendrum splendens (Rohitash, 2018) with 5% and 8% pollen germination on the stigmatic surface.

Different biochemical's such as sugar, starch, lipids, phytic acid, and mRNA are found in pollen grains; this stored product plays an important role in pollen germination and pollen tube growth (Patel et al., 2014). The osmotic pressure is maintained by sucrose, which also serves as a substrate for pollen metabolism (Linskens & Kroh, 1970). 7.5-20% sucrose solution is needed for optimal pollen germination (Kumari et al., 2009). The optimal germination rate was found to be 10% sucrose in Bambusa vulgaris (Koshy & Jee, 2001), Datura metel and Najas marina (Patel & Mankad, 2014), 15% sucrose in Bassia latifolia (Singh & Singh, 2001), 11–15% sucrose in Asclepias syrica (Kevan et al., 1989), 20% sucrose in Abelmoshus esculentus (Dabgar & Jain, 2001), and 30% sucrose in Catharanthus roseus (Patel et al., 1997). It was found that 5% of the Selix species and 15% of the water chestnut species had the best germination rates (Hoque & Arima, 2000). With increasing concentrations of boric acid, gibberellic acid, and IAA in basic sucrose and agar media (0.5-1.0 ppm), pollen germination and tube growth were enhanced (Bigdeli et al., 2016). At 15% sucrose concentration Cunninghamia lanceolata showed pollen germination and tube growth, and at 0.01% boric acid promoted pollen germination and tube growth (Fragallah et al., 2019).

External factors such as temperature, boric acid, fungicides, and the presence of heavy metals affect pollen germination (Radovic et al., 2016). Plant hormones affect pollen germination (Tosun & Koyuncu, 2007); they enhance the growth of pollen tubes (Sotomayor et al., 2012). After pollination, the IAA levels in the pistil increases, it was observed that IAA enhances pollen tube growth (Wu et al., (2008a). The effect of auxin (IAA) and gibberellin (GA3) on pollen germination and in vitro pollen tube growth was observed for five almond cultivars (Radovic et al., 2016). Pollen tube length increased from 23 to 86% when treated with auxin, and from 6 to 22%, pollen tube increased when treated with gibberellin. The germination of seeds, the growth of trichomes, lengthening of stems and leaves, induction of flowers, development of anthers, development of fruits and seeds, and many other aspects of plant development are impacted by GA3 (Hedden & Phillips, 2000). According to Acar et al., (2010) Pistacia vera pollen germinates at 20 percent sucrose with 10, 25, 50, 75, and 100 ppm of boric acid (H_3BO_3) and gibberellic acid (GA3). He concluded that GA3 inhibit pollen germination and boron promotes pollen germination slightly. The effect of IAA and IBA on two species of Bauhinia and GA3 and kinetin on Spathodea campanulata was found suitable for pollen germination, and during the first 24 hours, maximum germination was recorded (Sanjay et al., 2016). Ascorbic acid and indole butyric acid (IBA) promote distinct pollen germination and pollen tube growth in Nuomici litchi. Application of 5.0-10.0 mg/L ascorbic acid or 2.5-5.0 mg/L IBA to Nuomici litchi flowers can encourage pollination and fertilization (Zeng et al., 2018). Indole-3-acetic acid (IAA), regulates the growth of stamens and ovaries, maturation of egg cells, and induces axial polarity and polar development of the embryo, and it is vital for plant sexual reproduction (Wu et al., 2008b). IAA may be directly or indirectly involved in the pollen–pistil interactions during pollen germination and pollen tube growth of Nicotiana tabacum (Chen & Zhao, 2008). Growth regulators such as gibberellic acid, indole-3-acetic acid, indole-3-butyric acid, indole-3-propionic acid, 1-naphthaleneacetic acid, kinetin promote pollen germination in tomato (Karapanos et al., 2006). IBA concentration increases the number of female flowers in the cucumber cultivar Wisconsin MR28 (Diola et al., 2008). The addition of indole-3-acetic acid (IAA) to media containing buthionine sulfoximine (BSO) restored pollen germination and early elongation of the pollen tube to Arabidopsis thaliana (Zechmann et al., 2011). IAA plays an important role in pollen tube growth of Torenia fournieri (Wu et al., 2008b). Auxin appears to act at the germination stage, while gibberellin at the early and ethylene at the late pollen tube stages. Abscisic acid (ABA), ethrel (ETH) and indole-3-acetic acid (IAA) increased pollen germination while ABA and ETH enhanced pollen tube elongation in Arachis hypogea. Thus, pollen treated with IAA, GA3 and ETH caused maximum elongation of pollen tubes (Malik et al., 1976).

The asynchronous flowering among genotype cultivars frequently requires pollen storage for the genetic improvement of plant species. Short-term pollen storage is necessary for late and early flowering genotype hybridization because pollen quality quickly degrades at moderate temperatures and humidity (Towil, 2010). Cryopreserved pollen can be found in pollen banks, where it is simple and quick to obtain pollen for any purpose (Towill & Walters, 2000). For seed orchards and improvement programs, pollen banks have been established in germplasm systems (Walters & Pence, 2021). For studies on the basic physiology, biochemistry, and fertility, as well as for biotechnology projects involving gene expression, transformation, and in vitro fertilization, pollen is stored to facilitate crosses in breeding programs, distribute and exchange germplasm among locations, preserve nuclear genes of germplasm (Mondo et al., 2020). For germplasm banks, pollen preservation is a supplement to seed or clone preservation (Towill & Walters, 2000). Four storage temperatures (4°C, -4°C, -20°C, and -76°C) were used to keep herbaceous peony pollen for more than a year. It was observed that pollen stored at 4 °C could be used for hand-pollination during the asynchronized flowering season (Du et al., 2019). Before storage, a certain amount of drying must be done since pollen with high initial water content cannot be successfully preserved at extremely low temperatures (Barnabas & Rajki, 1976). According to Sedgley & Harbard, (1993), pollen of Acacia auriculiformis, Acacia iteaphylla, Acacia karroo and Acacia mangium was stored at 25, 5, -18, and -196°C for up to 3 years, and concluded that pollen should be vacuum dry before storing and pollen should begin storing from -18°C.

Chapter 3: Material and Methods

3.1. Geography and the forest

Mizoram is a region of global significance because it is located in northeast India, an important portion of the Indo-Myanmar biodiversity hotspot, and home to a variety of flora and fauna, as well as some of the world's biologically richest regions. However, the region is rich in biodiversity and natural resources; it simultaneously faces various threats, mainly anthropogenic-induced. Hence the study site is of prime importance for its rich natural heritage with conservation value. Mizoram, the 23rd state of India, with 11 districts, is situated between bordering countries like Myanmar in the east and Bangladesh in the west and touches it boundaries with neighboring states Assam and Manipur in the north and Tripura in the north-west. It is located at latitudes of 21°58' and 23°35'N and 92°15' and 93°29'E (Lalrinchhana et al., 2015). An altitude ranges between 21 and 2157 meters above mean sea level and receives rainfall between 2000 and 3200 millimeters yearly. The Tropic of Cancer passes across the center of Mizoram. Mizoram's topography consists of high hills and narrow gorges with six parallel hill ranges surrounded by deep river valleys, with a total geographical area of about 21081 km2. The climate of Mizoram is pleasant throughout the year. Temperature is mild during the summer, from 14.0°C to 30°C while in winter not too cold temperature varies from 6°C to 21°C. Soil is acidic in nature with rich in organic matter; soil texture is mainly sandy loam to loam. According to the 2015 India State of Forest Report, Mizoram has a total forest area covering about 18,748 km2 or 88.93% of the state's total geographical land area, i.e., 21,081 km2. Jhum cultivation or Shifting cultivation and home gardening are the major traditional agricultural practices for generating livelihood in Mizoram. The state is covered with 21 significant hill ranges or peaks. Phawngpui is the highest mountain rising 2,065 meters above sea level. Furthermore, there is a number of rivers flowing toward the north and south portion of the state. The rivers which flow towards the north portion of Mizoram fall into Barak river Assam which includes Tuirial (Sonai), Tlawng (Dhaleswari), and Tuivawl. And the rivers which drain into the south portion of Mizoram are Chhimtuipui(Kolodyne), Mat, Tuichang, Tiau, and Tuipui. Based on height, rainfall, and composition of the dominant species, Gogoi et al., (2022) has reported 6 forest type of Mizoram, i.e. (a)Tropical Wet Evergreen Forest (below 900 ma.m.s.l.) (b) Montane sub-tropical Forest (between 900-1500 ma.m.s.l.)) (c)Temperate Forests (above 1600 a.m.s.l.)) (d) Bamboo Forests (above and below 1600 m a.m.s.l.)) (e) Quercus Forests (f) Jhumland.

3.2. Study Sites

The study was conducted during the flowering seasons of C. coolebrookianum, C. infortunatum, and C. serratum in two successive years from 2018 to 2021 in a tropical moist natural forest of district Aizawl, Mizoram. The following study sites were selected for the study. Sairang: The study site is located at 23°48'18.53"N Latitude, 92°37'56"E Longitude, with an altitude of 179.832 m above sea level. Tanhril is situated in around forest area at 23°44'15"N and 23°43'37" N latitudes and 92°39'44" E and 92°40' 23" E longitudes and altitude range from 330 to 880 m above mean sea level. Hlimen: The study site is located at 23°40'29"N Latitude, 92°43'17" E longitude, with an altitude of 1140 m above sea level. Durtlang is situated at 23°46'27" N Latitude, and 92°43'47" E longitude at an altitude of 1161 m above mean sea level. Aizawl, Mizoram, experiences a humid and tropical climate with long, hot summers and mild, dry winters with little to no precipitation. The yearly rainfall averages 2162 mm, and the average annual temperature is around 24.9° C. The year-round average temperature is mild, ranging from 10 to 36 degrees Celsius, and does not vary considerably. Low-elevation site at Sairang forest are rich in bamboo species and following major tree species Schima wallichii, Mallotus sp. Albizzia chinensis, Alstonia scholaris, Callicarpa arborea, Toona ciliata, Cassia sp., Oroxylum indicum, Duabanga grandiflora, Dalbergia pinnata, Sterculia villosa, Terminalia bellerica. In mid-elevation site, Tanhril, the major forest trees found are Schima wallichii, Callicarpa arborea, Albizzia chinensis, Anogeissus acuminata, Albizzia procera, Castonopsis tribuloides, Sterculia villosa, Rhus semilata, Mallotus sp., Macaranga sp., Sepium sp., Dillenia sp., Duabanga grandiflora, Neolamarckia cadamba ,Ficus sp. Common tree species found at high elevation site of Hlimen are Castonopsis tribuloides, Schima wallichii, Sterculia villosa, Syzygium sp. Litsea sp. Lannea coromandelica, Phyllanthus emblica.

C. colebrookianum with white tubular flower. The flowering and fruiting phase is observed during the month between July – and November; the species is reported to be a vulnerable shrub (Gogoi& Nath, 2021), and the plant species are used as medicine by many local people of northeast India (Yadav, 2012).

In Mizoram C. colebrookianum, locally known as 'Phuihnam,' is traditionally used to control hypertension, blood pressure, and intestinal tapeworm infections (Temjenmongla & Yadav, 2005). Leaves and roots are used by tribes of Manipur for skin diseases, cough, and dysentery (Singh& Singh, 2006). Experimentally C. colebrookianum has been evaluated for antihypertensive activity (Lokesh & Amitsankar, 2012), antioxidant and hypolipidemic effect (Devi& Sharma, 2004), and anthelmintic activity (Yadav, 2012). The compound of pharmacological importance isolated is colebrosideA (1), colebrin A and B, C-29 sterols, and clerosterol (Yang et al., 2000).

Rotheca serrata (L.) (Synonym: Clerodendrum serratum (L.) flowered and fruited during March-July, and the plant species are found to be found in tropical regions of the world. The Chhattisgarh Medicinal Plant Board has recorded C. serratum L. as threatened species (Upadhyay &Koche, 2015). The plant's ethnomedical significance has been documented in numerous traditional medical systems, and Its parts, such as roots and leaves, are used to treat various human ailments across geographical regions. Possess antioxidant, antibacterial, and antifungal mainly found in the roots parts of C. serratum (Singh et al., 2012). The root of C. serratum contains sapogenins, D-mannitol, and stigmasterol, while the leaves contain high amount of flavonoids and phenolic acids (Apana et al. 2021).

Clerodendrum infortunatum is a gregarious shrub that flowered from March to April in the present study site. Locally leaves are tonic and used in Malaria, scorpion stings, and snake bites. The roots are boiled, and water is used for a bath in case of scabies and other skin diseases. The plant is useful in relieving thirst and burning sensation, foul orders, and blood diseases (Khatry et al., 2005). The plant's root is recommended for treating tumours, several skin conditions, and scorpion bites. (Bhattacharjee et al., 2011). Preliminary chemical research revealed that the leaves of C. infortunatum contain saponin, clerodin (a bitter diterpene) 4, 6, and a few enzymes. In addition, leaves have a fixed oil that is made up of glycerides of linoleic, oleic, stearic, and lignoceric acids. From the root sources, luperol and beta-sitosterol.

3.4. Methodology

3.4.1. Floral Development, anthesis pattern and duration of flowering

Floral morphology and structure were studied on twenty inflorescence units, each from five different individuals in each selected plant species. The complete developmental behavior of the flowers, from floral bud initiation up to the mature stage, was judged on randomly selected flower samples (n=50) in each plant species. The observations on the anthesis and time of anther dehiscence were recorded in the field conditions during the flowering seasons. For this purpose, flowers of different plant individuals (n=10) were labeled, and their anthers were examined at half-hour intervals, each time scoring the dehisced anthers and recording the prevailing weather conditions naturally. For determining the start of the flowering season, 20 fresh flowers per plant on a particular day were kept as standard. To track the length of the flowering season, the initial and last flowering dates were noted. When half of the plant's flowers were opened, the peak flowering time was observed.

3.4.2. Flower and Pollen Production

Ten chosen individuals in each studied plant species were used to estimate flowers and pollen production at their respective study locations. The Three Clerodendrum species' height ranges from 215 to 265 cm; thus, the total number of flowers in each branch and sub-branch is counted manually in selected randomly chosen plants. From each selected ten plants, 25 flowers were collected from each individual for stamen number counting. The floral characteristics, such as the number of sepals, petals, flower diameter (the length and width of the flower were measured using a digital caliper), style length, and ovule counts, were also counted from the collected flower. The ovule number was directly obtained from the dissection of ovaries under a stereoscopic microscope.

The pollen productivity was computed as per Tormo Molina et al. (1996). The total quantity of pollen grains per anther was counted from ten randomly chosen fresh flower buds in each individual to estimate the production of pollen grains per flower and per plant. The number of pollen grains was estimated using four anthers from each flower, a total of 40 anthers per plant (10 x 4 = 40). Pollen grains per anther were calculated using the average number of pollen grains from 40 anthers.

From each flower, one anther was taken in a glass slide and mixed with safranin stain; the pinkish colour of the safranin stain is absorbed by the pollen and is easily distinguished and visible under a stereoscopic microscope. The anther is a little crushed using a needle, and the outer cover of the anther is removed using pointed forceps then, it is mixed thoroughly and spread uniformly in the glass slide. Later the glass slide is left for drying; after the glass slide is fully dry, then is mounted with DPX and left for drying. Equal size of the small square box was drawn on the back of the glass slide, then using a stereoscopic microscope, each small square box was observed, and the numbers of pollens in each box were counted visually with a microscope.

further multiplied by the total number of flowers produced per plant, allowed determining the pollen grain production per plant. Fruit production was calculated by counting every single fruit set in each branch and sub-branch in a random selection of trees.

Cruden, (1977).

3.3.3. Diurnal rhythms of pollen concentrations

The diurnal rhythms of pollen concentrations in the ambient air or the occurrence of pollens in the atmosphere were observed by taking pollen air samples at different time intervals between 0500 to 1700 hours of the day for several days on jelly-coated microscopic slides (artificial trappers) as well as on the stigmatic surface of female flowers in relation to prevailing weather conditions. The emasculated flower with stigma was covered with very mesh nylon cloth, and the cover was opened for a particular interval of time; further, the stigma was cut from the flower and stained with safranin, and observed under a microscope for pollen deposition. The observation on the population was carried out from morning 0500 h to evening 1700 h; observation on each block was carried out at a time interval of 2 h, i.e., between 0500 – 0700, 0700 – 0900, 0900 – 1100, 1100 – 1300, 1300 – 1500, 1500 – 1700. The sample glass slide was then observed on the microscope to identify the pollen collected on the slide and count the number of pollen grains present on the 1 cm2 area of a glass slide. Prevailing weather conditions such as temperature and humidity were recorded.

3.4.4. Pollen Viability

The flower samples were taken in the morning (6-9 am) during anthesis from five individuals growing 100 meters apart from one another for experimentation. To test the pollen viability, freshly opened flowers and unopened flowers (just before anthesis) were selected. 0.5 percent 2, 3, 5-triphenyl tetrazolium chloride (TTC) was prepared in the sucrose solution to determine the pollen's vitality. A few pollen grains were dispersed in the TTC (0.5%) solution and protected from light with cover galas. For 60 minutes, the prepared slides were incubated in dark rooms. The preparation was examined under a light microscope (5 X and 10 X) after incubation; pollen grains dyed red were counted as viable. (Shivanna & Rangaswamy, 2012).

3.4.5. Assessment of pollen longevity

Fresh pollen grains were kept at three different temperatures, 6°C, - 4°C, and -20°C, in an airtight vial. The vitality of the stored pollen grains was routinely assessed with 0.5% TTC every 24 hours for another seven days; viability was then examined with a light microscope every week, at intervals of 14, 21, and 28 days, until the pollen grains were determined to be viable.

3.4.6. Pollen Germination

Studies on in vitro pollen germination used the basal media developed by Brewbaker&Kwack (1963). The impact of sucrose compared to the control (distilled water) was examined using 5% and 10% sucrose concentrations. Growth regulators such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), gibberellic acid (GA 3), and kinetin were added to the basal medium at concentrations of 100, 200, and 300 ppm were used to test their effects on in vitro pollen germination.

Five replications were blocked in time using a randomized complete block design for the experiment (Tuinstra& Wedel, 2000). A needle was used to transfer pollen grains from fresh anthers to germination material on cavity slides. The cavity slides were set up in a room environment and using a thermo-hygrometer, the average temperature (26.35°F/0.98°C) and average humidity (79.58°F/3.07°C) were recorded. After 24, 48, and 72 hours of incubation, cavity slides were examined under a light microscope. When

57%	MATCHING BLOCK 10/11	W

the length of the pollen tube was more than or equal to the diameter of the pollen grain, the

pollen grain was regarded to have germinated (Tuinstra& Wedel, 2000). Ten microscopic views were taken to evaluate the pollen grains in each germination cavity slide. The total number of germinated and non-germinated pollen grains was counted in each view and expressed as a percentage of in-vitro pollen germination. The statistical method, with the help of Excel 2016, the impact of hormones and their concentrations, sucrose concentrations, time, and plant species were evaluated for in-vitro pollen germination.

3.4.7. Stigma receptivity, mating system and fruit set

Using stigmatic surfaces to trap the pollen concentration was made to decipher the duration of pollination, time of the day best suited for pollination, and stigma receptivity. This was done by bagging hundreds of flowers (please specify exact number) at the initial stage of development (before anther dehiscence) and was exposed to wind in the batches for desired intervals, which then was removed from the source plant, and a number of pollen grains deposited on them were counted as per Ornduff, (1975).

The following treatments were used for evaluating the mating system (Modified from Dafni, 1992 and Boulter et al., 2006): (i) natural pollination, in which flowers were not modified; (ii) Spontaneous selfing, in which buds were bagged throughout their flowering period; (iii)Induced selfing, in which flowers anther was emasculated, bagged and hand pollinated with their own pollen; (iv) Geitonogamy, the pollen from the same tree is manually applied after the flower anthers have been emasculated and bagged, (v)Cross – artificial, in which flower buds which were bagged and pollinated with pollen from another plant; and (vi)Cross – natural, in which flower buds are removed and stigma are left open on air. Additionally, fruit setting between spontaneous selfing, induced selfing, geitonogamy, cross-artificial, and cross-natural was compared. The estimated selfing rate was determined in accordance with Charlesworth& Charlesworth, (1987).

3.4.8. Pollinators availability

During the peak flowering period, the frequency of pollinators and their visits were determined on ten randomly selected plant species. The study was conducted for 15 days during the peak flowering season after every alternate day in each site after the first bloom of the flower on the ten selected plants. A data sheet was prepared to collect the data according to its requirement. In the data set, the number of visits and the flowers that were visited, the frequency of visits, contact with the reproductive sections, and interactions with other visitors was all recorded.

The observation on the population was carried out from morning 0500 h to evening 1700 h; observation on each block was carried out at a time interval of 2 h, i.e., between 0500 – 0700, 0700 – 0900, 0900 – 1100, 1100 – 1300, 1300 – 1500, 1500 – 1700. Pollinator frequency was measured in terms of visits/inflorescence/hour. Butterflies, moths, bees, and bugs are the main pollinator recorded during the field observation. Photographs of pollinators were taken during the field observation using a high-resolution camera, and the image where identified using Butterfly of India (Antram, 1924); The book of Indian butterflies (Kehimkar, 2008); The dictionary of butterflies and moths in colour (Laithwaite et al., 1975), Butterflies and Moths of Pakke Tiger Reserve (Sondhi&Kunte, 2014); Butterflies and Moths of Pakke Tiger Reserve. Second Edition (Sondhi&Kunte, 2018) and Butterflies of the Garo Hills (Sondhiet al., 2013).

3.4.9. Statistical Analysis

MS Excel 2019 and SPSS statistics 25 were used for statistical analysis to estimate mean, standard deviation, and standard error from the data collected from various observations made during field surveys and laboratory observations. Pearson correlation was used to determine the association between floral characteristics, temperature, and humidity, as well as pollinators and temperature and humidity. ANOVA for the effect of year and population on the number of flowers, anthers, and pollen grains per plant and fruit set; plant species on the number of flowers, anthers, and pollen grains per plant and fruit set; distill water and sucrose 5% and 10%, concentration and time; and hormones, concentrations, time and species.

Chapter 4: Results

4.1.1. Floral biology: anthesis, anther dehiscence, stigma receptivity in C. colebrookianum

C. colebrookianum is a perennial deciduous gregarious erect shrub. The average height of the plant is 265.8±18.42 cm, and the average girth of the plant is up to 17±1.49 cm. The stem and branches are tender. The leaves are simple and opposite to each other. The leaves are large, soft, heart shape, and shiny in appearance with an unpleasant odour when crushed. The flowering begins in July and continues till December, while the peak flowering occurs between September and October. Flowers are white and borne in 4-6 branched corymbose cymes. The flowers are medium, 49.36 ± 0.14 mm long and 19.21 ± 0.16 mm wide, and bisexual. The Calyx is green and polysepalous, consisting of 5 sepals, 3.66 ± 0.04 mm long and 1.69 ± 0.04 mm wide. Corolla is white, with a floral tube (27.94 ± 0.11 mm), 5 lobed at the tip; each lobe is 8.11 ± 0.14 mm long and 2.88 ± 0.03 mm wide. The floral tube has deep-seated nectar (0.5-1 µl). The stamens are 4, epipetalous, 24.88 ± 0.17 mm long, and protrude from the flower. The anthers are purple-coloured, oblong, and 2.31 ± 0.01 mm long. The ovary is globose; the ovule is bi-locular due to the formation of a false septum; it is characteristically a 4-ovule. The ovules are erect, anatropous, and arranged on axile placentation. The style is white, 29.99 ± 0.03 mm, and ends with a simple bifid (two stigmatic lobes) slightly yellowish coloured stigma.

During the study period, it was observed that the time of the flowering phenological phase showed annual variability from one year to the following year, i.e., 2018 and 2019, and also from one population to another in the present study sites. The date of floral bud initiation of the selected study individual plants in C. colebrookianum starts in the year 2018 was 9th July, 11th July, and 5th July but in the year 2019, it began on 26th July, 29th July, and 25th July at study sites Tanhril, Durtlang, and Sairang respectively. Whereas in the population, the date of floral bud initiation starts in the year 2018 was 5th July, 6th July, and 2nd July and in the year 2019, it began on 23rd July, 25th July, and 22nd July at Tanhril, Durtlang, and Sairang respectively. The flowering duration of the sample plant during 2018 was 156 d, 154 d, and 160, and in the year 2019, the duration of flowering was 139 d, 136 d, and 138 d at study sites,Tanhril, Durtlang, and Sairang respectively. Whereas at the population, the duration of the flowering in the year 2018 was 167 d, 164 d, and 166 d; in the year 2019, the duration of a flower was 154 d, 144 d, and 147 d at Tanhril, Durtlang, and Sairang respectively. After the floral bud initiation, the anthesis starts on an average of 10-12 days.

The opening of the flower i.e. anthesis started in the morning at 0500 h, and between 0700 and 0900 of the day, the anthesis of the flower reached its maximum level (Fig. 1 & 2). During the beginning of anthesis, the stamen remains coiled, and when the flower is fully opened, the stamen stands erect (Fig. 3 & 4). A distinct physical separation between stamen and pistil was observed during anthesis (Fig. 4). The pistil length is very short and confined to the base of the flower on the first day of anthesis. At noon time, the relative humidity decreases due to an increase in temperature between 1100 and 1300; there is also a decrease in the anthesis of the flower, and it reaches its minimum level. Again, as the day proceeded, when it reached the afternoon, i.e., the time between 1500 and 1700, the slight increase in relative humidity and the temperature decreased. To complete one full flower opening took 44 minutes. There was no opening of flowers during evening and night-time. The correlation analysis also verified this trend as there is a negative (= -0.538; p = 0.271) and positive (= 0.537; p = 0.271) relationship of anthesis with temperature and humidity, respectively (Fig. 2).

Anther dehiscence started in the morning hours and coincided with anthesis, and peak pollen release was observed during 0700-0900 hours and continued till 1300-1500; a distinct powdery white appearance of anthers during peak (Fig.2 & 5). Pollen was released from anthers through a longitudinal slit and after complete anther, dehiscence stamen bends downwards (Fig. 5). There is very weak positive (= 0.003; p = 0.995) and negative (= -0.079; p = 0.882) relationship of anther dehiscence with temperature and humidity, respectively (Fig. 2).

The receptivity of the stigma was recorded after 3-4 days from anthesis, it was observed that the length of the stigma becomes longer when it is receptive and the bifid pistil splits into acute V shape stigmatic lobes (Fig. 4 & 6). Maximum receptivity of the stigma was recorded between 0900-1100 hours of the day (Fig. 2). It was observed that the receptivity lasted only for one day the tip of the stigma colour turns yellowish to brown colour (Fig. 6). There is positive (= 0.487; p = 0.328) and negative (= -0.533; p = 0.277) relationship of receptivity with temperature and humidity, respectively (Fig. 2).

4.1.2. Floral biology: anthesis, anther dehiscence, stigma receptivity in C. infortunatum

C. infortunatum is a perennial gregarious deciduous erect shrub that can reach a height of up to 259.9±9.86 cm and girth of up to 7.88±0.62 cm on average. Leaves are simple and opposite to each other. The leaves are large, elliptical ovate, pubescent, soft, and release an unpleasant odour when crushed. Flowering starts from mid of February and continues till April, and the peak flowering occurs during March. The flower possesses sweet fragrant white with nectar guide pink in colour at the center with peduncle panicle cymes. The flower starts to mature from the base with the shape of a pyramid consisting of 32 to 138 pedicellate flowers. The flowers are medium 57.96 ± 0.49 mm long and 31.43 ± 0.66 mm wide bisexual and distinct herkogamy and dichogamy (protandry) with deep-seated nectar 3-4 µl at the base of the ovary. The calyx is yellowish green, polysepalous consisting of 5 sepals, 13.83 ± 0.36 mm long, 5.96 ± 0.14 mm wide. Corolla is white, with a floral tube (17.49 ± 0.40 mm), 5 lobed at the tip; each lobe 14.90 ± 0.42 mm long and 6.91 ± 0.17 mm wide. The stamens are 4, epipetalous, 37.28 ± 0.97 mm long, and protrude from the flower. The anthers have purple-coloured, oblong, 2.78 ± 0.04 mm long. The ovules are erect, anatropous, and arranged on axile placentation with 4 ovules. The style is white 43.48 ± 0.36 mm with a bifid stigmatic lobe yellowish coloured at the tip of the stigma (Fig. 10. 11 & 12).

Between 2019 and 2020, the timing of the flowering phenological phase varied from one year to the following year. And also, from one population to another. The date of floral bud initiation of the selected study plants starts in 2019 was on the 25th of February and the 2nd of March, while in the year 2020, it was started on the 3rd of March and the 7th of March at the study sites Tanhril and Sairang, respectively. In contrast, in the population, the date of floral bud initiation began in the year 2019 on the 26th of February and the 4th of March, and in the year 2020, it was started on the 25th of February and the 6th of March at Tanhril and Sairang respectively.

After the floral bud initiation, on an average of 7-9 days, the anthesis starts. The flowering duration of the sample plant in 2019 was 46 days and 39 days, and in the year 2020, the duration of flowering was 43 days and 35 days at Tanhril, and Sairang, respectively. Whereas in the population, the duration of the flowering in the year 2019 was 48 days and 34 days, and in the year 2020, the duration of the flower were 41 days and 37 days at Tanhril and Sairang, respectively.

Anthesis of the flower begins from morning 0500 h, and between 0700 and 0900 h of the day, the anthesis of the flower reaches its maximum level (Fig. 8 & 9). Before the anthesis, the stamen and pistil are coiled inside the flower, but when the anthesis starts, the coiled stamen and pistil start to uncoil (Fig. 9 & 10). The stamen and pistil are physically separated when the flowers are fully opened. Stamens are positioned at the front with a little curve, and the pistil at a back position (Fig. 10). At noon time, the relative humidity decreases with an increase in temperature between 1100 and 1300 h. There is also a decrease in the anthesis of the flower, and it reaches its minimum level. Again, as the day proceeds, the relative humidity increases, and the temperature decreases when it reaches the afternoon, i.e., the time between 1500 and 1700. To complete one full flower opening, it took 30-40 minutes. There was no opening of flowers during evening and night time. The correlation analysis also verified this trend as there was a negative (= -0.658; p = 0.155) and positive (= 0.350; p = 0.496) relationship of anthesis with temperature and humidity, respectively (Fig. 8).

Anther dehiscence starts in the morning hours and coincides with anthesis, and peak pollen release was observed during 0700-0900 hours and continued till 1300-1500(Fig. 8). A distinct powdery white appearance of anthers during peak dehiscence. Pollen was released from anthers through a longitudinal slit. After complete anther dehiscence stamen bent downwards (Fig. 11). There was a negative (= -0.435; p = 0.389) and negative (= -0.201; p = 0.703) relationship of anther dehiscence with temperature and humidity, respectively (Fig. 8).

The receptivity of the stigma was recorded on the following day from anthesis. When the bifid pistil split with an acute V shape stigmatic lobe, the stigma became receptive (Fig. 12). Maximum receptivity of the stigma was recorded between 0700-1100 hours of the day (Fig. 8). It was observed that the receptivity lasted only for one day the tip of the stigma colour turns yellowish to brown colour (Fig. 12). There was weak negative (= -0.090; p = 0.865) and negative (= -0.557; p = 0.251) relationship of receptivity with temperature and humidity, respectively (Fig. 8).

4.1.3. Floral biology: anthesis, anther dehiscence, stigma receptivity in C. serratum

Clerodendrum serratum is a perennial deciduous woody shrub with an average height of the plant is up to 215.8±13.76 to 226.7±13.80 cm and an average girth of the plant is up to 1.88±0.12 to 2.29±0.12 cm. The stem and branches are so tendered that they can easily break while pulling. The leaves are simple and opposite to each other. The leaves are oblong and thorny, with an unpleasant odour when crushed. The flowering starts in March and continues till July, while the peak flowering occurs between April and May. Flowers are light purple in colour in compound cymes inflorescence consisting of 27 to 60 flowers.

The flowers are medium, 44.29 ± 0.40 mm long, and 22.43 ± 0.16 mm wide. Bisexual with distinct herkogamy and dichogamy (protandry) with hair around the base of the ovary. Nectar is present in the form of the droplet in very minute quantities on the tip of that hairy structure. The calyx is green, polysepalous consisting of 5 sepals, 5.43 ± 0.05 mm long, 2.89 ± 0.08 mm wide. Corolla is creamy white light purple, with 4 lobed at the tip, each lobe 16.28 ± 0.09 mm long and 6.87 ± 0.10 mm wide, and one lip (nectar guide) with distinct purple color 23.46±0.06 mm long and 3.51± 0.05 mm width. The presence of a lip on the flower is used as a landing platform by insect floral visitors. The stamens 4 epipetalous, purple colour 32.25 ± 0.17 mm long and protrude from the flower. The anthers have purple-coloured, oblong, 2.43 ± 0.05 mm long. The ovules are erect, anatropous, and arranged on axile placentation with 4 ovules. The style is purple colour 37.08± 0.09 mm bifid at the tip of the stigma.

It was noted that between 2019 and 2021, the timing of the flowering phenological phase varied from one year to the next year. The date of floral bud initiation of the sample plant starts in the year 2019 was 15th March, but in the year 2021, it starts on 22nd March, whereas in the population, the date of floral bud initiation begins in the year 2019 was 12th March and in the year 2021 it was started from 19th March. The flowering duration of the sample plant in 2019 was 87 days, and 94 days in the year 2021. Whereas in the population, the duration of the flowering in the year 2019 was 83 days and 89 days in the year 2021. After the floral bud initiation, on an average of 15-18 days, the anthesis starts.

Anthesis of the flower starts from morning 0500 h, and at the time between 0700 and 0900 of the day, the anthesis of the flower reaches its maximum level (Fig. 14 & 15). Before the anthesis, the stamen and pistil are coiled inside the flower, but when the anthesis start, the coiled stamen and pistil start to be uncoiled (Fig. 15 & 16). When the flower is open, the stamen and pistil are physically separated; the stamen they are positioned at the front with a little curve, while the pistil is positioned back straight and erect with a little curve at the tip of the stigma (Fig. 16). At the noon time, the relative humidity decreases due to increase in temperature between 1100 and 1300 h there is also decreased in the anthesis of the flower, and it reaches its minimum level. Again, as the day proceeds, when it reaches the afternoon, i.e., the time between 1500 and 1700, the relative humidity increases, and the temperature decreases. To complete one full flower opening, it took 50-60 minutes. There was no opening of flowers during nighttime. The correlation analysis also verified this trend as there was a significant negative (= -0.258; p = 0.621) and positive (= 0.621; p = 0.188) relationship of anthesis with temperature and humidity, respectively (Fig. 14)

Anther dehiscence started in the morning hours and coincided with anthesis, and peak pollen release was observed during 0900-1100 hours and continued till 1500-1700; a distinct powdery white appearance of anthers during peak (Fig. 14). Pollen was released from anthers through a longitudinal slit and after complete anther dehiscence stamen bends downwards (Fig. 17).

There was a significant positive (= 0.481; p = 0.334) and negative (= -0.114; p = 0.830) relationship of anther dehiscence with temperature and humidity, respectively (Fig. 14)

The receptivity of the stigma was recorded on the following day from anthesis; by visual eye receptivity of the stigma can be understood when the bifid pistil curved at U/hook shape the stigma become receptive (Fig. 18). Maximum receptivity of the stigma recorded between 0900-1100 hours of the day (Fig. 14). It was observed that the receptivity lasts only for one day the tip of the stigma colour turns purple to brown colour (Fig. 18). There was significant positive (= 0.378; p = 0.460) and positive (= 0.055; p = 0.918) relationship of receptivity with temperature and humidity, respectively (Fig. 14).

4.1.4. Pollinators availability in C. colebrookianum.:

Lepidoptera, Hymenoptera, Coleoptera, and Hemiptera are the insect orders observed in the C.colebrookianum as floral visitors. Twenty-six insect species of the order Lepidoptera have been identified as a floral visitor and has the maximum number of floral pollinators, 4 species of the order Hymenoptera and 1species each from the order Coleoptera and Hemiptera. Butterflies were observed as the most frequently visiting pollinator of C. colebrookianum followed by moths and bee species. A total of 16 butterflies and 10 moths belonging to the order Lepidoptera were observed as floral visitors in the flower of C. colebrookianum during the flowering period. Maximum visitation of butterflies was observed between 0800-1500 of the day, and they follow a diurnal pattern, which coincides with anther dehiscence and nectar production. Papilio polytes, Papilio helenus, Delias descombesi, and Eurema andersonii were observed with the highest visitation frequency among the butterflies. Moths follow a uniform visitation frequency, and it was observed that they follow a bimodal pattern. Maximum visitation of moths was observed between 0700-1700 of the day. No pollinator was observed during the morning hours between 0500-0700 for both the insect groups (butterflies and moths), and fewer butterflies were observed in the evening, i.e. between 1500-1700 but moth pollinators were still observed in evening hours between 1500-1700. Macroglossum stellatarum, Koruthaialos butleri, and Matapa aria were observed as the maximum visitor during this hour. Nectar present in minute quantities is taken as a floral reward by these pollinators.

Three bee pollinators Amegilla cingulata, Bombus albopleuralis and Trigona carbonaria, where observed as a floral visitors in the flower of C. colebrookianum during the flowering period. They follow proclaimed forenoon patterns; maximum visitation was observed during the morning hours between 0700-1100 of the day, coinciding with anthesis, anther dehiscence, and nectar production of the flower. Amegilla cingulata and Trigona carbonaria received pollen as a floral reward, and Bombus albopleuralis try harvest nectar as a floral reward. Fewer visitations were observed during the afternoon and evening time. Each species of the order Hemiptera i.e. Halyomorpha halys and Order Coleoptera Charidotella sexpunctata were observed as a floral visitor in the C. colebrookianum.

Temperature and humidity enhance the pollinator activity on flowers. The visitation frequency and activity of butterflies and moths increase with the rise in temperature between 0900-1500. With the increase in temperature, the visitation frequency of the bee's pollinator increased, and maximum visitation frequency was observed between 0900-1100 but at very high temperatures between 1100-1300, visitation frequencies of the bee's pollinator decreased. In addition, bugs pollinator was observed to increase with an increase in temperature between 1100-1300 of the day. In all the five different groups of pollinators (butterflies, moths, bees, beetle, and bugs) positive (+ve) correlation at (0.05*) was observed (r = 0.879*, 0.903*, 0.297 and 0.877*; p = 0.021, 0.014, 0.567 and 0.022) with temperature and negative (-ve) correlation at (0.05*) was observed (r = -0.794, -0.884*, -0.150 and -0.732; p = 0.061, 0.019, 0.776 and 0.098) with humidity.

4.1.5. Pollinator's availability in Clerodendrum infortunatum:

Lepidoptera and Hymenoptera are the insect order which was observed in the Clerodendrum infortunatum as floral visitors. 16 species of the order Lepidoptera have been identified as floral visitors with a maximum number of floral pollinators, and 3 species of the order Hymenoptera. Butterflies were observed as the most frequently visiting pollinator of C. infortunatum. A total of 13 butterflies and 3 moths belonging to the order Lepidoptera were observed as floral visitors in the flower of C. infortunatum during the flowering period. Maximum visitation of butterflies was observed between 0700-1300 of the day, and they follow a diurnal pattern, which coincides with anther dehiscence and nectar production. Delias descombesi, Catopsilia florella, Papilio memnon and Papilio helenus were observed as the highest visitation frequency among the butterflies.

Moths followed the bimodal pattern, it was observed that maximum visitation of Pelopidas mathias and Koruthaialos butteria was observed during morning and afternoon hours of the day i.e., between 0900-1300 hours, whereas pollinator Macroglossum stellatarum was observed maximum during the evening hours between 1500-1700 hours of the day. By using a proboscis length of 2.8 cm Macroglossum stellatarum collect nectar as a floral reward. No pollinator was observed during the morning hours between 0500-0700 for both the species (butterflies and moths), and no floral visitor was observed in the evening hours for butterflies between 1500-1700. Nectar which is present in minute quantities, is taken as a floral reward by these pollinators.

Three bee pollinator Apis cerana indica, Xylocopa micans and Xylocopa virginica were observed as floral visitors in the flower of C. infortunatum during the flowering period. Among the three pollinators Apis cerana indica was observed as the most frequently visiting pollinator with proclaimed forenoon pattern; maximum visitation was observed during the morning hours between 0900-1300 of the day, which coincided with anthesis, anther dehiscence and nectar production of the flower.

Maximum visitation of Xylocopa micans and Xylocopa virginica were observed when the stamen curled downward and the anther was fully dehisced, i.e., between 1100-1300 hours of the day. The two species received nectar as a floral reward. During the evening hours, no floral visitation was observed i.e. between 1500-1700.

Temperature and humidity enhance floral activity on flower and pollinator activity. The visitation frequency and activity of butterflies and moths increased with the rise in temperature, i.e., between 0900-1100 and 1100-1300. With the increase in temperature, the visitation frequency of the bee's pollinator increased, and maximum visitation frequency was observed between 0900-1300. But with the decrease in temperature around 1500-1700 hours, the visitation frequency of pollinators decreased. All the three different groups of pollinators (butterflies, moths, and bees) positively showed (+ve) correlation at (0.01**) observed (r = 0.649, 0.928** and 0.793; p = 0.163, 0.008 and 0.060) with temperature while negative (-ve) correlation at (0.01** and 0.05*) was observed (r = -0.835*, -0.891 and -0.924**; p = 0.039, 0.017 and 0.009) with humidity.

4.1.6. Pollinators availability in Clerodendrum serratum:

Lepidoptera and Hymenoptera are the insect order which is observed in the Clerodendrum serratum as a floral visitors. Five species of the order Lepidoptera and five species of the order Hymenoptera have been identified as floral visitors. Five butterfly species Anthene lycaenina, Celaenorrhinus aurivittata, Hesperia sassacus, Notocrypta curvifascia and Pelopidas mathias were observed as floral visitor of C. serratum. Maximum visitation was observed during 0900- 1600 hour. Highest visitation frequency was observed for Pelopidas mathias, Notocrypta curvifascia and Hesperia sassacus, following a diurnal pattern.

Moreover, 5 bee pollinators Apis cerana indica, Bombus albopleuralis, Polistes spp, Xylocopa virginica, and Xylocopa violacea, were observed as a floral visitors in the flower of C. serratum during the flowering period. Among the pollinators, Apis cerana indica was observed as the most frequently visiting pollinator with proclaimed forenoon pattern; maximum visitation was observed during the morning hours between 0500-1100 of the day, coinciding with anthesis, anther dehiscence and nectar production of the flower. Bombus albopleuralis, Xylocopa virginica and Xylocopa violacea were observed to squeeze the nectar, which is present on the tip of the hairy like structure present at the base of the ovary. In addition, when they have squeezed the nectar, the stamen of the flower touches the dorsal site of these three pollinators. During the evening hours, no floral visitation was observed, i.e., between 1500-1700.

Temperature and humidity enhance the floral activity of flowers and pollinator activity. The visitation frequency and activity of butterflies increase with the rise in temperature, i.e., between 0900-1300. With the increase in temperature and humidity, the visitation frequency of bee pollinators increased, and maximum visitation frequency was observed between 0700-0900 hours. But with the decrease in temperature around 1500-1700 hours, the visitation frequency of pollinators decreased.

Pollinators (butterflies and bees) positive (+ve) correlation was observed ($r = 0.744$ and 0.087; $p = 0.869$ and 0.090) with temperature, but a negative (-ve) correlation at (0.01**) was observed ($r = -0.931^{**}$; $p = 0.007$) for butterflies and (+ve) correlation ($r = 0.414$; $p = 0.414$) for bees with humidity was observed.

4.1.7. Diurnal rhythms of pollen concentrations on stigma and in air:

Clerodendrum colebrookianum and Clerodendrum infortunatum maximum concentration of pollen in the air was observed during 1100-1300 hours of the day, while in Clerodendrum serratum it was observed during 0900-1100 hours of the day. With the increase in temperature and reduction in humidity, the concentration of pollen in the air increases. Increased in time from anther dehiscence increased the concentration of pollen in the air in all three Clerodendrum species. Less pollen concentration was observed during the morning hours 0500-0900, which coincides with anthesis and anther dehiscence of the three Clerodendrum species; low temperature and high humidity were observed during this hour. Less pollen concentration was observed during the evening hours, 1500-1700, since no anthesis and anther dehiscence occurred during this hour and the anther is fully dehisce.

Clerodendrum colebrookianum maximum concentration of pollen on the stigma was observed during 1100-1300 hours of the day, but in Clerodendrum infortunatum and Clerodendrum serratum, the maximum concentration of pollen on the stigma was observed during 0900-1100 hours. During this hour's maximum visitation of pollinator and their pollination activity were high for all three Clerodendrum species, which helped in supplying and increasing pollen on stigma during these hours. It was observed that with the increase in temperature and reduced humidity, pollen on the stigma increases. Less pollen on the stigma was observed during morning and evening hours since fewer floral visitors for pollination during these hours in all three Clerodendrum. In-vivo pollen germination on the stigmatic surface of the stigma was observed in all three Clerodendrum species under a fluorescent microscope (Figure). Aniline blue stain was used to observe the germinated pollen on the stigma with cross-pollination treatment.

Pollen on stigma and pollen in air positive correlation (0.01** and 0.05*) was observed ($r = 0.880^{*}$ and 0.967^{**}, $p = 0.021$ and 0.009); ($r = 0.356$ and 0.700, $p = 0.489$ and 0.121) and ($r = 0.580$ and 0.583, $p = 0.227$ and 0.225) with temperature, but negative correlation (0.01** and 0.05*) ($r = -0.889^{*}$ and -0.883^{*}, $p = 0.018$ and 0.021); ($r = -0.958^{**}$ and -0.939^{**}, $p = 0.003$ and 0.006) and ($r = -0.067$ and -0.158, $p = 0.900$ and 0.764) was observed with humidity for C. colebrookianum, C. infortunatum and C. serratum (Table).

4.2.1 Assessment of pollen production, pollen/ovule ratio in three Clerodendrum species.

The average girth and average height varied with geographical locations in C. clerodendrum and C. infortunatum within the years, with slight variation between years among selected individuals of all three study plant species (Table). The stamen per flower was observed to be the same, i.e., 4 in C. clerodendrum, C. infortunatum and C. serratum. The production of flowers and pollen grains per plant in all the selected plant species substantially varied among geographical locations. It was observed that among the three chosen sites, the production of pollen grain per plant was more at Tanhril (medium altitude) and Sairang (low altitude) compared to Durtlang (high altitude) in C. colebrookianum. While among selected two laoctions, the production of pollen grain per plant was more at Tanhril (mid-altitude) and less at Sairang (low altitude) for C. infortunatum. Among plant species, the magnitude of pollen grain production per plant was highly varied and highest in Clerodendrum infortunatum (10.44-82.09 × 105), followed by C. clerodendrum (21.54-54.33 × 105) and C. serratum (45.15-53.81 × 105). The maximum percentage of fruit setting was recorded in C. clerodendrum (65.30%) at Tanhril (mid-altitude), followed by C. serratum (56.23%) at Hlimen (high altitude) and C. infortunatum (42.15%) at Tanhril (mid-altitude).

The statistical analysis revealed significant effect of plant species on flower production (p=0.0001; F=16.81), pollen grains per anther (p=0.0001; F=11.32), pollen grains per flower (p=0.0001; F=11.32) and pollen grain production per plant (p=0.0001; F=49.06). There is significant effect of geographical locations, i.e. plant populations varied in three altitudes (low, mid & high), on flower production (p=0.002; F=6.55), pollen grain production per plant (p=0.0001; F=20.53) and fruit set (p=0.0001; F=27.92) while there was non-significant effect of years on flower production (p=0.97; F=0.022), pollen grain production per plant (p=0.97; F=0.023) and fruit set (p=0.70; F=0.35) in C. colebrookianum. There is significant effect of geographical locations i.e. plant populations in varied two altitudes (low & mid) on flower production (p≤0.001; F=15.08), pollen grain production per plant (p=0.005; F=8.81) but non-significant effect on fruit set (p=0.264; F=1.28), while there was non-significant effect of year on flower production (p=0.79; F=0.069) and fruit set (p=0.47; F=0.51) but significant effect of year on pollen grain production per plant (p=0.005; F=8.81) in C. infortunatum. In C. serratum, there is non-significant effect of year on flower production (p=0.44; F=0.61), pollen grain production per plant (p=0.34; F=0.96) and fruit set (p=0.54; F=0.37). No effect of geographical location was evaluated in C. serratum since the plant population was located and studied in only one high-altitude location, i.e., Hlimen.

The correlation coefficient revealed that there is a weak correlation between pollen grain per plant to fruit set percentage in C. colebrookianum at Sairang (low), Tanhril (mid), and Durtlang (high) altitudes. There is a significant correlation between flower/inflorescence and fruit setting percentage (p=.021) in C. colebrookianum at Tanhril (mid-altitude site). In C. infortunatum, there is a weak correlation between pollen grain per plant and fruit setting percentage at Tanhril (mid-altitude), while there is a significant correlation between pollen grain per plant to fruit setting percentage (p=0.016); the number of inflorescences/plants to fruit setting/plant (p=0.016) at low altitude site Sairang. Again in C. serratum there is a significant correlation between pollen grain per plant to fruit setting percentage (p=0.025); the number of inflorescences/plants to fruit setting/plant (p=0.027) at Hlimen, a high-altitude site in Mizoram.

Regression analysis revealed that there is a weak to average positive relationship observed between the production of flower per plant and fruit set percentage per plant (r2 = 0.255), and total pollen grain production per plant and fruit set percentage per plant (r2 = 0.402) in C. colebrookianum. In C. infortunatum, there is an average positive relationship observed between the production of flower per plant to fruit set percentage per plant (r2 = 0.457) and total pollen grain production per plant and fruit set percentage per tree (r2 = 0. 457). Again, there is an average positive relationship observed between the production of flower per plant and fruit set percentage per plant (r2 = 0.482), and total pollen grain production per plant and fruit set percentage per plant (r2 = 0.484) in C. serratum. In all three-study species, four numbers of ovules per ovary were observed in the cross-section. The pollen-ovule ratio ranged from 726.3-1190.7 in C. clerodendrum (, 950.2-1218.9 in C. infortunatum, and 1300-1368.1 in C. serratum. For all the three-study species range value occurred within the range of 224.7-2588.0 (Table) of facultative xenogamy as per the Cruden (1977).

4.2.2. Mating system evaluation of three Clerodendrum species.

The evaluation of the mating system of all three-plant species of Clerodendrum revealed that the species are capable of being partially self-compatible. Fruit setting percentage was less in spontaneous selfing, induced by selfing, and geitonogamy in C. colebrookianum (24 ± 15.05, 21 ± 10.48, and 25.5 ± 13.42); C. infortunatum (15±10.54, 17.5±12.96 and 19±10.48) and C. serratum (12.5±9.78, 15.5±9.55 and 19±10.48). Nevertheless, fruit setting percentage was high in cross–artificial, cross-natural and control (open-pollinated flowers) in C. colebrookianum (66.5±9.73, 64.5±11.65 and 63.5.5 ± 12.70); C. infortunatum (43.5±9.14, 41.5±10.08 and 39.5±5.98) and C. serratum (52.5±11.84, 54.5±11.16 and 48.5±9.44), respectively (Table).

was calculated; the result showed all three Clerodendrum species ascertained the index value of 0.38 (C. colebrookianum), 0.36 (C. infortunatum) and 0.29 (C. serratum), (Table) which occurred within the range of <0.2 but >1 of partially self-compatible as per Zapata and Arroyo (1978). The value of the outcrossing index was calculated on the basis of variables such as the diameter of the flower, temporal separation of anther dehiscence and stigma receptivity, and spatial positioning of stigma and anthers. All three Clerodendrum species scored 5 points which falls within the range <4, indicating partially self-compatible and required pollinator services for outcrossing as per Cruden, (1977).

4.3. Pollen viability, the effect of growth regulators on in vitro pollen germination with pollen longevity tests.

Pollen viability tested with TTC for the three Clerodendrum species is as follows, pollen grain viability percentages at the pre-anthesis stage (un-opened flower) are 28.57%±2.61%, 19.37%±1.73% and 18.85%±1.38% in C. colebrookianum, C. infortunatum and C. serratum respectively. While, pollen grain viability percentage at the anthesis stage (opened flower) are 71.97%±4.30 %, 81.63% ±3.23 and 76.62%±2.63% in C. colebrookianum, C. infortunatum and C. serratum respectively. The above result shows that the pollen viability percent is higher at the anthesis stage (opened flower) compared to pre-anthesis (unopened flower) in all three species of Clerodendrum.

The result of in vitro pollen germination in sucrose concentrations (5% and 10%), distilled water (control), with growth regulators (IAA, IBA, GA3, and Kinetin), and their varied concentrations in basal media with 10% sucrose and time outline showed a differential response in all three species Clerodendrum. Distilled water (control) showed a poor percentage of germination 0.85±0.23, 0.78±0.21 and 0.49±0.11 in C. colebrookianum, C. infortunatum and C. serratum, respectively, in the first 24 hours (Figure). Later after 48 and 72 hours, there was no germination in all three Clerodendrum species. It showed that the presence of only moisture germination capability and viability is low for the selected three Clerodendrum species.

Sucrose concentrations (5% and 10%) were found to induce in vitro pollen germination and acted as a fundamental substrate compared to the control (distilled water). In 5% sucrose concentration, low percentage of pollen, germination was recorded with 5.19±0.60% in C. colebrookianum, 10.65±1.14% in C. infortunatum and 6.18±1.94 in C. serratum while at 10% sucrose concentration still low germination of 7.54±1.21 was recorded for C. colebrookianum, fair germination (17.92±4.93%) in C. infortunatum and 12.44±2.73 in C. serratum at the initial 24 hours. Further, the germination percentage relatively declined as time passed, i.e., at 48 hours and 72 hours (Fig.) in all three species. Additionally, it is also observed that in all three Clerodendrun species, with the increment in the concentration of sucrose, the percentage of pollen germination also increased (Figure). All the examined pant species ascribed that sucrose is a fundamental substrate for the induction of pollen germination. A significance difference was observed at (p>0.0001) (Table) between distilled water (control) and sucrose 5% & 10%; concentration in all three Clerodendrum species. There is a significance effect of time on in vitro pollen germination in C. colebrookianum and C. infortunatum except in C. serratum.

Maximum pollen germination was recorded in the first 24 hours, which decreased with time, i.e., 48 and 72 hours. The lowest germination percentage was observed after 72 hours in 100, 200, and 300 mg L-1 concentrations in all the selected growth hormones, i.e., IAA, IBA, GA3, and Kinetin in all three Clerodendrum plant species (Table).

In C. colebrookianum highest in vitro pollen germination of 52.10±5.30% was recorded in GA3 (200 mg L-1), followed by 46.56±4.59 % in IBA (100 mg L-1), 44.48±3.26% in GA3 (300 mg L-1) and least 23.37±1.67% in Kinetin (200 mg L-1) respectively. In the case of C. infortunatum, the highest in vitro pollen germination of 61.91±1.76% was also recorded in GA3 (200 mg L-1), followed by 48.61±1.79 % in IBA (200 mg L-1), 43.42±2.37% in Kinetin (100 mg L-1) and least 25.45±2.89% in IAA (100 mg L-1) respectively. While in the case of C. serratum highest in vitro pollen germination of 55.81±4.97% was recorded in IAA (100 mg L-1), followed by 52.50±6.61 % in IAA (200 mg L-1), 34.23±9.89% in GA3 (100 mg L-1) and least 16.50±3.05% in Kinetin (100 mg L-1) respectively. Hence GA3 (200 mg L-1) was found to be the most suitable growth hormone concentration, followed by IBA (200 mg L-1 and 100 mg L-1) for inducing in vitro pollen germination in C. colebrookianum and C. infortunatum (Table 1). In contrast, IAA at (100, 200 mg L-1) and GA3 (100 mg L-1) was found to be the most suitable growth hormone concentration for inducing in vitro pollen germination in C. serratum (Table).

Statistically, the response of all the treatments, viz. hormones, their concentrations, and time, on in vitro pollen germination of C. colebrookianum, C. infortunatum, and C. serratum was found to be significantly different (p>0.05) (Table).

It was observed that among the treatments, different hormones and times gave higher significant responses (p>0.0001), followed by the hormone concentrations application (p>0.05). A non-significant difference was recorded between the study plant species for in vitro pollen germination (Table).

Under varied temperatures, storage conditions such as -20°C, -4°C, and 6°C, pollen viability of all three Clerodendrum species decreased with increased storage durations. It was observed that pollen grains of all three Clerodendrum species stored at -20°C and 6°C showed relatively longer pollen viability duration; pollen grains lost their viability after 28 days. It was also observed that the pollens which were stored under -4°C lost their viability within 14 days of storage in all three Clerodendrum species. Hence, storage conditions of C. colebrookianum, C. infortunatum and C. serratum followed a similar trend (Figures). There is a significant difference between storage days for all three studied plant species (p>0.0001).

Chapter 5:

Discussion

5.1.1. Flowering phenology

C.colebrookianum, C. infortunatum and C. serratum have a regular annual flowering season like other tropical flowering plant species. C. colebrookianum and C. infortunatum mostly found along roadsides, in forest edges, moist, shady places and amidst bushes habitat of North Eastern region of India (Kalita et al., 2012 and Kumar et al., 2017). As reported above the C. colebrookianum and C. infortunatum is found mostly in similar habitats in our study sites too, apart from it C. colebrookianum was found to grown in the local home garden of Mizoram due its traditional medicinal uses. C. colebrookianum flowered during July to December in the present study site. C. infortunatum flowered during January to April and maximum during mid-February and mid-March in Araku Valley Reserve Forest in Visakhapatnam, Andhra Pradesh (Kumar et al., 2017) while our study site flowering starts from mid of February and continues till April and the peak flowering phase occurred during March. C. serratum are found in forest sites at an altitude up to 1500 m and annual flowering season during the month of August to September (Patel et al., 2014 and Poornima et al., 2015). In the present study site it was observed that C. serratum was found to grow at an altitude of 1140 m and flowered during March to July. It was observed that the flowering phenology varied from year to year within population of C. colebrookianum, C. infortunatum and C. Serratum. These three-plant species of Clerodendrum species exhibited well defined seasonality and temporal separation in annual flowering time with each other. Such separation and variation in closely related flowering plant species is of ecological and evolutionary significance. Thus, such variation in floral biological rhythms helps in avoiding pollinator's competition. The flowers bloomed in an umbel pattern in C. colebrookianum while pyramid shape in C. Serratum and C. infortunatum (Kumar et al., 2017). Different reproductive developmental stages viz. budding, flowering and fruiting found to co-occur in the same inflorescence all the three study Clerodendrum species. Such pattern of reproductive developmental pattern was observed in Clerodendrum inerme (Aluri et al., 2016). Difference in development of flowering stages among inflorescence within same plants shall helpful in promoting xenogamy and geitonogamy through floral visitors.

5.1.2. Anthesis, Anther dehiscence and Stigma receptivity

Differential pattern of anthesis was recored in Clerodendrum species. The flower opens during morning hours' in C. infortunatum (Kumar et al., 2017), C. inerme flowered during evening hour (Aluri, et al., 2016), C. molle during evening time (Mc Mullen, 2011), while C. phlomidis open during night time (Rohitash, 2016). During the study period it was observed that the flower opens during morning hours in C. colebrookianum, C. infortunatum and C. Serratum. All the three Clerodendrum species with the increased in temperature during morning hours it help in the developmental and maturation of flower during anthesis. Weather fluctuations such as temperature and humidity are prime importance in predicting behaviour floral development in plants. Both anthesis and anther dehiscence were significantly regulated by temperature and relative humidity (Khanduri et al., 2013). Increased in the temperature during and before the anthesis of Allium sativum help in the development of flower (Mayer et. al., 2015). Anther dehiscence of Euphorbia pulcherrima varied according to time, temperature and humidity (Vargas et al., 2017). The anthesis of the flower is more dependent to temperature (Shahbaz et al., 2021) while anther dehiscence was more strongly influenced by relative humidity (Khanduri et al., 2013; Bera et. al., 2018). Extent of anthesis i.e. the amount flower opens per unit time is played crucial role in pollination, fertilization and gene flow in plant species.

The receptivity of C. infortunatum observed on the next day of anthesis (Kumar et al., 2017) and receptivity of C. indicum observed on the third day from anthesis (Ghosh & Pal, 2017). In the present study of three Clerodendrum species it was observed that receptivity of stigma occurred after 3-4 days from anthesis in C. colebrookianum and after the next day from anthesis in C. infortunatum and C. serratum. It was observed that receptivity of stigma correlated with respect to time, temperature and humidity in all three species of Clerodendrum. The receptivity of the female part of the flower is influenced by both temperature and humidity (Vargas et al., 2017; Khanduri et al., 2013). Thus, manipulating the receptivity could be of importance in promoting fertilization and fruit set. In Douglas fir, receptivity of stigma was extended by artificial overhead water-cooling treatment (Sk Lai et al., 2010).

In our present finding all the three Clerodendrum species exhibited distinct protandous, dichogamous and herkoganous maturation of male reproductive part i.e. stamen first and maturation of female later with physical and temporal separation of male and female during anthesis of flower (Figure). The stigma is receptive only for one day all the three species. In C. infortunatum, C. inerme and C. serratum is reported to be protandous, herkogamous and dichogamous (Singh et al., 2012; Aluri et al., 2016 and Mukhopadhyay & Quader, 2022). Flower with typical adaptations of herkogamy and dichogamy becomes potential donor to conspecific flowers and at the same time the donor flower itself become a site for pollen reception (Waines & Hegde, 2003). Since the anthesis, anther dehiscence, and receptivity differed significantly within the same inflorescence and at population in all the three-study species will influence pollination, mating system and potential gene flow at population level.

5.1.3. Flower pollinators

During the present study, it was observed that all three species of Clerodendrum are visited by moths and butterflies, bees, ants, and bugs that belong to the order Lepidoptera, Hymenoptera, and Hemiptera. As the anthesis begins, the floral features such as flower color, nectar, pollen, and odour/fragrance of the flower attract different pollinators. The order Lepidoptera and Hymenoptera are the primary floral visitors in all three Clerodendrum species, which include moths, butterflies, and bees (Figure). As pollen is an essential source of proteins and lipids for many insects, thus all three-plant species of Clerodendrum are attracted by many insect pollinators.

C. colebrookianum with morning anthesis, white corolla, unpleasant order, landing platform, and floral tube with deep-seated concentrated nectar appeared to adapt toward Lepidoptera (butterflies and moths). C. infortunatum morning anthesis, with the white corolla, distinct nectar guide, sweet odour, landing platform, and floral tube with deep-seated nectar, appeared to adapt toward both Lepidoptera (butterflies and moths) and Hymenoptera(bees), while C. serratum exhibited morning anthesis, with the purple corolla, pubescent nectar guide, landing platform, and short floral tube, minute droplet nectar secreted on hairy nectar guide appeared to adapt toward more Hymenoptera (bees) and less to Lepidoptera (butterflies and moths).

Subba & Solomon (1997) reported that C. infortunatum with morning anthesis is exclusively pollinated by papilionoid butterflies; they also observed that pollination occurred due to the striking of anthers and stigma with the wings of butterflies. Aluri et al. (2016) reported that C. inerme with evening anthesis, long white corolla, hairy interior, and strong fragrance appeared to attract hawk-moth Macroglossum gyrans as the main pollinator. And he further mentioned that this plant species is visited by insect orders belonging to Lepidoptera and Hemiptera as pollinators. Guddeti (2014) reported that the typical butterfly pollination in C. infortunatum is because of the non-promiscuity of floral rewards to others foragers. She also noted that this pollination syndrome is a necessary pre-condition for the rise of the floral isolating mechanism.

Wadhwa & Sihag (2012) reported that Rauvolfia serpentina, Lepidopterans, and Hemipterans were observed as pollinators. They concluded that all these pollinators species were not equally and sufficient pollinators for this crop. In our finding, the floral adaptation of the flower C. colebrookianum and C. infortunatum is highly suited for Lepidopterans, while the floral adaptation of the flower C. serratum is suited for Hymenopterans apart from other groups of floral visitors, seems to assist in playing a role in the pollination.

Proboscis length is a crucial organ of Lepidoptera for harvesting nectar from flower C. colebrookianum and C. infortunatum, while harvesting nectar, landing and sitting posture (Zhang et al., 2010b) of insect play a crucial role in pollen transfer. While harvesting nectar from the flower, the head of the insect is pressed against the floral tube of the flower, resulting in contact with the flower's reproductive organs. Cruden et al. (1983) reported that the flowers pollinated by the insect order Lepidoptera are rich in nectar sugar concentration ranging from 15-25%. From our findings, the two species, C. colebrookianum and C. infortunatum are pollinated mainly by the insect order Lepidoptera, and the flower is also rich in nectar sugar concentration ranging from 18-23%.

Butler & Johnson (2020) reported that the wing of butterfly help in the pollination of Scadoxus multiflorus, a South African Amaryllidaceae plant species. He observed that the umbel inflorescence flower shape of Scadoxus multiflorus gives a perfect landing platform for the butterfly species and helps facilitate pollination. The striking of butterflies and moths' wings helps pollinate C. colebrookianum and C. infortunatum. The presence of pollen on the wings, proboscis, and legs of butterflies and moths and the deposition of pollen on the stigmatic surface prove that they help in the pollination of the flower during their visitation, The elongated filament and styles with umbel and pyramidal inflorescence shape with compact flower arrangement provide a perfect landing platform for the butterflies and moths pollinator, which supports that C. colebrookianum and C. infortunatum is well adapted to Lepidoptera for pollination. The presence of deep-seated nectar in C. colebrookianum and C. infortunatum was very little amount (0.5-1µl and 3-4 µl respectively), promote and force butterflies and moths move from one flower to another flower to quench their thirst for nectar, during this movement, they help in pollen transfer which leads to cross-pollination. Whereas C. serratum they don't show uniform inflorescence shape, which does not provide a proper landing position for butterflies and moth's pollinator, and the nectar are present in the form of the minute droplets (Figure); this shows that C. serratum is not well adapted to Lepidoptera for pollination.

Bees often have dense body hairs, long or short proboscis, and pollen baskets (Wojtaszek & Maier, 2014). Observation showed that the flower of C. colebrookianum was visited by Amegilla cingulata, Bombus albopleuralis, and Trigona carbonaria. C. infortunatum was visited by Apis cerana indica, Xylocopa micans and Xylocopa virginica; pollen grains, sweet odour, nectar guide, and deep-seated nectar attracted the bee’s pollinator. However, these pollinators are symmetrically not fit for deep-seated nectar from the floral tube since their proboscis length is shorter than the floral tube length. The above finding supports that C. colebrookianum and C. infortunatum flower are not well adapted for bees.

While the floral tube length of C. serratum flower is relatively short; bees' pollinators Apis cerana indica, Bombus albopleuralis, Polistes spp, Xylocopa virginica and Xylocopa violacea can easily be squeezed out the nectar, which are present in the form of minute droplets in pubescent nectar guide by using their proboscis. Aluri et al. (2016) observed Xylocarpa and Anthophora bee species bite the corolla and suck out the nectar from the plant species C. inerme. Witter et al. (2015) reported that during pollen collection, the bees landed on the corolla, walked on the flower, and touched the reproductive organs of the flower Brassica napus. From our field observation, the lip of the C. serratum flower gives a perfect landing platform for the bee's pollinator to sit and squeeze out the nectar. While sitting on the lip and squeezing out the nectar from the flower the dorsal site of the bee pollinator touches the curve/bending filament and style which help in the cross pollination of flower since the nectar are present in the form of droplets in less quantity pollinator move from one flower to another flower for the same. Hu et al. (2022) reported that bee pollinators are attracted to flowers with red and yellow filaments. From our result, it can be said that C. serratum flowers with purple colour filament are visual attractiveness to bee visitors for pollination. The above result supports that C. serratum is well adapted to the insect order Hymenoptera (bees). High vibration and clinking onto the flower by bees increased pollen released (Pereira & Vallejo, 2022). A similar finding was observed for the bee's pollinator in C. colebrookianum, C. infortunatum, and C. serratum. This bee's pollinator collects pollen through vibration with a pollen basket, which is present on the hind tibia body parts of bees.

In addition to nesting locations, floral phenology and nectar volume also influence insect flower visits. According to Faheem et al. (2004), the number of insect pollinators was influenced by the size, shape, colour, volume of the nectar secreted, the number of flowers that bloom, and pollen content. From our findings, it can be said that the white and purple colour flowers of C. colebrookianum, C. infortunatum, and C. serratum attract a greater number of insect pollinators. Duara & Kalita, (2013) reported that the inflorescence with blue-yellow flower colour attracts Hymenoptera, Diptera, and Lepidoptera. However, from our finding, it can be said that inflorescence with white and purple flower colour attract Lepidoptera, Hymenoptera, Coleoptera, and Hemiptera. Rianti et al. (2010) reported the effectiveness of insect pollination using basic data such as flowering periods, flower nectar volume, and environmental factors in the seed set of Jatropha curcas. He observed that insect diversity is not influenced by temperature, light intensity, and wind velocity. The contrary result might be due to the site's location and anthropogenic activity, which might reduce the insect visitation on a flower. From our diverse insect study, we found the highest number of individuals is from Hymenoptera (Figure), and the highest species numbers (diversity) was from Lepidoptera (Figure). C. colebrookianum, was observed to be visited by insect order, belonging to Hemiptera (bugs), but these floral visitors seem to be of minor importance. Though floral features adapted to relatively primitive group pollinators (Lepidoptera), but seems to be highly important in conserving huge diversity of Lepidoptera pollinators since moths and butterflies they solitary nature, don't preserve food materials like social bees, they seem to be more prone to biotic and abiotic stress factors or climate change.

Kumar et al. (2017) reported that C. infortunatum is visited by bees, ants, thrips, and butterflies during the day but is mainly pollinated by papilionid and pierid butterflies. Meerabai (2014) reported the typical butterfly pollination in C. infortunatum is because of the non-promiscuity of floral rewards to other foragers. She also noted that this pollination syndrome is a necessary pre-condition for the rise of the floral isolating mechanism.

Begum et al. (2014) mentioned that C. infortunatum is an important source of nectar for certain butterflies such as the Papilionid, Papilio polytes, the Nymphalids, Danaus chrysippus, Pierids, Eurema blanda, E. hecabe, and the Lycaenid, Zizinaotis. In all three species of Clerodendrum presence of nectar in flower was observed, the presence of nectar in flower attracts; the nectar foraging insect species of the order Hymenoptera and Lepidoptera. Two ant species Tapinoma melanocephalum and Trichomyrmex destructor, were reported as floral visitors of C. infortunatum as nectar larceny and herbivory (Mukhopadhyay & Quader, 2018). Raju & Kumar (2016) reported that C. inerme flowers are rich in nectar, so they attract pollinators such as hawk-moth (Macroglossum gyrans), bees (Xylocopa and Anthophora), and butterflies. Aluri et al. (2016) reported that pollinator hawk moth, Macroglossumgyrans, bees Xylocopa pubescens and Anthophora bicincta, and butterflies Pareroniavaleria, Danaus genutia and Borbocinnara were observed to pollinate on C. inerme, pollen and nectar are received as a floral reward. Gautam, (2012) reported that C. splendens were pollinated by Xylocopa(carpenter bee), Eumenes sp.(wasp) and Componotus campestris (black ant).

The presence of nectar guide was observed in C. infortunatum and C. serratum (Figure), which attracts the insect species of the order Hymenoptera. Clerodendrum flower pollinators frequently make inter-floral visits b from one flower to another since the amount of nectar present in the flower is very low. The morphological character of flowers in all three Clerodendrum species differs, such as flower color, presence of nectar, nectar guide, and pollen attracts different groups of insect pollinators. Sakamoto et al., (2012a) reported that C. trichotomum is pollinated by the carpenter bees; Xylocopa species, butterflies, Papilio species, and the hawk-moth Macroglossum sp. Primack et al. (1981) reported that C. inerme is never visited by daytime visitors but is visited by one large hawk-moth during dusk hours. As it is reported that C. trichotomum and C. inerme is visited by hawk-moth, during the study period it was observed that C. colebrookianum and C. infortunatum is visited by hummingbird hawk-moth Macroglossum stellatarum during the evening hours of the day. It was observed that the hummingbird hawk-moth sucked only the flower's nectar with the help of its long proboscis. The anthesis of the C. colebrookianum, C. infortunatum, and C. serratum is in the morning followed by the release of pollens from anther, so the pollinator's visits and pollination mainly occurred from morning to afternoon through different pollinating agents.

A study (Mizusawa et al., 2014) reported that Clerodendrum izuinsulare and C. trichotomum were visited by diverse insect floral visitors. C. trichotomum and C. izuinsulare were reported to share common pollinators, diurnal hawk-moths, bees, swallow tails and nocturnal hawk-moths (Miyake & Inoue, 2003), and they also mention that pollen transfer might happen between the two species, but hybridization does not occur. During our study period, visitation of common pollinators was observed among the three Clerodendrum (Figure) species. Among the butterflies, Anthene lycaenina was recorded as a common floral visitor in C. colebrookianum and C. serratum. And among C. colebrookianum and C. infortunatum Delias descombesi, Papilio helenus, Papilio memnon, Tirumala septentrionis, and Vindula erota erota were recorded as common floral visitors. The hummingbird hawk-moth Macroglossum stellatarum was recorded as a common floral visitor C. colebrookianum and C. infortunatum. Pelopidas mathias was recorded as common moths' floral visitors who visited all three Clerodendrum species. And among the bee pollinator, Bombus albopleuralis in C. colebrookianum and C. serratum; Xylocopa virginica and Apis cerana indica in C. infortunatum and C. serratum. The flowering seasons of all three Clerodendrum are quite different, but they start flowering one after another. Sharing of common pollinators among three species of Clerodendrum species might be important from an evolutionary and hybridization point of view. Butler et al. (2020) observed pollen deposition on the butterflies' wings when pollinating on the flower of C. infortunatum. The result of pollen storage from three Clerodendrum (Figure), i.e., pollen is viable for up to 28 days; this support that pollen which was attached to the body part of the pollinator might pollinate the flower during floral visitation this might lead to cross pollination among the species.

Both pollination and subsequent fertilization play an essential role in successful fruit set and subsequent fruit development in plants with seeded fruit (Zhang et al., 2010a). Pollination plays an important role in the deposition and transfer of pollen on the stigmatic surface of a flower (Cheung, 1996). During the study period, it was observed that pollen deposition on the pollinator's body part and on the stigmatic surface after insect visits in open flower in all three Clerodendrum species proves that pollen deposition on the stigmatic surface (Figure) of flower take place during the insect visits leads pollination. Pollen germination and tube growth in-vivo in many plant species depend on the density of pollen deposited on the stigmatic surface (Chen et al., 2000). All three Clerodendrum species are visited by a significant number of insect pollinators (Table); the movement and visitation by insect pollinators in flower increased the pollen density and deposited sufficient pollens on the stigmatic surface of the flower (Figure). in-vivo and in-vitro pollen germination of Pyrus pyrifolia was reported by Zhang et al. (2010a), and they also mention an increase in pollen density, increased pollen tube growth, and higher pollen germination rate. In the present study, in-vivo and in-vitro pollen germination for all three Clerodendrum species (Figure) was observed. The concentration of pollen on the stigma and in the air was recorded as high during noon time between 2 pm for Tectonagrandis (Khanduri, 2012); he also observed 4-8 pollen grains per stigma for fruit development. During our study period, it was observed that pollen on the stigma and in the air was recorded high after anthesis for all three Clerodendrum species between 0900-1300 hours (Figure).

Increased fruit set production in cross–artificial, cross-natural, and control treatment support high pollen density and allows for a higher frequency of ovule fertilization. An increase in pollen number increased the rate of fruit set production. Varieties of biotic and abiotic (insect and wind) mechanism help in the transportation of pollen on the stigma of a flower (Edlund et al., 2004). Aerial pollen concentration depends upon pollen production, transportation, and pollen uptake into the air by wind, temperature, and humidity. Low pollen concentration in the air was reported when the relative humidity was high (Erkara, 2008). Our finding supports that pollen concentration varies high in the afternoon hours when the relative humidity is low and less pollen concentration during morning and evening hours. Change in wind direction, temperature and humidity change the concentration of pollen in the air throughout the day (Figure). The accumulation of pollen on the stigmatic surface of the flower and on the glass slides at hourly intervals reflects the amount of pollen released into the air. Many authors have reported that pollen in the air cause seasonal pollen allergy (Garcia, 2017), and pollen concentration varies from season to season (Schramm et al., 2021). Different concentrations of Clerodendrum pollen during the study period support that this pollen might cause pollen allergy during that particular flowering season. The pollen content in the air varies due to climatic and weather conditions (Velasco et al., 2015). Our finding supports that temperature and humidity influence the content of Clerodendrum pollen (Figure) in the air. Increased pollen content in the air during the daytime with an increase in temperature. This pollen might go along with the wind flow and help in reproductive success. In addition, this pollen might also go along the wind flow and add to the pollen allergy.

5.2.1. Flower and pollen production

Year-on-year variations in the production of pollen, flowers, and inflorescences (Table) may be attributable to year-on-year variations in weather conditions (Ribeiro et al., 2005; Khanduri et al., 2015b). The results support that with the variation in the geographical area, there is a variation in climatic conditions in all the selected sites, and the production of the flower, pollen, and fruit set varied in all three Clerodendrum species between years and geographical locations. Pollen production per anther differs between the two populations; the data showed some inter-population variability that may be related to differences in environmental conditions affecting pollen production (Delph et al., 1997 and Guardia & Belmonte, 2004). Annual variation in pollen production between the years in Ambrosia artemisiifolia is associated with an increase in temperature (Ranpal et al., 2022). Ziello et al. (2012) and Ranpal et al., (2022) reported the effect of pollen production at higher and lower elevations might be the effect of temperature. During the study period, it was observed that the production of flowers and pollen was recorded as high at a lower elevation than higher elevation (Table).

The variation in pollen production per flower in different plant species and even in closely related species belonging to the same family is attributed to male reproduction variables (Garcia et al., 2020). Pollen productivity data is valuable in selecting male mates in plants (Vankin et al., 2003). The amount of pollen produced in flowers is a genetically determined characteristic; it has been frequently documented that this characteristic can vary significantly between species and between cultivars or varieties (Anton & Denisow, 2018).

Pollen production is not a constant character and strongly depends on environmental conditions and can vary significantly between individuals of the same species and across growing seasons (Alonso et al., 2013). From the result of this study, it can be said that the environmental conditions and flowering seasons for all three Clerodendrun species are different. It supports that pollen, flower, and fruit production vary according to the seasons and differ with climatic conditions in all three Clerodendrum species. Low temperature, drought stress, and soil conditions also have an impact on pollen production (Garcia et al., 2020). Pollen production is directly proportional to the number of flowers and inflorescence on a plant and the condition in which it grows. And the production of flowers per tree and pollen grains per flower is directly related to the production of fruit set. Hidalgo et al., (1999) reported that the density of flowers (flower per plant) has an effect on pollen production further; he also mentioned that the distribution of flowers over the crown is affected by environmental and genetic factors. Our finding supports that at low and mid elevations, Clerodendrum flowers have high flowering density and produce more pollen than at higher elevations, and a greater number of flower inflorescence per plant was observed at mid and lower elevations in the present study sites. Large and high-quality pollen production acts as potential donors in plant mating success. Flowers with high pollen production are visited by a great number of pollinators which makes reproduction successful (Khanduri et al., 2015a). Our finding support that all three Clerodendrum are visited by a great number of pollinators (Table). This proves that all three Clerodendrum flowers have high pollen production, which helps for successful reproduction.

Hidalgo et al., (1999) reported the shape of the crown and the radius of the tree affect pollen production.

The height of a tree varied with the variation in elevation. It was observed that the tree with good height produced a greater number of flowers and fruits. Production of flowers and fruit was more in large trees than smaller trees. Our finding supports that plant height for Clerodendrum species which were found at low and mid-altitude, was taller produced more branch numbers, a high amount of flower, and pollen grains than those found in higher elevations.

The production of pollen is related to the length and size of the anther (Bhowmik & Datta, 2013). Different research has reported the correlation between the size of the anther and the quality of pollen grains (Mondal et al., 1992). During our present study, no correlation was established between the anther size and pollen production among the species.

Rojo et al., (2015) reported that the production of flowers branch, inflorescences, flowers, and pollen varies from year to year in Olea europaea with variation in the onset pre-flowering period and with environmental factors such as temperature, rainfall, elevation, and northern vs. southern exposure. According to Aguilera & Valenzuela, (2012), changes in the microclimate have an impact on the development of Olea europaea fruiting branches, inflorescences, flowers, and pollen grains production. Khanduri, (2012) reported the onset and end of Tectona grandis flowering, as well as the amount of pollen grains and fruit produced by each tree, varies throughout the year. The reproductive phenophases and the production of flowers and production of pollen vary from year to year for Lagerstroemia speciosa (Khanduri, 2014). Flower production, pollen production, fruit production, and seed production of Schima wallichii vary from year to year (Khanduri et al., 2013).

Pollen production significantly affects reproductive success in plants (Khanduri et al., 2015a). Low pollen production can negatively affect fruit and seed set in distantly located conspecific individuals at a population level. In Taxus canadensis seed set was correlated with pollen and ovule production (Allison, 1990). The relationship between pollen availability and fruit and seed production is crucial in gene flow among populations of wind and animal-pollinated taxa (Allison, 1990 and Khanduri et al., 2015b). A large amount of pollen grain production in outcrossing plant taxa is attributed to compensation loss due to the uncertain fate of pollen grains during pollen transfer. Pollen transfers are affected by a variety of biotic (pollinators) and abiotic (rainfall, temperature, humidity, etc.). In the present site, high amounts of rainfall, humidity, and dense canopy covers are impeding factors for pollen transfer. Naturally, less than 1% of pollen produced is transported conspecifics stigma (Minnaar et al., 2019). Henceforth, pollen grains produced a much higher magnitude than ovules in xenogamous plant species (Cruden, 2000). Magnitude pollen production per flower is significantly related to the number of ovules to be fertilized (Jürgens et al., 2002). Wind-pollinated plants produce a huge amount of pollen grains, and high pollen production in tropical plants is related to pollinator limitations. Thus, pollinator limitation acts as a selective for huge pollen production outcrossing plant species (Cunha et al., 2022).

Apart from the role of pollen production in mating success, pollen production and seasonal variability could be useful in apiculture and aerobiological monitoring. By knowing the pollen production of a plant, we can estimate the number of pollen grains that will present in the air ambient for that particular season; this will add knowledge to the aerobiological data, which are crucial in allergy (Ghitarrini et al., 2017). Even the pollen production capability of the plant could be utilized in choosing appropriate plant species as food sources for bees in apiculture, since C. coolebrokianum is also planted in a home garden in Mizoram and other states of North east India hence could a valuable plant species for aforesaid.

5.2.2. Pollen ovule ratio (P/O):

A facultative xenogamy type of mating system was reported for C. infornatum (Kumar et al., 2017) since pollen ovule ratio falls at the range between 244.7-2588. Pollen ovule ratio of C. splendens is 540:1 (Jai, 2010). During our study period all three Clerodendrum species show facultative xenogamy type of mating system as per pollen ovule ratio (Table). McMullen, (2011) reported C. mole to show a facultative xenogamy type of mating system. A high ovule number per flower is crucial when pollinators are limited by biotic and abiotic factors. The higher magnitude of pollen production compared to ovule production per flower reflects Clerodendrum species reproduction could be limited by pollinators.

5.2.3. Mating system

Open pollination, autonomous autogamy, facilitated autogamy, and facilitated cross-pollination of C. mole shows no difference in fruit and seed production, but cross-pollination showed higher fruit and seed set production (McMullen, 2011). C. infornatum (Kumar et al., 2017 and Mukhopadhyay & Quader, 2022) recorded a higher percentage of fruit and seed set percentage in xenogamy and lesser fruit and seed set the percentage at open pollination and geitonogamy, but no fruit formation was observed with spontaneous autogamy and facilitated autogamy. (Sakamoto et al., 2012a) reported C. trichotomum show less fruit and seed set percentage with self-pollination and a higher percentage of fruit and seed set with cross-pollination. Aluri et al., (2016) and Raju & Kumar, (2016) reported C. inerme showed high fruit and seed set percentage with xenogamy and lesser fruit and seed set percentage with open pollination and geitonogamy. C. viscosum (Liza et al., 2010), C. splendens (Jai, 2010) and C. splendens (Rohitash, 2018) show fruit set when the flower is cross-pollinated, but when bagged, no fruit set was observed, which indicates self-incompatibility breeding system. C. trichotomum and C. izuinsulare show higher fruit set when it is outcrossing but fewer fruit set with self-pollination and open-pollination (Mizusawa et al., 2014).

During the study period, it was observed that the flower was found to set fruit at different test levels of treatment. Cross artificial, cross natural, and control shows higher fruit set percentage but spontaneous selfing, induced selfing, and geitonogamy show less fruit set percentage (Table). From this result, it can be said that a partially self-compatible type of mating system is recorded in all three Clerodendrom species (Table). Therefore, pollinator plays an important role in supplying pollen on the stigma of the flower and helping in the fertilization of the ovary to form fruit and seed. The lower fruit setting percentage after the treatment suggests that there is an occurrence of pollen limitation in flowers. Reduced supply of pollen and blocking of pollinators in flower causes pollen limitation, which leads to less fruit setting in plants.

Wheeler et al., (1992) noted that fruit or seed dispersal occurs in the Clerodendrum genus through birds. Keng, (1990) reported that C. laevifolium fruits are probably dispersed by birds. Lorence & Flynn, (1997) stated that C. macrostegium is spread by fruit-eating birds. Solomon & Rajendra (2016) stated that C. inerme fruits are dispersed by birds such as Acridotherestristis, Corvussplendens, Corvusmacrorhynchosand Turdoidescaudatus.

During our study, there was no observation recorded with the dispersion of fruit and seed by birds, and there is no previous study found on the dispersal of fruit and seed for these three Clerodendrum species. In all three Clerodendrum species, the fruit is a drupe with a fleshy mericarp. After ripening, the fruit's color turns purple, and the colour of the calyx, which is green during the flowering period, turns red during the ripening phase for C.colebrookianum and C. infortunatum.

5.3. Effect of growth regulators on in vitro pollen germination with pollen longevity tests.

TTC test was found to be a dependable test for assessing pollen viability in different flowering stages, such as pre-anthesis and anthesis, to distinguish between alive and dead pollen in C. colebrookianum, C. infortunatum and C. serratum. A colour distinction in viable pollen stained with red while non-viable pollen with no stain was observed in all three Clerodendrum species (Figs.). The colour differentiation between alive and dead pollen using TTC was observed for Jatropha curcas (Abdelgadir et al., 2012), Prunus armeniaca (Yaman &Turan, 2021), Bursera hybrids (Rico & Reyes, 2019), and Fraxinus excelsior (Buchner et al., 2022). Yang et al. (2021) found that the TTC test for pollen viability is reliable for Amomum villosum and Amomum longiligulare. The finding of a high viable percentage after anthesis in all three Clerodendrum species (Table) is similar to the findings observed for Jatropha curcas (Abdelgadir et al., 2012), Passifloracincinnata, Passiflora edulis, Passifloraedmundoi, Passifloragalbana, Passifloragibertii, and Passiflorasuberosa (Soares et al., 2013). Shekari et al. (2016b) observed low viable and low germination percentages of pollen for Leonurus cardiaca before anthesis. Pollen viability is often correlated with pollen quality to be used in artificial pollination and breeding (Dafni & Firmage, 2000). Selection of appropriate anthesis stage for notably viable pollen grains is paramount in pollination, fertilization, and breeding of Passiflora sp. (Soares et al., 2013); improved cultivars of banana (Soares et al., 2015) and Leonurus cardiaca (Shekariet al., 2016b).

Pollen germination under in vitro experiments helps to recreate the in vivo environment of pollen tube germination on the pistil. Sucrose helps to increase pollen germination and tube growth, thereby providing nutrients to the culture media (Lin et al., 2017). Appropriate sucrose concentration is a source of nutrition, osmotic balance, and vital carbon energy to induce pollen germination (Dong & Beckles, 2019). A high sucrose concentration may inhibit pollen grain germination (Lin et al., 2017). Within the same culture and media variations, pollen germination might occur due to unbalanced osmotic pressure (Youmbi et al., 2015). Significant variation in pollen germination and tube growth was seen between the sucrose concentrations in all three Clerodendrum species. An increase in sucrose concentration from 5 % to 10 % enhanced the pollen germination in all three Clerodendrum species (Fig.). A similar result in Cunnighamialanceolata (Fragallah et al., 2019), Psidium guajava (Sarkar et al., 2018), Impatiens balsamina (Patel & Mankad, 2014), and Leonurus cardiaca (Shekari et al.,2016 a) were recorded for the effect of sucrose concentration on the percentage of pollen germination. A low sucrose concentration gives low pollen germination, while above <10% sucrose concentration gives high pollen germination in Momordica subangulata (Naik et al., 2016). The pollen germination rates varied significantly between incubation times and between treatments. Pollen germination rates were higher at 24 hours and less at 48 hours (Table). The results revealed that the pollen grains of all three species of Clerodendrum need just 24 hours to start growing for germination (Fig.); a similar effect was observed for Prunus laurocerasus (Sulusoglu & Cavusoglu, 2014); Cunnighamia lanceolata (Fragallah et al., 2019) and Spathodea campanulata, Bauhinia purperea and B. racemosa (Sanjay et al., 2016). The rate of pollen germination determines the effectiveness of pollen germination media.

All four-growth hormones influenced pollen germination, but their rate differed (Table and Figs.). Different concentrations of hormones give different germination percentages (Table). An increase in the concentration of GA3 enhanced the pollen germination in C. colebrookianum and C. infortunatum; thus, a high concentration of GA3 plays a vital function in pollen germination. A similar result was observed for Accasellowiana (Xiong et al., 2016), Prunus dulcis (Maita and Sotomayor, 2015), and functional male flower of pomegranate (Engin & Gokbayrak, 2016). However, with the higher concentration of (GA3), i.e., at 200 mg L-1 and 300 mg L-1 the germination percentage decreased but with the decrease in the concentration of (GA3) at 100 mg L-1, the germination percentage shown increased for the plant species C. serratum (Table). A similar result has been observed on (GA3) for strawberry pollen (Voyiatzis & Paraskevopoulou-Paroussi, 2002) and Pistacia vera (Acar et al., 2010) plant species. In some species, the application of (GA3) and the concentration used can promote, inhibit or show no effect on pollen germination and pollen tube growth in-vitro or in-vivo (Acar et al., 2010). GA3 promotes amylase activity, acid phosphatase, and β-glucosidase. It enhances the leaching of amylase and acid phosphatase enzymes to stimulate pollen germination (Sanjay et al., 2016). GA3 significantly promoted in vitro pollen germination of Vitis vinifera, Spathodeacampanualata, and Momordica charantia (Gokbayrak & Engin, 2015; Sanjay et al., 2016). Gibberellins (GA3) are endogenous plant growth regulator hormone which involved in many aspects of plant development for many species; the application of (GA3) help in the development of pollen tube growth in-vivo or in-vitro (Singh et al., 2002).

Indoles-3-acetic acid (IAA) plays an important role in plant sexual reproduction by controlling the development of stamens and ovaries by promoting egg cell maturation and inducing embryonic axial polarity and polar development (Abdelgadir et al., 2012). IAA promotes pollen germination and tube growth of Nicotiana tabacum L. in-vitro directly or indirectly (Chen & Zhao, 2008). IAA promotes pollen tube growth in pistils (Chen & Zhao, 2008). The study shows that IAA promotes pollen germination and tube growth for C. serratum species (Table). At 100 mg L-1 and 200 mg L1, IAA pollen germination percentage and tube growth increase significantly compared with IAA at 300 mg L1. The higher concentration of IAA at 300 mg L-1 inhibits pollen germination and tube growth. In addition, it can be said that the application of IAA at an appropriate concentration can improve the rate of pollen germination and tube growth, but with a higher concentration of IAA, it can hinder pollen germination and tube growth.

Kinetin regulates pollen germination and tube growth at different concentrations (Manonmani & Mekala, 2016 and Marchioretto et al., 2019). A high concentration of Kinetin reduced pollen germination (Table) in C. colebrookianum and C. infortunatumspecies, while a lower concentration of Kinetin was suitable for pollen germination and tube growth. Dziurka et al. (2019) and Usman et al. (2022) reported that low content of Kinetin in the plant improves regeneration, thereby increasing the efficiency of doubled haploid production; this supports our finding. Kinetin is reported to influence the germination of pollen and tube growth of Prunus dulcis (Maita & Sotomayor, 2015). It was observed that Kinetin promotes pollen germination and tube growth for C. serratum at different hormone concentrations, i.e., at 100 mg L-1,200 mg L-1 and 300 mg L-1 (Table). Kinetin observed the least effect on pollen germination and tube growth for C. serratum (Table). A study by (Sotomayor et al., 2012) has obtained a similar observation for Prunus dulcis species; they also obtained the least effect of Kinetin on pollen germination.

In C. colebrookianum, IBA and IAA at higher concentrations reduced pollen germination, but in C. infortunatum, IBA and IAA increased pollen germination at higher concentrations (Table). Thus, suitable concentrations of IAA and IBA are needed for C. colebrookianum and C. infortunatum species (Table). IBA promoted in vitro pollen germination in four Hibiscus species (Li et al., 2015); B. purpurea and B. racemose (Sanjay et al., 2016); Litchi chinensis (Zeng et al., 2018); and Actinidiadeliciosapollen (Marques, 2018). Abdelgadir et al. (2012) reported that a suitable IAA concentration is needed for proper pollen germination and tube growth; this supports our finding, too for C. colebrookianum and C. infortunatum. In addition, Kovaleva et al., (2005) reported that low concentrations of IAA promoted pollen germination while higher concentrations inhibited it. IAA stimulated pollen germination in vitro of male Petunia hybrida through osmoregulation by activating K+ channels (Kovaleva et al., 2016).

IBA was found to regulate pollen germination and pollen tube elongation for the plant species C. serratum (Table). IBA activates the catalytic activity of peroxidase, an enzyme essential for pollen germination and pollen tube growth (Qiu et al., 2021). IBA shows less pollen germination and tube growth at concentration 100 mg L-1 and 300 mg L-1, and at concentration 200 mg L-1 IBA shows good pollen germination and tube growth for C. serratum. From the result, it can be said that a decrease or increase in the concentration of hormone also had an adverse effect on pollen germination and tube growth; appropriate concentration is required for pollen germination and tube growth. IBA promotes pollen germination and tube growth on Litchchinensis (Zeng et al., 2018), Salix lapponum (Pogorzelec et al., 2015), Bauhinia purperia and Bauhinia (Sanjay et al., 2016), Torreyagrandis (Aihua et al., 2001) and also for other different plant species.

IAA, GA3, IBA, and Kinetin influenced and regulated in vitro pollen germination for both Clerodendrum species. But their response in three selected Clerodendrum species differed with growth hormones and their concentrations, suggesting that the pollen grain of the three Clerodendrum species react differentially with different growth hormones.

Long-term pollen storage is essential for plant breeding, especially in asynchronous flowering species and germplasm exchange. The longevity of pollen differs from plant species to species and from minutes to months. Thus, there is a practical need to evaluate and standardize storage conditions of pollen grains to maintain their vitality for an extended period for making crosses between two varieties/ species which flower at different times. From the result of pollen storage, pollen grains of three Clerodendrum species which were stored (Fig.) at various temperatures (-20°C, -4°C and 6°C) for varying lengths of time (0-28days) revealed that the pollen viability decreased after storage at different temperatures with an increase in the interval of time. This would be due to the fact that the metabolic activity of pollen depends on temperature and time (Du et al., 2019). Among the stored temperatures (-20°C, -4°C and 6°C), viability percentage of pollen varies significantly in all three Clerodendrum species. Temperature and humidity influence the viability of pollen (Sidhu, 2019 & Du et al., 2019). Therefore, an appropriate temperature is needed to be screened through more experimentation to extend the longevity of pollen viability up to 70 days so that species hybridization could be done between C. colebrookianum(as flowering occurred between July to December) and C. infortunatum (flowering occurred between February to April); hybridization between C. infortunatum and C. serratum (flowering occurred between March to July) and hybridization between C. serratum and C. colebrookianum to develop new species with novel importance.

Pollen grains of Fraxinus excelsior lose their viability in warmer conditions, and they can be stored for a longer duration at temperatures of -20°C and -80°C than at 4°C; the viability of stored pollen grains could be used to overcome crucial problem concerning ash dieback disease through a future breeding program (Buchner et al., 2022). Pollen grains of Juniperus communis (a tree with meager seed sets due to pollen limitation under natural conditions) can be stored suitably with significant pollen viability at – 20 °C as compared to -4°C. The stored pollens were valuable for pollen supplementation experiments to enhance seed sets in the tree species (Kormutak et al., 2021). Hence pollen storage is crucial in pollen handling that can act as a valuable tool for breeders to overcome problems associated with differences in flowering time, pollen shedding, stigma receptivity, and pollen limitation in controlled pollination experiments. Further, the studies of long and short-term pollen viability and storage could help to make crosses among individuals of sub-populations growing geographically separated and adapted to biotic and abiotic gradients in racial hybridization to improve traits of interest.

Chapter: 6

Conclusions:

Floral features such as anthesis, anther dehiscence & stigma receptivity are associated and liable with the variation in climatic factors such as temperature and humidity in all three-study species of Clerodendrum. A distinct herkogamy i.e. physical separation of stamens and stigma, and dichogamy i.e. temporal separation in maturation of male (anther dehiscence) and female (stigma receptivity) reproductive parts and mainly protandry i.e. maturation of stamens well before stigma receptivity was observed all three species of Clerodendrum. Variation in anthesis, anther dehiscence and stigma receptivity among inflorescences of plants and spatial (herkogamy) and temporal (dichogamy) variation in male and female reproductive parts of flowers in all three species of Clerodendrum are important adaptations in floral biology features for promoting xenogamy and geitonogamy.
Distinct floral adaptions were observed in C. colebrookianum, C. infortunatum and C. serratum. White color flowers with floral tube length (2.7 cm) with deep seated minute quantity rich nectar is well adapted to hummingbird hawk-moths, moths and butterflies compared to bees due to their synchrony with nectar harvesting organ proboscis and its length in C. colebrookianum. Proboscis is almost similar to C. colebrookianum floral tube length i.e. 2.8 cm in hummingbird hawk-moths. White colour flower with distinct light purple coloured nectar guides and floral structures adapted for bees, and as well as floral tube length (1.9 cm) with deep seated minute nectar is well synchronous and adapted to hawk moth, moths and butterflies, hence bees, moths and butterflies seems are important in pollination. Light purple flower with distinct pubscent nectar guides, landing platform, shallow nectar in form of minute droplets in is well adapted to carpenter and bumble bees compared to butterflies due to their synchrony with flower in C. serratum. Diversity i.e. number species (species richness) of butterflies and moths floral visitors are high compared bees in C. colebrookianum. Butterflies and moths are ascertained important pollinators based their visitatation and synchrony with floral adaptation in facilitating pollen transfer in C. colebrookianum. Again, in C. infortunatum species richness of butterflies and moths are high compared to bees. However, both Lepidoptera (butterflies and moths) and Hymenoptera (Carpenter bees) were ascertained as important pollinators on basis visitation frequency and pollinators synchrony with floral features of C. infortunatum. But in C. serratum both bees and butterflies are found to be equally rich in species recorded. However, based floral adaptation bumble bees and carpenter bees were found to be most pollinators due synchrony with floral adaptations, foraging behaviour and visitation frequency. Floral visitors followed a diurnal pattern in all three species of Clerodendrum. Increase in temperature during day time enhanced pollinator activity on flower. Temperature positively correlated with insect floral visitors while humidity is negatively correlated with pollinator's visits in all three species of Clerodendrum. Concentration pollens in air nearby to flower and deposition of pollen grains on stigma are highly correlated and followed diurnal pattern. Maximum concentration in air and deposition on stigma was found between 0900 to 1400 hours coincided high visitation frequency of pollinators compared early morning and evening hours. Thus, the rate and quantity of pollinator's visitation in the study plant species is linked with deposition of pollen on stigma. Lepidoptera and large solitary bees are most vulnerable to climate change compare to social bees due to long-term fluctuations in resource availability, because they do not store food or switch diets henceforth C. colebrookianum, C. infortunatum and C. serratum could be a valuable plant species for butterflies, moth and bees conservation. Based index of self incompatibility and out crossing index mating system all three plant species of Clerodendrum was found to partially self compatible.
Pollen production revealed that each species has significant differential production capacity. There is significant difference for flower and pollen production among populations but insignificant inter annual variability was recorded. The production of flower and pollen was recorded high at lower elevation than higher elevation. Pollen production was significantly positively correlated with fruit settings in different years.
TTC staining test is suitable to determine the viability of pollen grains for C. serratum. Anthesis stage pollen grains will be valuable in future breeding and hybridization experiments for the plant species due to their high viability. Moisture and sucrose were found to be initiating factors for pollen grain developments. Pollen grains germination under in vitro conditions exhibited a differential response to different growth hormones and their concentrations and time with respect to Clerodendrum species. GA3 (200 mg L-1) was found to be the most suitable growth hormone concentration, followed by IBA (200 mg L-1 and 100 mg L-1) for inducing in vitro pollen germination in C. colebrookianum and C. infortunatum during the first 24 hours of incubation. While in case of C. serratum IAA was found most suitable growth hormone in inducing in vitro pollen germination, followed by IBA, GA3, and Kinetin in the first 24 hours of incubation. Among the treatments, different hormones and times gave a higher significant response (p>0.0001), followed by the hormone concentrations application (p>0.05). There was a non-significant difference between the plant species for in vitro pollen germination. Pollen grains of both Clerodendrum species remained viable up to 28 days at -20°C. Thus, pollen grains of both Clerodendrum species should be collected at anthesis stage for short-term storage of pollen grains which shall be valuable for future application in pollination, supplementation, hybridization, and breeding experiments in both Clerodendrum species.

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the size of the pollen grains and the length of the

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pollen vigour during storage, genetic and pollen-stigma interaction studies, crop improvement and breeding, incompatibility and fertility studies. Short-term storage of pollen

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5/11	SUBMITTED TEXT	25 WORDS	66%	MATCHING TEXT	25 WORDS

Flowering plants are hermaphrodites; the amount of seeds fertilized by self and outcross pollen varies greatly among species, from predominate self-fertilization to exclusively outcrossing (

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6/11	SUBMITTED TEXT	41 WORDS	62%	MATCHING TEXT	41 WORDS

of pollen biology. Such as monitoring pollen vigour during storage, genetics and pollen-stigma interaction, crop improvement and breeding programmes, maintaining gene banks, incompatibility and fertility studies, assessing pollen germinability after exposure to specific conditions, and assessing dispersal and gene flow

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7/11	SUBMITTED TEXT	28 WORDS	75%	MATCHING TEXT	28 WORDS

High humidity (<95% RH) and temperature (38°C) or storage stress of Nicotiana tabacum, Agave sp., Tradescantia virginiana and Iris sp. affected pollen vigour before affecting pollen viability (

high humidity (<95% RH) and temperature (38 °C) or storage stress of Nicotiana tabacum, Agave sp., Tradescantia virginiana, and Iris sp. Both high RH and temperature, as well as storage stresses, affected pollen vigor before affecting pollen viability.

W https://www.researchgate.net/publication/7598630_The_Effect_of_Temperature_on_Pollen_Germination_ ...

8/11	SUBMITTED TEXT	17 WORDS	68%	MATCHING TEXT	17 WORDS

The average number of pollen per anther multiplied by the average number of stamens per flower,

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9/11	SUBMITTED TEXT	24 WORDS	75%	MATCHING TEXT	24 WORDS

The pollen-ovule ratio was calculated by dividing the expected amount of pollen grains per flower by the projected number of ovules per flower

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10/11	SUBMITTED TEXT	20 WORDS	57%	MATCHING TEXT	20 WORDS

the length of the pollen tube was more than or equal to the diameter of the pollen grain, the

the aperture of the pollen wall by reaching less than or equal to the diameter of the pollen. The

W https://www.researchgate.net/publication/322390312_Pollen_Germination_and_Pollen_Tube_Elongation_ ...

11/11	SUBMITTED TEXT	17 WORDS	57%	MATCHING TEXT	17 WORDS

Based on the ratio of fruit set in open-pollinated to cross-pollinated, the Index of self-incompatibility (ISI)

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POLLINATION BIOLOGY OF THREE *CLERODENDRUM* SPECIES IN A TROPICAL FOREST OF MIZORAM

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DEPARTMENT OF FORESTRY

SCHOOL OF EARTH SCIENCES AND NATURAL RESOURCE MANAGEMENT

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ABSTRACT

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SUBMITTED

IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN FORESTRY OF MIZORAM UNIVERSITY, AIZAWL.

Abstract:

North East region of India is rich in biodiversity and ethnicity. The diversified landscape of North East India is a vast reservoir of medicinal plants used by local people in their primary health care management. But those medicinal plants, which are relatively common in their natural habitat in the region, are now quickly diminishing due to forest fragmentation, forest fire, jhum cultivation, logging, and landslide, thus threatening the valuable plant biodiversity of the forest area. Climate change, urban development, industrialization, pollution, destructive harvesting practices, and indiscriminate use have decreased the wild population of medicinal plants.

Understanding the pollination biology of wild and domesticated plant genetic resources is essential for the management of the rapidly diminishing tropical biodiversity and increasing plant species diversity, evenness and productivity. Medicinal plants have received much less attention in reproductive and genetic studies and species improvement programs than agricultural and forestry crops. There is an urgent need to develop a database that is essential for the conservation and management of medicinal plant resources since there is a severe lack of baseline data on pollination biology for tropical, medicinal plant species, especially from North East India.

Floral biology enables us to understand the various phenophases of a species as well as the processes that take place from the creation of gametes to the germination of seeds as well as the limitations on the natural reproduction of the plant species. Floral and pollination biology of vulnerable medicinal plant species will offer useful

knowledge and information, such as the timing of flowering, fruiting, and plant-pollinator interactions. This will help in the hybridization, cultivation, sustainable use, and conservation of that particular plant species in their habitat. Scientific studies on floral features and pollinators responsible for the mating system will help to formulate conservation measures for rare, vulnerable, and endangered plants of ecological and economic importance.

The germination of pollen and pollen tube growth is essential for fertilization and seed development. The biotic and abiotic factors help in the success of sexual reproduction, which plays an important part in the successful mating and fruit set. The study on pollen grains production is essential because it determines the pollen availability in and across the populations by gene flow which impacts mating success. The production of pollen is influenced by many biotic and abiotic factors. Pollen production of a particular species will help to aero biologists to predict the pollen season and amount of pollen emission to the air, Plant and tree breeders to make timely crosses, foresters and horticulturists to improve the quality of the wood through provenance testing and mating design, trait of interest and ecologists to study natural selection and evolution of plant species.

Pollen viability, germination, and storage are essential parts of the reproductive biology and breeding of a plant species because effective sexual plant reproduction depends on viable and fertile pollen. To understand sterility issues, hybridization programs, fruiting, breeding programs, evolutionary ecology, pollen viability, artificial pollination, and inbreeding experiments are crucial. During reproduction process plant face many environmental factors. *in vitro* pollen germination provides

a wealth of information about the physiology and biochemistry of pollen germination and pollen tube growth for their present and future applications.

The current study was done to discern detailed information about the anthesis, anther dehiscence, female receptivity, pollen production interactions, pollen production, *in vitro* pollen germination, and pollen storage of three important *Clerodendrum* species in the tropical forest of Mizoram.

Objectives:

The three-plant species selected for the study are as follow *Clerodendrum colebrookianum* Walp., *Clerodendrum infortunatum* L. and *Cleroldendrum serratum* (L.) Moon.

The study was designed to achieve the following major objectives:

1. Anthesis, anther dehiscence, female receptivity and pollen-pollinator interaction in relation to the time of the day and associated weather conditions.
2. Assessment of pollen production, pollen/ovule ratio, and its impact on reproductive success.
3. Effect of growth regulators on *in vitro* pollen germination with pollen longevity tests.

Methods

Floral Development, anthesis pattern, and duration of flowering: Floral development, anthesis, anther dehiscence, and stigma receptivity were observed during the flowering season with temperature and humidity, from 0500-1700 hours of the day with the time interval of two hours.

Pollen Production: Total pollen production per was estimated as per the protocol of Molina et al. (1996). Total pollen grains/plant = Number of inflorescences/plants*average number of flowers/inflorescences*average number of anthers/flowers*average number of pollen grains/anther.

Diurnal rhythms of pollen concentrations: Concentration of pollen in the air and stigma was recorded during the flowering season from 0500-1700 of the day with two hours intervals.

Pollen Viability: the viability of pollen was tested on freshly opened flowers and unopened flowers (just before anthesis) with 0.5 percent 2, 3, 5-triphenyl tetrazolium chloride (TTC).

Assessment of pollen longevity: three different temperatures 6°C, - 4°C and -20°C were used to store pollen; viability was examined every week, at intervals of 14, 21, and 28 days using 0.5 percent 2, 3, 5-triphenyl tetrazolium chloride (TTC).

Pollen Germination: *In vitro* pollen germination was conducted under different hormones (IBA, IAA, GA3, and Kinetin) with concentrations of 100 mg L-1, 200 mg L-1, and 300 mg L-1 with the time interval of 24, 48, and 72 hours, Brewbaker & Kwack (1963).

Stigma receptivity, mating system, and fruit set: stigma receptivity was checked using hydrogen peroxide from 0500-1700 of the day with 2 hours time intervals. The mating system and fruit set were conducted as per (Modified from Dafni, 1992 and Boulter et al., 2006).

Pollinators availability: The types and behaviors of pollinators were assessed by: counting the total number of visits by insects during day time (05:00-18:00) per field visit/flower in the peak flowering season; foraging rate (number of flowers visited/minutes); and average time spent per flower. The frequency of pollinator visits was also determined in terms of visits/flower/hour, and visits/inflorescence/hour.

Results:

During the study period, it was observed that the time of the flowering phenological phase showed annual variability from one year to the following year. The flowering of *C. colebrookianum* was observed from June-December, *C. infortunatum* February-April, and *C. serratum* March-July.

All three *Clerodendrum* exhibit a distinct dichogamy(protandry) and herkogamy (spatial separation between stamen and pistil). The opening of the flower, i.e., anthesis, started in the morning at 0500 h, and between 0700 and 0900 of the day, the anthesis of the flower reached its maximum level for all three *Clerodendrum* species. A distinct physical separation between the stamen and pistil was observed during anthesis. Anther dehiscence started in the morning hours and coincided with anthesis, and peak pollen release was observed during 0700-0900 hours and continued till 1300-1500 for all three *Clerodendrum* species. The receptivity of the stigma was

recorded after 3-4 days from anthesis in *C. colebrookianum*, and receptivity was observed on the next day for *C. infortunatum* and *C. serratum*.

Twenty-six insect species of the order Lepidoptera, 4 species of the order Hymenoptera and 1 species each from the order Coleoptera and Hemiptera are observed as floral visitor of *C. colebrookianum*. 16 species of the order Lepidoptera and 3 species of the order Hymenoptera in *C. infortunatum*. Five species of the order Lepidoptera and five species of the order Hymenoptera have been identified as floral visitors in *C. serratum*. Maximum visitation was observed between 0700-1700 of the day. A postive correlation with temperature and a negative correlation was observed with humidity among the pollinators.

Clerodendrum colebrookianum and *Clerodendrum infortunatum* maximum concentration of pollen in the air was observed during 1100-1300 hours of the day, while in *Clerodendrum serratum* it was observed during 0900-1100 hours of the day. Pollen on stigma and pollen in air showed a positive correlation with temperature, and a negative correlation was observed with humidity.

Pollen grain production per plant was highly varied and highest in *C. infortunatum* (10.44-82.09 × 105), followed by *C. clerodendrum* (21.54-54.33 × 105) and *C. serratum*(45.15-53.81 × 105). The maximum percentage of fruit set was recorded in C. *clerodendrum* (65.30%) at Tanhril (mid-altitude), followed by *C. serratum*(56.23%) at Hlimen (high altitude) and *C. infortunatum* (42.15%) at Tanhril (mid-altitude). The evaluation of the mating system of all three-plant species of *Clerodendrum* revealed that the species are capable of being partially self-compatible.

Pollen viability percent is higher at the anthesis stage (opened flower) compared to pre-anthesis (unopened flower) in all three species of *Clerodendrum*. Sucrose concentrations (5% and 10%) were found to induce *in vitro* pollen germination and acted as a fundamental substrate compared to the control (distilled water). The highest *in vitro* pollen germination percentage was observed in the first 24 hours in all the observed hormones IBA, IAA, GA3, and Kinetin. GA3 (200 mg L-1) with (52.10±5.30% and 61.91±1.76%) was shown to be the most effective growth hormone concentration for *in vitro* pollen germination in *C. colebrookianum* and *C. infortunatum* while in *C. serratum* IAA (100 mg L-1) with (55.81±4.97%) show most effective *in vitro* pollen germination. Statistically, the response of all the concentrations of treatments, sucrose, and hormones, with their time on *in vitro* pollen germination, all three *Clerodendrum* species, was found to be significantly different ($p<0.05$). In contrast, non-significant differences were recorded for *in vitro* pollen germination between the selected medicinal plant species. Pollen storage under temperature gradient conditions exhibited a similar trend in the viability for all three *Clerodendrum* species, the pollen remained viable for up to 28 days at -20°C and 6°C, respectively.

Conclusion:

Distinct floral adaptions were observed in *C. colebrookianum*, *C. infortunatum* and *C. serratum*. Anthesis, anther dehiscence & stigma receptivity of the flower are associated and liable with the variation in climatic factors such as temperature and humidity in all three-study species of *Clerodendrum*. A distinct herkogamy, i.e. physical separation of stamens and stigma, and dichogamy i.e.

temporal separation in maturation of male (anther dehiscence) and female (stigma receptivity) reproductive parts and mainly protandry i.e. maturation of stamens well before stigma receptivity was observed all three species of *Clerodendrum*. Floral tube length (2.7 cm) with deep-seated minute quantity rich nectar is well adapted to hummingbird hawk-moths, moths, and butterflies in *C. colebrookianum*. Light pink colored nectar guides and floral structures are adapted for bees, and a floral tube length of (1.9 cm) with deep-seated minute nectar is well synchronous and adapted to hawk moth, moths, and butterflies in *C. infortunatum*. Light purple flower with distinct pubescent nectar guides, landing platform, and shallow nectar in the form of minute droplets is well adapted to a carpenter and bumble bees in *C. serratum*.

Pollen production revealed that each species has a significant differential production capacity. There is a significant difference in flower and pollen production among populations, but insignificant inter annual variability was recorded. The production of flowers and pollen was recorded as high at a lower elevation than higher elevation.

TTC staining test is suitable to determine the viability of pollen grains for all three *Clerodendrum* species. Anthesis stage pollen grains would be valuable in future breeding and hybridization experiments for the plant species due to their high viability. IBA, IAA, GA3, and Kinetin induced *in vitro* pollen germination in all three *Clerodendrum* species. This study on pollen storage will be helpful in future breeding, hybridization, and conservation efforts for all three *Clerodendrum* species.