EMBRYOLOGICAL STUDIES ON *GAULTHERIA FRAGRANTISSIMA* WALL., AN IMPORTANT MEDICINAL PLANT

(ABSTRACT)

BY

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THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

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ABSTRACT

The genus *Gaultheria* comprises about 130 species are native to a wide range of geographical areas ranging from Andes, North America, Australia and nearby islands to eastern Asia and Himalaya (Airy-Shaw 1941; Middleton 1991a; 1991 b; Mabberley 2008). The genus *Gaultheria* is named by Kalm after Dr. Gaulthier, a physician in Quebec and the species epithet *fragrantissima* is described by Wallich (1820). *G. fragrantissima* Wall. is medicinally and economically important plant because of the presence of an essential oil in its leaves. Oil extracted from the leaves of *G. fragrantissima* Wall. is similar in its physical and chemical properties to the oil of Wintergreen obtained from *Gaultheria procumbens* Linn. and *Betula lenta* Linn, both natives of North America and thus it is commonly known as Indian Wintergreen Oil (Anonymous 1956). The oil contains methyl salicylate as the chief constituent which is used for rheumatic arthritis, sciatica, neuralgia and in most of the proprietary balms as liniments or ointments (Chopra 1932).

Gaultheria fragrantissima Wall. (Indian wintergreen oil) belongs to family Ericaceae also known as Heath family, is an evergreen ericaceous shrubs found in Indo-Malaya, North-East India; common at higher elevation in Shillong about 1500m a.s.l., particularly under pine forests and open places. (Haridasan and Rao 1985; Meher - Homji 1972). Locally it is known as Jirhap, Jirhapiong, Soh-lingthrait, Dieng-lashyrhap and it has been used in indigenous medicine for a long time in the treatment of rheumatism and arthritis (Hynniewta and Kumar 2008).

The reproductive biology of flowering plants is an important aspect for determining barriers to seed and fruit set, for understanding pollination and breeding mechanisms that regulate the genetic structure of populations. India's progress in improving traditional crops is a matter of pride for us. However, little attention seems to have been given to medicinal plants, which occupy a unique place in Indian socio-economic conditions (Raina *et al.* 1998; 2011; Bernardello *et al.* 1999).

Despite the medicinal, aromatic, ecological important and the unusual aspects of its sexual dimorphism of *G. fragrantissima,* not much information is available on floral biology, developmental studies of microsporogenesis, megasporogenesis, breeding system, pollination mechanism and seed germination in *G. fragrantissima*. A perusal of literature shows that the embryology has not been investigated in *G. fragrantissima* so far. Therefore, the present study aims to investigate the embryology of *G. fragrantissima* an economically important medicinal plants growing in Meghalaya.

The objectives of the proposed investigation are to study the following aspects in *G. fragrantissima* Wall.

- Floral biology – morphometric details of flower and inflorescence, sex ratio in both hermaphrodite and male sterile plants.
- To study the microsporogènesis, megasporogenesis and formation of embryo sac in both hermaphrodite and male sterile plants.
- Pollen viability and pollen germination of hermaphrodite plants.
- Pollination mechanism, pollinators and rewards to the pollinators.

- Post-fertilization changes in the embryo sac.
- Seed biology – Seed morphology, seed germination and viability

The thesis comprises nine chapters. The first three chapters are pertaining to the introduction, review of literature and materials and methods respectively.

The fourth chapter deals with the study of floral biology of hermaphrodite and male sterile plants of *G. fragrantissima* Wall. This chapter reveals the morphological difference of reproductive structure between hermaphrodite and male sterile plant in two populations viz. Nongkrem and Lum Shyllong population. Statistical analysis showed several floral traits like length of pedicel, length of bract, length and breadth of corolla and length of style revealed significant difference between hermaphrodite and male sterile plants in both populations. There is high variation in sex ratio and female frequency in both population studied.

The fifth chapter is concerned with microsporogenesis of hermaphrodite and male sterile plants. In hermaphrodite flower of *G. fragrantissima* the number of stamens is ten. There is normal development of anther. Anther wall development is of dicotyledonous type. Tapetal cells characterized by the presence of binucleate sometimes contains polyploidy prominent nucleus within the dense cytoplasm. The tapetum is secretory type. A special callose wall layer is secreted around each microspore mother cell, meiosis is of simultaneous type. The pollen tetrad surface is uneven and rugged, primary apocolpial exine sculpture moderate to coarsely rugulate

psilate. The apocolpial exine is composed of ektexine and endexine. Sexine is thick, endexine is very thin. The septum is thick. Intine is almost evenly thick around the pollen tetrad, but sometimes comparatively thicker near the colpus region and showed low electron density than the endexine at both apocolpial and septal exine in the hermaphrodite plant.

On the other hand male sterile flower, all the ten stamens released degenerated sporogenous tissues and formed a white unorganized mass of tissues with a tuft of hairy outgrowth at the tip therefore viable pollen are absent, awn or horns are absent. Abnormal differentiation of archesporial initials lead to the sterility of sporogenous tissue in male sterile plant. Pollen grains in male sterile plants are with irregular projection of exine and presence of thick electron dense layer below the intine which prevents the exchange of substances and formation of pollen tube. The exine wall which primarily composed of biopolymer sporopollenin dissolved and disorganized due to polymerization of sporopollenin. Topographically, the ektexine and endexine are distinct, however, tectum and baculae of sexine completely fused and lost their integrity and developed a continous layer of radially oriented membranous granular material above the intine. Intine composed primarily of cellulose and pectin.

The six chapter deals with pollination mechanism, pollen viability and pollen germination. Pollination is entomophilous where *Apis sp.* (Honey bee) are primary pollinators. The pollen tetrads range from 27.8- 30.0 µm in diameter and the pollen grain are tricellular. Pollen tetrads of hermaphrodite

flower showed 75 % of viability with the Fluorochloromatic Reaction (FCR) test. Based on modified Brewbaker and Kwacks medium, the optimum pollen germination was with the following concentration (i.e 10% sucrose, 120 ppm boric acid, 200 ppm calcium nitrate, 200 ppm potassium nitrate, 150 ppm magnesium sulphate) at pH 7.0 at 25 °C for 19 hours in dark at 90% relative humidity in BOD incubator.

The seventh chapter deals with the development of female gametophyte and post-fertilization changes in embryo sac. The ovary is globular, ovules are unitegmic, tenuinucellate and anatropous. The ovule is dizonate. The nucellus develops out of the apex of the ovular primordium as a small protuberance. The archesporial does not undergo further mitotic divisions, it enlarge, elongate and function as megaspore mother cell. The development of the embryo sac confirms to the Polygonum or Normal type Lateral view of embryo sac shows at the extreme micropylar pole the wall is strongly thickened, forming a lunar or sickle shaped structure known as "filiform apparatus". Endothelium surrounded middle portion of the megagametophyte except at the micropylar and chalazal ends. The zygote is distinctly an elongated ovoid in structure and located at the micropylar pole of the embryo sac with its basal portion attached to the embryo sac wall, while the apical portion projects into the central cell. Embryogeny follows the Solanad type. The embryo of *G. fragrantissima is* straight, cylindrical, about two-thirds the length of the seed with two inconspicuous cotyledons, and is surrounded by endosperm.

The endosperm is initially nuclear and later become cellular in the mature seeds. The micropylar haustorium has dense cytoplasm with two nuclei. The lower portion of endosperm elongates gradually and formed a sac like chalazal haustorial structure. The chalazal portion of the endosperm is smaller than the micropylar portionand extends beyond the antipodals and eventually the chalazal cell also undergoes longitudinal divisions, forming the chalazal haustorium, the chalazal haustoria of *G. fragrantissima* is composed of densely cytoplasmic cells to form finger like projection. In *G. fragrantissima* the suspensor is large, comprising of four to five cells which pushes the embryo towards the middle of endosperm.

Chapter eight deals with seed morphology and germination of *G. fragrantissima*. Seeds in hermaphrodite and male sterile plants have both obliquely pyramidal and trapezoidal seeds in the same fruits. Seeds are glossy and are either yellow or light brown in colour. Both hermaphrodite and male sterile seeds showed reticulate ornamentation of the seed coat however, in male sterile the seeds are narrowly elongated. Seed coat of *G. fragrantissima* shows a characteristic features in which the seed coat is lignified thickening wall projections which subsequently lead to the formation of pores that would facilitate exchange of water molecule and gases during seed germination. Gibberellic acid increased the seed germination and rate of germination in both hermaphrodite and male sterile plants. 200 ppm gibberellic acid treatment showed highest percentage of seed germination in both hermaphrodite (87.39 ± 2.50 %) and male sterile plants (89.23 ± 2.84

%) and significantly the duration of duration of seed germination is reduced from 19 days to 11-12 days in the gibberellic acid treatment.

In *G.fragrantissima* the percentage of seed germination is very low. Seed germination is hypogeal and seedlings of both hermaphrodite and male sterile plants when transplanted in the soil established very well but rate of seedling survival is comparatively low in hermaphrodite plants. It can be substantiated that the seed derived from hermaphrodite plants are self pollinated while the seed of male sterile plants are the product of cross pollination.

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DEDICATED

TO

MY LOVING BROTHERS

NORTH-EASTERN HILL UNIVERSITY

SHILLONG

APRIL 2014

DECLARATION

I, **Wympher Langstang**, hereby, declare that the thesis entitled **'Embryological studies on *Gaultheria fragrantissima* Wall., an important medicinal plant'** is a record of original and independent research work carried out by me in the Department of Botany, North-Eastern Hill University, Shillong, under the supervision of Prof. N. Venugopal. The work is original and no part of the thesis has been submitted for any other degree or diploma of any University.

This is being submitted to the North-Eastern Hill University, Shillong for the award of the degree of Doctor of Philosophy in Botany.

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(Wympher Langstang)

28 th April, 2014

Prof. S. K. Barik
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Department of Botany

28/4/14

Prof. N. Venugopal
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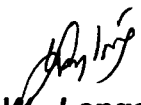


W. Langstang

CONTENTS

CHAPTER - 1

Introduction

India is one of the 17th identified mega diverse country of the world. India, with a varied terrain, topography, land use, geographic and climatic factors can be divided into ten recognizable bio-geographic zones namely, the Trans-Himalayan, the Himalayan, the Indian desert, the semi- arid zone(s), the Western Ghats, the Deccan Penninsula, the Gangetic Plain, North- East India and the islands and coasts (Rodgers *et al.* 2002). These zones encompass a variety of ecosystems: mountains, plateaus, rivers, forests, deserts, wetlands, lakes, mangroves, coral reefs, coasts and islands. India is also situated at the tri-junction of the Afro-tropical, the Indo- Malayan and the Paleo-Arctic realms, which displays significant biodiversity.

Forest Survey of India (FSI) started undertaking systematic and periodic assessment of the forest cover of the country using remote sensing technology. As per the assessment in 2005, the forest cover of the country is 67.71 million hectare, which is 20.60% of its geographic area. Of this, 5.46 million hectare (1.66%) is very dense forests, 33.26 million hectare (10.12%) is moderately dense and the rest 28.99 million hectare (8.82%) is open, including 0.44 million hectare mangroves. The percentage of forest cover in the hilly region of the country is 38.85%.

"Global change" typically conjures images of climate change, biodiversity loss, sea level rise, and the other biophysical changes that include prominently conversion and fragmentation of natural habitat (Barber

et al. 2004). The effects of changing environments on organisms are one of the foci of contemporary science (Intergovernmental Panel on Climate Change 2007) and it is essential to understand and predict them. The negative effects of climate change on biodiversity and food production have been sources of concern (Intergovernmental Panel on Climate Change 2002; Hedhly *et al.* 2009). Plant reproductive success determines the levels of resources that support both biodiversity and the food supply, therefore the effect of climate change may depend largely on the responses of plants in terms of sexual reproduction (Hedhly *et al.* 2009; Eckert *et al.* 2010).

The drivers of socio-economic change *viz.,* human population and economic growth, trade and consumption, and poverty and inequality have significantly altered the composition of the Earth's land cover, with a significant net global change from natural habitats (e.g. forests, grasslands, wetlands) to agricultural, pastoral, urban, and other human land uses (Tilman *et al.* 2001; Tscharntke *et al.* 2005). This has resulted in the inclusion of the two Indian regions namely Western Ghats and the Indo- Burma region (covering the Northeastern Himalayas) in the 25 global biodiversity hotspots (Myers *et al.* 2000). Western Ghat is also known as the Malabar rain forest province and is one of the major tropical evergreen forested regions in India which exhibit enormous plant and animal diversity and it is estimated to harbor approximately 5,500 species of flowering plants (Nair and Daniel 1986). According to Myers *et al.* (2000) they are also home to 2,180 endemic plants, constituting 0.7% of global plant species.

North East India (located between 87°32 to 97° 52 E longitude and 21° 34 to 29° 50 N latitude) form a unique transitional zone between the Indian, Indo- Malayan and Indo- Chinese biogeographical zones as well as the confluence of the Himalayan region with peninsular India (Rao 1994). Aptly called "Cradle of Angiosperm" by Takhtajan (1969) and as assets of 'Pleistocene Refugee', the North East India is one of the hotspots of the world and is a prime one among the two identified for the Indian sub-continent (Myers *et al.* 2000). The region harbours as many as 5000 to 7000 species of flowering plants, which includes several endemics and primitive angiospermic plants and wild relative of economically important plants that spans from timber to non-timber category, medicinal, aromatic to food and industrial gene pools (Takhtajan 1969; Rao 1994). The Eastern Himalayas is one among the 150 important botanical sites identified for conservation action by the World Conservation Monitoring Center (IUCN 1987).

The World Health Organization has estimated that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs (Vines 2004). In India, about 2500 species are used for medicinal purposes, and about 90% of the medicinal plants provide raw materials for the herbal pharmaceuticals, which are collected from the wild habitats (Rajasekharan and Ganeshan 2002). An estimated number of about 70,000 such medicinal plants have been reported worldwide (Prajapati *et al.* 2003). Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious

diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. A good number of medicinal plants, particularly those used in Ayurveda, about 35-45% are cross pollinated plants and therefore there exist a large number of genetic variants in population, which account for the variation in chemical composition. The ancient Ayurvedic physicians made elaborate descriptions on habitat, edaphic, climate conditions as well as the specific stage of growth and developmental stage of the plants (such as pre flowering, post flowering, dormancy period etc.) for collection of medicinal plants.

North East India with its unique floristic diversity is endowed with rich treasure of medicinal plants, the regionalize accounts for the occurrence of about 60% of medicinal plants found in whole India. Many valuable plants like *Zingiber officinale*, *Z. zerumbet*, *Rauvolfia serpentine*, *Zanthoxylum armatum* etc. are quite common and widely occurring in entire North East India. About 300 species of angiosperm plants having medicinal and aromatic properties are grown in large quantities in North East India and 400 additional species are used by the villager for curing ailments (Dhar *et al.* 2000). Meghalaya has a high magnitude of landscape heterogeneity and climatic gradient making the state a key biotic sub-province and life supporting system for a myriad of life forms. The state is endowed with rich genetic resources of horticultural, medicinal and aromatic plants. Meghalaya is well known for the existence of large variety of plant species, many of which have medicinal properties (Haridasan and Rao 1985). More than 200 forest plants are used by the people of Meghalaya for food, medicine, dye

and for ornamental and constructional purposes (Tiwari and Tynsong 2004). Ethnomedicines and medicinal plants of Meghalaya have received some attention of researchers (Rao 1981; Dolui *et al.* 2004). However, the rich biodiversity in the region is being eroded due to severe alterations in land use due to shifting cultivation, indiscriminate felling and illegal deforestation (Behera *et al.* 2001). These coupled with socio-economic changes have caused loss of natural habitats and complex assemblies of species, accelerating the process of degradation. There is a need to conserve the gene pools while utilizing them for sustainable economic development of Meghalaya in particular and North East in general.

The medicinal plants are confined in certain pockets and their population is very small in size (Pushpangadan and Nair 2005). The propagation, conservation and sustainable utilization can be done through the study of reproduction biology including the study of pollen biology, operational mechanism of sterility factors at various levels like stigma, style, ovary and fertilization and this will enhance the better seed set, multiplication and conservation (Bernardello *et al.* 1999; Moza and Bhatnagar 2007). There is a need to conserve the gene pools while utilizing them for sustainable economic development of the country (Jain and Shastri 1980; Roy and Tomar 2000).

Gaultheria fragrantissima Wall.

Gaultheria fragrantissima Wall. (Indian wintergreen oil) belongs to family Ericaceae also known as Heath family is an evergreen ericaceous shrub found in Indo-Malaya, North-East India and South India; common at

higher elevation in Shillong about 1500m a.s.l., particularly under pine forests and open places. (Haridasan and Rao 1985). It occurs only in the two biodiversity hotspots in India *viz.* Western Ghats also known as the Malabar rainforest and the Northeastern Himalayas (NE). (Meher - Homji 1972). Locally it is known as Jirhap, Jirhapiong, Soh-lingthrait, Dieng-lashyrhap and it has been used in indigenous medicine for a long time in the treatment of rheumatism and arthritis (Hynniewta and Kumar 2008).

The genus *Gaultheria* is named by Kalm after Dr. Gaulthier, a physician in Quebec and the species *fragrantissima* is described by Wallich (1820). *G. fragrantissima* Wall. is medicinally and economically important plant because of the presence of an essential oil in its leaves. Oil extracted from the leaves of *G. fragrantissima* Wall. is similar in its physical and chemical properties to the oil of Wintergreen obtained from *G. procumbens* Linn. and *Betula lenta* Linn, both natives of North America and thus it is commonly known as Indian Wintergreen Oil (Anonymous 1956). The oil contains methyl salicylate as the chief constituent which is used as a prescription for rheumatic arthritis, sciatica, neuralgia and in most of the proprietary balms as liniments or ointments (Chopra 1932). It is applied externally in the form of a liniment or an ointment in rheumatism, sciatica and neuralgia. External application may cause eruptions. The oil is also given internally and is best administered in the form of an emulsion. It has vermicidal action against hookworm. Experiments on tumor susceptible mice showed that the onset of cancer is delayed when small amounts of oil of gaultheria are administered (Anonymous 1956).

Apte (2004) reported that there is also variation in average oil yield content from two biodiversity hotspot of India. North East region gave high yield of oil in the range of 1.40 - 1.49% and high methyl salicylate content in the range of 98.2 - 99.4% as compared to the plants from Western Ghat where oil yield ranged from 0.056 - 0.086% and methyl salicylate content ranged from 89.9- 92.3%. Furthermore, within population variation in oil yield was also observed for plants from Cherapunji road and Shillong peak road populations where the yield ranged from 1.10 - 1.67% and 1.23 - 1.79%, respectively.

The reproductive biology of flowering plants is an important aspect for determining barriers to seed and fruit set, for understanding pollination and breeding mechanisms that regulate the genetic structure of populations. India's progress in improving traditional crops is a matter of pride for us. However, little attention seems to have been given to medicinal plants, which occupy a unique place in Indian socio-economic conditions (Raina *et al.* 2011; Bernardello *et al.* 1999). Thus selection of high yielding trees and genetically improved varieties for cultivation can be achieved through understanding of sexual reproduction in the species.

Despite the medicinal, aromatic, ecological important and the unusual aspects of its sexual reproductive biology of *G. fragrantissima,* no investigation on the structural or developmental studies published on microsporogenesis, megasporogenesis, breeding system and floral biology in *G. fragrantissima*. A perusal of literature shows that the embryology has not been investigated in *G. fragrantissima* so far.

Therefore, the present study aims to investigate the embryology of *G. fragrantissima* an economically important medicinal plants.

The thesis deals with the following objectives in *G. fragrantissima*:

- ❖ Floral biology.
- ❖ Development of anther, ovule and differentiation of embryo sac.
- ❖ Embryogenesis and endosperm formation.
- ❖ Pollination, pollen germination and pollen viability tests.
- ❖ Seed viability and germination.

CHAPTER - 2

Review of Literature

The order Ericales comprise about 25 families most of which have been placed in Dilleniidae, a few in Rosidae (Balsaminaceae, Roridulaceae), and one in Asteridae *sensu stricto* (s.s.) (Polemoniaceae) (Cronquist 1981; Thorne 1983; Dahlgren 1989; Takhtajan 1997). The ericalean clade was referred to as order Ericales in the classification of the Angiosperm Phylogeny Group (APG 1998). The Ericaceae, generally confined to high altitude regions in the tropics, constitute ecologically significant communities on moors, swamps and peaty soils (Willis 1973). It is the eight largest family nearly cosmopolitan distributions (Wood 1961; Stevens 1971; Wilbur and Luteyn 1978; Luteyn 1991a; Oliver 1991) of angiosperms; it comprises eight subfamilies, approximately 125 genera and 4100 species (Kron and Luteyn 2005). It is widespread in temperate, cool, subtropical regions and in tropical regions in the mountains. In tropical climates they prefer montane localities, and everywhere acid habitats are favoured. Ranging in life form from trees, epiphytes and small shrubs to herbs without chlorophyll (e.g *Monotropa uniflora*) and dependent on an association with mycorrhizal fungi for their existence, they form an important element in the vegetation in many areas of the world, except lowland tropical rain forests and deserts (Stevens 1971; Wallace 1975).

Sleumer (1966) briefly discuss various aspects of the Ericaceae and gives an account of the world distribution of *Gaultheria* (America, Southeast

Asia, Malesia, Southeast Australia, including Tasmania and New Zealand). Middleton (1991b) recognized fourteen species of *Gaultheria* in Australiana (here defined as Australia and New Zealand, with New Guinea exclude). The New Zealand species of *Gaultheria* readily hybridize, with mixed species populations often forming apparent hybrid swarms. Burtt and Hill (1935) described six putative hybrid combinations among the species with a dry capsule (treated as *Gaultheria sensu stricto*), and five putative hybrids between capsular and berry-fruited species (treated as *Pernettya*). Hybrid formation is apparently restricted to disturbed habitats, as in road cuts or braided river systems (Parsons and Hermanutz 2006) and thus the New Zealand species can still easily be discerned as distinct in ecologically stable environments.

In tropical America, there is a prominent disjunct distribution of *Gaultheria* between the Andes and southeast Brazil. Forty-three species of *Gaultheria* occur in Latin America (primarily in the Andes) and of these only eight occur in Brazil (Luteyn 1991b). There are two species of *Gaultheria* in Brazil that are also found in other areas of tropical South America. *Gaultheria erecta* is very common in the Andes, from Venezuela to northern Argentina (Luteyn 1991b). In India, the genus is represented by 21 species (Panda 2008) where it is restricted to the Himalayas (mostly in the eastern Himalaya), northeastern India and the hilltops of southwestern Ghats. Clarke's (1882) treatment of the Indian species reported six species in Northeast India viz *G. mummularioides, G. trichophylla, G. pyrolaefolia, G. fragrantissima, G. hookerii* and *G. griffithiana*. In Meghalaya, Kanjilal (1937)

reported three species of *Gaultheria* viz *G. fragrantissima*, *G. griffithiana* and *G. mummularioides*.

Studies using light and electron microscopy have provided detailed account on the morphological and ultrastructural changes that characterized embryonic development (Maheshwari 1950; Wardlaw 1955; Natesh and Rao 1984; Raghavan 1986). A survey of literature concerned with the aspects of floral biology in the order Ericales to which family Ericaceae belongs, showed that considerable works have been done, particularly on the organography and vascular anatomy (Mathews and Knox 1926; Copeland 1935; 1954; Chou 1952; Paterson 1961). Maheshwari (1950) and Palser (1961) enumerate several embryological features of Ericales: absence of fibrous layer in anther wall except in Lethraceae and Enkianthus (Ericaceae), secretory anther tapetum, compound pollen grains, pollen shed either in tetrads or singly, two celled at the shedding stage, a fluted hollow style along which the pollen tubes make their way into the ovary, ovules anatropous, unitegmic,tenuinucellar ovule with endothelium, single celled archesporium, polygonum type of embryo sac, mature embryo sac broader at the micropylar end and narrower at the chalazal end, cellular type of endosperm with micropylar and chalazal haustoria; considerable elongation of zygote, asterad type of embryogeny in Clethraceae, Caryophyllad in Pyrolaceae, Caryophyllad and Solanad in Ericaceae and Epacridaceae and Caryophyllad in Empetraceae, albumous seed with fleshy endosperm and straight embryo, and single layered seed coat formed from the outer most layer integument (Safijowska 1960; Palser 1961; Ganapathy and Palser 1964).

Stamen and pollen development is a highly conserved process in angiosperms (Hong 2005; Feng and Dickinson 2007; Dickinson and Grant-Downton 2009) with an equally highly conserved genetic control (Borg *et al.* 2009). The developmental events leading to anther formation and pollen release are exquisitely timed and choreographed (Koitunow *et al.* 1990; Scott *et al.* 2004). During the past few years, there has been an explosive burst of interest in anther biology, both as a system to dissect plant developmental processes at the molecular and genetic levels (Koltunow *et al.* 1990; Gasser 1991; Preuss *et al.* 1993) and for practical genetic engineering studies to improve crop plants (Mariani *et al.* 1990; Schmulling *et al.* 1993). One of the major mysteries of anther development is how a differentiated anther switches from a histo differentiation program to a cell degeneration and dehiscence program that leads ultimately to pollen release and stamen senescence at flower opening. The dehiscence program begins after the formation of tetrads, results in the sequential destruction of specific anther cell types, and is coordinated temporally with the pollen differentiation process. Anther development initiates with the emergence of the stamen primordia in the third whorl of the floral meristem and concludes with the release of pollen grains at dehiscence (Goldberg *et al.*1993). Within the stamen primordia cell-specification and differentiation events give rise to mature anther cell types and generate the morphology of the anther and the filament. In many flowering plants, the anther has a four-lobed structure containing a stereotyped cell-type pattern that is repeated in each lobe (Koltunow *et al.* 1990; Goldberg *et al.* 1993). Histospecification,

morphogenesis, and meiotic events constitute phase one of anther development (Goldberg *et al.* 1993). By contrast, phase two of anther development involves the functional programs that occur within differentiated anther cell types after tetrads have formed in the locules (Koltunow *et al.* 1990; Goldberg *et al.* 1993). The microspores differentiate into pollen grains, the filament elongates, the anther enlarges and expands, cell degeneration occurs, and the anther enters a dehiscence program that ends with flower opening (Goldberg *et al.* 1993).

The Ericaceae are characterized by the presence of staminal appendages which varies in position, shape and size (Mathews and Knox 1926; Stevens 1971; Stevens 1995). The stamens of the Ericaceae have a strong tendency toward terminal, poricidal dehiscence and the rotation of the anther during early stamen development occur at varying times during development in most other members of the Ericales (Matthews and Knox 1926; Ganapathy and Palser 1964; Hermann and Palser 2000). Anthers of some species of Arbuteae, Cassiopeae, and Enkiantheae invert from an initially extrorse to an introrse position at about the time of anthesis (Matthews and Knox 1926; Palser 1951). A few developmental studies of species in which the anthers always appear introrse, such as *Erica arborea, Vaccinium vitis-idaea* (Artopoeus 1903), *E. hirtiflora* (Mathews and Taylor 1926), *V. myrtillus* (Mathews and Knox 1926), and *V. angustifolium* (Bell and Burchill 1955) show that these also invert, although very early in development. In several species of *Erica* some anthers appear basifixed

(Palser and Murty 1967). Stamen dimorphism occurs in *Rhododendron ferrugineum* (Escaravage *et al.* 2001).

Ericaceous anther commonly dehisces by apical pores or clefts (Luteyn and Wilbur 1977; Luteyn 1987) and rarely by lengthwise or longitudinal split as in *Leiophyllum* and *Loiseleuria* characters shared with the families Epacridaceae and Clethraceae (Ganapathy and Palser 1964). The dehiscence of anthers has been reported as resulting from the formation of a "collapse" tissue, or a tissue variously called "resorption" (Artopoeus 1903), "disjunctive" (Mathews and Knox 1926), or "calcium oxalate package" (D'Arcy *et al.* 1996). The basic progress of microsporogenesis in Ericaceae follows the normal pattern except that, for the most part, the microspores do not separate from one another and the pollen grains are shed as tetrads. The anther wall comprises the epidermis, two or three middle layers and glandular tapetum with multinucleate cells. The resorption tissue responsible for anther dehiscence in also found in the Ericales. An exothecium develops at the extreme apical region of anther in *Kalmia, Phyllodoce* and *Rhodothamnus* (Ganapathy and Palser 1964). The tapetum is of parietal origin but it is said to differentiate from the sporogenous tissue in three species of *Vaccinium* (Batygina *et al.* 1963). An extensive endothecium, such as occurs in the anther wall of most angiosperm, is rare in the Ericaceae, occurring only in *Eikanthus*, but a local one is found in *Kalmia*, *Phyllodoce* and *Rhodothamnus.* The development of the distinct endothecium in *Enkianthus* (Safijowska 1960) is an unusual feature; the tapetal cells are binucleate in *Vaccinium* (Venkateswarlu and Maheshwari 1973). The

microspore mother cells undergo meiotic division, simultaneous cytokinesis is followed by furrowing, and the tetrads are tetrahedral, occasionally decussate.

The pollen grains are three colporate, and two celled at the dispersal stage (Safijowska 1960). They either remain united in tetrads, or are shed singly. In Vaccinioideae (Venkateswarlu and Maheshwari 1973) they always remain attached in tetrad, all the pollen grains of a tetrad do not germinate at the same time (Wallace 1975). The dimorphic pollen grains of *Enkianthus* vary in size and number of germpores, are three celled, and shed singly; occasionally they germinate in situ (Safijowska 1960). Pollen grains in *Gaultheria* are both in normal and compact tetrahedral tetrad; rarely in other configurations, often or sometimes broken along colpi in *G. erecta* and *G. prostrate* ; endocracks present and distinct, but sometimes indistinct; endoaperture lalongate; apocolpial exine 1.4 – 2.3 µm thick, septum 0.6 – 1.7 µm thick; tectate, exine sculpture from verrucate to rugulate or psilate. In SEM studies, pollen surface is uneven and rugged, primary apocolpial exine sculpture moderate to coarsely rugulate-psilate, the rugulae with minute (diam. < 0.2 µm) striate surface somewhat flat, primary exine sculpture moderate to coarsely rugulate without any secondary sculpture ; surface somewhat flat, primary exine sculpture moderate to coarsely rugulate-psilate, the rugulae with moderate (diam. > 0.2 µm) granulate to short striate or intermediate type; colpus membrane from granulate to smooth. TEM studies of *G. itatiae*, *G. insane* and *G. rigida showed* that the apocolpial exine is composed of ektexine and endexine. The septum is ca. 0.4 – 1.2 µm thick.

Intine is almost evenly thick around the pollen tetrad, but sometimes comparatively thicker near the colpus region (Sarwar 2007). The tetrads of the largest pollen grains in *Gaultheria shallon* are ca. 51 to 53 µm in diameter, and the smallest ones in G. *hispidula* and *G. oppositifolia* are less than 23 µm (Lu *et al.* 2009).

Studies of pollen morphology in *Gaultheria* and its related genera in relation to its taxonomic and evolutionary significance have attracted the attention of scientific community (Erdtman 1952; McGlone 1978; Hesse 1979; Sarwar and Takahashi 2006; Lu *et al.* 2009; Sarwar *et al.* 2009). Many of the palynological characters eg dispersal units and presence or absence of viscin threads have also been used in different classification schemes of the Ericaceae from long time (Wodehouse 1935; Erdtman 1952; Kron *et al.* 2002). With the advances in microscopic techniques, both in transmission (TEM) and scanning electron microscopies (SEM), new dimensions have been added to palynological research in solving many taxonomic problems (Cole and Behnke 1975). SEM studies of apocolpial exine sculpture have proved an important source for taxonomic relationship among and or within the families (Lens *et al.* 2005) as well as identification purpose of Ericaceous pollen (Foss and Doyle 1988).

An unusual feature of anther, usually associated with several genera of Andromedeae (Mathews and Knox 1926; Chou 1952; Palser 1951; Dorr 1981) is the occurrence of two pouches of cells containing small crystals (granules or disintegrating tissue) on the abaxial side of the anther, which eventually breaks down completely. The presence of various protrusions

called as appendages is another unusual and characteristic feature of the Ericaceae; however there are some ericaceous taxa which also lack these staminal appendages (Mathews and Knox1926; Stevens 1971). Those appendages called 'awns', which are elongations of thecae, occur at the apparent apex, two per anther and can be hollow(in which they are called tubules) or solid , single or bifurcated, or apparently fused to become one per anther 'Spurs'. In addition to spurs and awns, in a few scattered species there are other projections or overgrowth of the base of the thecae as in *Vaccinium reticulatum* (Palser 1961).

Viscin thread occurs only in few distantly related angiosperm families: Onagraceae, Ericaceae, and Caesalpinaceae (Skvarla *et al.* 1978; Graham *et al.* 1980; Sarwar *et al.* 2005). In Ericaceae, viscin threads are present on the pollen grains of tribes Bejarieae, Phyllodoceae, and Rhodoreae of subfam. Ericoideae and the genus *Gaylussacia* of the tribe Vaccinieae, Sub-fam. Vaccinioideae (Waha 1984; Kron *et al.* 2002). Lu *et al.* (2009) recently reported that viscin threads are also present in tetrahedral of *G. fragrantissima*, *G. lancifolia* and *G. pyrolifolia* from the tribe Gaultherieae. The origin of the viscin threads was rather obscure until now. Evidently the threads are neither sticky nor viscous like the pollenkitt, they contain sporopollenin, and they are attached to the exine (Skvarla *et al.* 1978; Hesse 1980). The size of the threads and their number are very variable. In Onagraceae they are long, numerous, thin and sculptured, while in Ericaceae they are also long, numerous, thin, but smooth; in Caesalpiniaceae there are short, thick and smooth threads (Hesse 1981). The sculpture of the threads

varies widely. Sometimes the threads are smooth throughout, as in most Ericaceae and Caesalpiniaceae, while in Onagraceae there are knobs, furrows etc. The three dimensional arrangement of the threads is very similar in Onagraceae and Ericaceae (Hesse 1980), while there are fewer threads in the Caesalpiniaceae.

Female gametophyte development in angiosperms takes place in two key phases: megasporogenesis and megagametogenesis (Johansen 1950; Gifford and Foster 1989; Johri *et al.* 1992). Megasporogenesis refers to the developmental stages through which megaspores (haploid spores) are produced, whereas megagametogenesis refers to the developmental stages through which the female gametophyte is formed from the "functional megaspore" to produce the female gametes, the egg cell and the central cell (Gifford and Foster 1989; Johri *et al.* 1992). These processes encompass several variations during growth. For example, cell wall formation during megasporogenesis and the number of mitotic divisions during megagametogenesis are factors affecting female gametophyte development (Yadegari and Drews 2004). Additionally, the genetic composition of nuclei and cells varies among developmental pathways. As a consequence, more than fifteen different patterns of female gametophyte ontogeny have been described (Maheshwari 1950; Gifford and Foster 1989; Johri *et al.* 1992).

In Ericaceous taxa the ovule is anatropous or slightly campylotropous, unitegmic, tenuinucellar, with a narrow micropyle, and devoid of vasculature, except in *Enkianthus* (Palser *et al.* 1971; Vilamil and Palser 1980) the integumentary epidermis contains tannin; the hypostase is densely

"cytoplasmic" (Ganapathy and Palser 1964; Stushnoff and Palser 1969; Palser *et al.* 1971). The single archesporial cell functions as megaspore mother cell in *Vaccinium retusum*. Rarely, two archesporial cells, lying aside by side, may enlarge and divide in *Rhododendron yunnanense* (Palser *et al.* 1971) and *V. leschnaultii* (Reddy *et al.* 1966). The megaspore undergoes meiotic division, and the chalazal megaspore of a linear, T or inverted T shaped tetrad develops into a polygonum type of embryosac (Venkateswarlu and Maheshwari 1973).

The embryo sac is broader at the micropylar end, and narrower at the chalazal. A prominent endothelium develops around the chalazal region of the embryo sac. It is biseriate in *V. retusum* and *V. serratum* (Venkateswarlu and Maheshwari 1973). The synergids are hooked and extended into the micropyle. The large egg projects beyond the synergids. The two polar nuclei fuse prior to or at the time of fertilization (*Rhododendron* and *Vaccinium*). The antipodals are T shaped, and usually ephemeral. Supernumerary antipodals occur in *Leucothoe racemosa* and *L. recurva.* The mature embryo sac contains starch. Allium type of development is reported in *Cassiope mertensiana* (Palser 1952).

The development of endosperm is either of the Nuclear or Cellular type. Nuclear type occur in *Rhododendron japonicum* x *R. mucronatum* (Creech 1955), *Vaccinium augustifolium* (Bell 1957), *V. leschenaultia* (Reddy *et al.* 1966). According to Ventakeswarlu and Maheswari (1973), the division of the primary endosperm nucleus is followed by two free nuclear, and the eight daughter nuclei arrange in three groups, two at each pole and four in

the centre. Walls are laid down separating the two terminal cells, each two nucleate, which develop into haustoria. The central four nucleate cells give rise to cellular endosperm proper. Cellular type of endosperm occurs in *Daboecia, Kalmia, Keiophyllum, Loiseleuria, Phyllodoce* and *Rhodothamnus* (Ganapathy and Palser 1964), *Rhododendron ferrugineum*, *R. yunnanense* (Veillet Bartoszewska 1959; Palser *et al.* 1971) and *Vaccinium* spp (Stushnoff and Palser 1969). The division of the primary endosperm nucleus separates a micropylar chamber from the chalazal chamber. A transverse division in each chamber results in a row of four cells; a vertical division in each cell form four superposed tier, each of two cells. The terminal tiers develop into haustoria, while the two central tiers produce the cellular endosperm proper (Ganapathy and Palser 1964; Palser *et al.* 1971). The micropylar haustorium is larger than the chalazal, and shows extensive growth except in *Daboecia* (Ganapathy and Palser 1964). The intervening wall of the chalazal cells disintegrates and the haustorium become two to four nucleate.

In *Tripetalleia brateata* and *T. paniculata* (Yamazaki 1975), the primary endosperm mucleus divides transversely; another transverse division occurs in each cell and a row of four cells is formed. A vertical division occurs in each of the upper two cells, while the lower tow undergoes transverse division. Therefore, the endosperm is composed of eight cells in six tiers. The micropylar haustorium develops into a large balloon shaped cell with a vacuolated cytoplasm, and eight to ten nuclei in *T. bracteata* and 10-16 in *T. paniculata*. The chalazal haustorium is two nucleate, and ellipsoidal with

vacuolated cytoplasm. The number of nuclei may increase up to four to six. Stevens (1919) observed both Nuclear and Cellular type of endosperm in *Vaccinium corymbosum*. Otherwise, Stushnoff and Palser (1969) described Cellular type of endosperm development in five taxa of *Vaccinium*- diploid, tetraploid, and hexaploid species. The embryogeny conforms to the Solanad type (Ganapathy and Palser 1964; Stushnoff and Palser 1969). The suspensor is linear and four to five celled. It is Caryophyllad type in *Calluna vulgaris* (Veillet Bartoszewska 1961), *Erica tetralix* (Veillet -Bartoszewska 1960), and *Gaultheria shallon* (Veillet- Bartoszewska 1959). A similar destination of initials occurs in the embryogeny of *Tripetaleia brateata and T. paniculata* (Yamazaki 1975).

The process of pollination is fundamental to the long-term sustainability of a plant. It is through pollination that seed set occurs and on which depends the genetic future of the individual. It has been clear, at least from the time of Sprengel's pioneering work in 1793 that animals are involved in a majority of angiosperms as the agents by which male gametes (pollen) are transferred to female receptive surfaces (stigma). The interactions between plants and pollinators are thought to be responsible for much of the diversity in angiosperm flower morphology, with many floral traits associated with particular animal behaviours (Lawrence *et al.* 2001). Animals, primarily insects, are known to pollinate over 90% of plants in tropical forests, and a high proportion of plant species are obligate outcrossers (Bawa 1990; Bawa *et al.* 1985). This high degree of dependence on animals for reproduction

brings many tropical plants at risk from anthropogenic disturbance such as loss of habitats and changes in land-use patterns (Kearns *et al.* 1998).

About 20,000 species in 72 families of flowering plants are presumed to be buzz pollinated by bees (Buchmann 1983). Buzz pollination occurs when a bee vibrates its thoracic flight muscles over the anthers, vibrating dry pollen onto its body (Harder 1998; Thorp 2000; Houston and Ladd 2002). These buzz-pollinated flowers share a number of floral traits viz., often open bowl-shaped or reflex petals, small to average sized and often lack of nectar (Buchmann 1983). Buzz pollination are common in Ericaceae (Buchmann 1983; Knudsen and Olesen 1993; Mahy and Jacquemart 1998; Houston and Ladd 2002; Escaravage and Wagner 2004; Loose *et al.* 2005; Johnson and Mcquillan 2011).

The small seed is albuminous, with a wing in Rhododendrioideae. The seed coat is exotestal. The outer epidermis is generally show thick pitted and lignified inner and radial wall; short hair like outgrowths occur in *Daboecia*. The mesophyll and inner epidermis get crushed but in, *Daboecia* the outer hypodermis is persistent and slightly thick walled (Corner 1976). Stevens (1911) published the first accurate description of the development of seeds in Ericaceae, *Epigaea repens*. In the Ericales, seeds are unitegmic, tenuinucellate, albuminous, and form both micropylar and chalazal haustoria (Maheshwari 1950; Palser 1961). Later studies by Ganapathy and Palser (1964) and Stushnoff and Palser (1969) have indicated that most ericaceous seeds contain linear embryos with two cotyledons, with exceptions being found in the subfamilies Pyroloideae and Monotropoideae. Copeland (1933;

1947) studied seeds and embryos from certain members of the Pyroloideae and found that the embryos had failed to differentiate distinct cotyledons. Pyykko (1968), after examining seeds from several members of the Pyroloideae growing in Finland, concluded that mature embryos remain undifferentiated and embedded in endosperm. The fruit of wintergreen is a many seeded capsule surrounded by a persistent, thickened and pulpy calyx that forms a fleshy pseudoberry (Hitchcock *et al.* 1959). Fruits ripen from mid-summer on and are persistent on the plants into winter, thus providing food for birds, mammals, the main dispersers (Van Dersal 1938; Van Dersal 1980).

Cronquist (1981) considered Ericaceae, Empetraceae and Epacridaceae to be closely related, and he placed them in the order Ericales (also including Cletheraceae and Cyrillaceae). Cronquist also recognized the non green ericad unigeric family Monotropaceae, and the green herbaceous taxa as Pyrolaceae. Subdivisions within Ericaceae and the recognition of the segregate families Monotropaceae and Pyrolaceae varied with different authors (Drude 1889; Watson *et al.* 1967; Stevens 1971; Wallace 1976; Thorne 1992). Anderberg (1992; 1993), Judd and Kron (1993) addressed the cladistic relationship among members of Ericales based on morphological data and by Kron and Chase (1993) who analysed rbcL sequence data. These phylogenetic studies have shown that Ericaceae was not paraphyletic in the traditional sense but also that Empetraceae and Epacridaceae were derived from Ericaceae. However, Kron *et al.* (2002) came out with the new classification of Ericaceae based on molecular and morphological data in

which eight- subfamilies and twenty tribes are recognized. The herbaceous taxa previously recognized as Pyrolaceae and Monotropaceae by some authors are also included within Ericaceae, in the subfamily Monotropoideae. Affinities of the Ericaceae with other families have also been variously discussed (Drude 1889; Takhtajan 1997).

The Vaccinioideae is a very heterogeneous subfamily, with the highest number of genera, and consists of five tribes viz. Andromedeae *s.s.*, Gaultherieae, Lyonieae, Oxydendreae and Vaccinieae, comprising 45 genera and about 1600 species (Kron and Luteyn 2005). Vaccinieae is the largest tribe (ca. 32 species and 1270 species) among the tribes of subfamily Vaccinioideae as well as the family Ericaceae (Kron and Luteyn 2005). *Vaccinium* L. is the largest (ca. 500 species) genus of this subfamily followed by *Gaultheria* and *Cavendishia*. Early comprehensive treatments of Ericaceae prepared by Hooker (1876) and Drude (1889) are different in their placement of the Vaccinioideae. Hooker (1876) separated the taxa of the tribe Vaccinieae from Ericaceae and recognized as a separate family Vacciniaceae, emphasizing the presence of an inferior ovary in Vacciniaceae. However, the most subsequent workers described it as a tribe within the subfamily Vaccinioideae of the Ericaceae (Drude 1889; Watson *et al.* 1967; Stevens 1971). In the classification of Stevens (1971), the circumscription of Vaccinioideae was largely enlarged by the inclusion of Arbuteae, Andromedeae, Cassiopeae and Enkiantheae. In the most recent classification of the Ericaceae (Kron *et al.* 2002), Vaccinieae are sister to Andromedeae *s.s.* and Gaultherieae, which form together with Lyonieae and

Oxydendreae the rest of the subfamily Vaccinioideae. This subfamily contains many species that are sources of economically important plants e.g., blueberries and cranberries, wintergreen oil etc. Many species are medicinal or used as herbal remedies. There are also many ornamentals in this subfamily: wintergreen, *Gaultheria*, sourwood, *Oxydendrum*, fetterfush, *Leucothoe*, bog-rosemary, *Andromeda*, and staggerbush, *Lyonia* (Luteyn 2002).

The tribe Gaultherieae comprises of about a total of approximately 250 species. The important characters of the tribe are its four appendaged anthers and a base chromosome number of eleven (Kron *et al.* 2002). Kron *et al.* (2002) conducted a comprehensive study of the Ericaceae based on phylogenetic analyses of nuclear and chloroplast DNA sequence data, morphology, anatomy, and embryology and concluded detailed account of the phylogenetic relationships of the genera in the tribe Gaultherieae. A strongly supported subclade, informally named wintergreen group comprising *Diplycosia, Tepuia, Gaultheria,* and *Pernettya* is diagnosed by the presence of methyl salicylate, although this compound has apparently been lost in many species (Powell and Kron 2001). Several studies have assessed the phylogenetic placement of the wintergreen group and the relationships of its species. In a study of five wintergreen group species as part of an Ericaceae-wide phylogenetic estimate based on morphological and DNA sequence data (18S, *matK* , and *rbcL*), the group was recovered as monophyletic (Kron *et al.* 2002). Lu *et al.* (2009) studied pollen morphology in the context of the phylogenetic estimate of Kron *et al.* (2002). Recently Fritsch *et al.* (2011)

provide strong molecular evidence of reticulate evolution at both shallow and deep levels of the wintergreen group phylogeny that, together with assessments of morphology both here and elsewhere (Powell and Kron 2001; Bush *et al.* 2009)

The genus *Gaultheria* comprises about 130 species are native to a wide geographical areas ranging from Andes, North America, Australia and nearby islands to eastern Asia and Himalaya (Airy-Shaw 1941; Middleton 1991a; 1991 b; Mabberley 2008). The characteristics features of this genus are characterized by having straight filaments and 4-awned anthers (Luteyn 1995; Middleton 1991a), superior ovaries, dry capsular fruits surrounded by a fleshy, often brightly colored, accrescent calyx (Middleton 1991a). Initially *Gaultheria* is placed within the tribe Andromedeae (Watson *et al.* 1967; Stevens 1970).

It is evident from the review of literature only a few works has been carried out on embryological and reproductive biology of *G. fragrantissima*. Therefore, the thesis is aims to study the embryology as well as some aspects of floral biology, pollination, pollen germination, seed germination and its viability in *G. fragrantissima*.

CHAPTER- 3

Materials and Methods

3.1 Study Area and Climate

The study was carried out in Meghalaya, Northeast India which is a landlocked territory lying between 25°47' and 26°10' North latitudes and 89°45' and 92°47' East longitudes. It is bounded by Assam in the North and the East and the plain of Bangladesh in the South and the West. It is divided into 3 hilly regions - the Garo Hills (Western Meghalaya), the Khasi Hills (Central Meghalaya) and the Jaintia Hills (Eastern Meghalaya). Climatologically, this study area belongs to the sub-tropical wet climatic region (Champion and Seth 1968). This region receives abundant southwest monsoon from June to October. The mean temperature ranges from 6 °C to 17°C in winter and from 15 °C to 24°C during summer. The maximum rainfall was recorded during the study period in the month of June i.e. 442.90 mm and minimum rainfall was recorded during the month of November to February i.e. less than 40 mm. The data on the climatic factors for the year 2008 - 2010 were collected from Central Seismological and Meteorological observatory, Shillong station, Government of India.

Floral morphology of male sterile and hermaphrodite flowers of two natural populations of *G. fragrantissima viz.* Nongkrem forest (25°31¢ N Latitude 91°52¢ E Longtitude, 1833 m AMSL) and another population *viz* Lum Shyllong (25°34¢0 N Latitude 91°52¢60 E Longitude, 1965 m AMSL) (Plate 3.1) were thoroughly investigated and flower were collected during three

consecutive years of flowering period i.e. April 2008 to May 2010. *G. fragrantissima* is a bushy shrub, up to 4 m high, bark brown, branchlets angled, reddish; leaves 3-9 x 1.3- 4 cm, broadly elliptic, oblong or oblong-lanceolate or oblanceolate-elliptic, sometimes orbicular, base rounded shining, nerve impressed above, punctuate beneath; flower in raceme axillary and terminal, 1.5-4 cm long, sometimes branched or fascicled, glandular hairs in the leaf surface; flowers creamy white or pinkish, 0.3-0.5 cm width, calyx lobes ovate, triangular, corolla tube urceolate, angled, apical lobes reflexed; capsules globose, calyx accrescent, 0.4-0.6 cm across length. In Meghalaya, G. *fragrantissima* flowers bloom from March to late June and fruiting till late August.

Floral morphometric variations in male sterile and hermaphrodite individual were examined. Fruit set and fruit weight were also compared in both male sterile and hermaphrodite plants. Sex ratio and male sterility were calculated based on the observation of open flowers in the field as well as the racemes that collected during the flowering stage within the population investigated and the individual was considered as female (male sterile) if the anther did not produce pollen grains and if the plant with fertile male and female organ are considered hermaphrodite. Statistical analysis of mean data (ANOVA) was done by using origin 7; Turkey's post hoc test was done to interpret significant difference between mean values.

Floral visitors were observed in both hermaphrodite and male sterile flowers in Lum Shillong during three flowering seasons (May-June) from 2009 to 2011. Flower visitors were recorded in both the plants on sunny days

between 0900 to 1500. For each insect group the forage times per flower were recorded and insects were caught by sweep netting as well as spraying of 10 % chloroform for species identification with the help of Zoological Survey of India, Shillong.

3.2 Developmental aspects of flowers

To study the microsporogenesis, megasporogenesis and female gametophyte development and post fertilization changes in embryo sac, flower of various developmental stages of both hermaphrodite and male sterile plants were collected from the field and fixed in FAA [Formalin (5ml): Acetic acid (5ml): 70% Alcohol (90ml)], 3% Glutaraldehyde in 7.2 pH phosphate buffer and Cornoy's fluid (Johansen 1940; Krishnamurthy 1988).

The plant samples fixed in FAA were used for microtomy by the usual dehydration method using tertiary butyl alcohol series followed by impregnation with paraffin wax (Johansen 1940; Sass 1958; Berlyn and Miksche 1976). The paraffin block were trimmed and sectioned at a thickness of 7-10µm using Leitz rotatory microtome.

The sections were stained by following the staining procedures:

1. Safranin fast green (Johansen 1940).
2. Total insoluble polysaccharides: Periodic acid Schiff's (PAS) method (Jensen 1962; Fedder and O'Brien 1968).
3. Callose: Decolorized aniline blue and Cotton blue (Johansen 1940; O'Brien and McCully 1981; Shivanna and Rangaswamy 1993).

4. Total proteins: Mercuric bromophenol blue method (Mazia *et al.* 1953).

For whole mount studies, seed were soaked with 2 ml of modified Franklin's fluid and left in the solution at room temperature for about 36-48 hours. The softening solution was carefully removed and the seeds were washed in four changes of distilled water, after the last washing the seeds were dried at 35 °C for 2- 4 hours. Herr's fluid was added, and seeds were maintained in this solution for 48-72 hours. The seeds were directly observed under microscope in a drop of Herr's fluids (Vega and Oliveira 2007).

Photomicrographs were taken by using Olympus microscope (BX 43) and Zeiss Axio Imager Al fluorescence microscope.

3.3 Scanning Electron Microscopy (SEM)

The following methods were employed for SEM studies:

1. Flowers parts of *G. fragrantissima* such as anther, pollen grains and ovules were dissected longitudinally with razor blades and fixed in 2-3% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) at 4°C for 4 hrs. The samples were thoroughly washed in 0.1 M phosphate buffer and post fixed in 1% OsO_4 for 2 hrs. The pollinator that was present inside the corolla tube was also fixed and processes for SEM studies.
2. After fixation, the plant materials were dehydrated using increasing concentration of acetone (30%, 50%, 70%, 80%, 90%, 95% two changes in every 15 min. in each step).

3. Dehydrated materials were dried in a Jeol JCPD-5 critical point dryer, 3-methyl butyl acetate solution as the exchange liquid.
4. Dried materials were fixed on Eikon ion sputter, JFC-1100 and were coated with thin layer of gold vapour (300 Å layer).
5. Gold coated plant materials were observed under Scanning electron microscope Joel (JSM- 6360).

3.4 Transmission Electron Microscopy (TEM)

The following procedure was employed for the TEM studies:

1. Flower parts such as anther lobes, pollen grains, ovules were fixed in 2-3% glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.2 at 4°C for 8 hours, thoroughly washed in 0.1 M phosphate buffer and post fixed in 1% OsO_4 for 2 hours.
2. The samples were gradually dehydrated with acetone for 10-15 min in each step. Three changes were made in absolute acetone.
3. The plant materials were embedded in Araldite CY 212. Ultrathin sections were cut at approximately 60-90 nm (600Å-900Å) through a Sorvall MT-2 ultramicrotome using a glass or diamond knife, and then stained with 2% aqueous uranyl acetate and lead citrate. Samples were observed under Zeiss EM-109 TEM.

3.5 Pollen viability

Fluorochromatic Reaction (FCR) Test (Heslop-Harrison and Heslop-Harrison 1970; Shivanna and Rangaswamy 1993). To 2-5ml of 10% sucrose solution in a small glass vial drops of stock solution of FDA (2mg/ml) were added until the resulting mixture shows persistent turbidity. A drop of sucrose-FDA mixture was taken on a slide. Sufficient amount of fresh pollen grains were suspended in the preparation and incubated in a humidity chamber (>90%RH) for 5-10 min. At the end of the incubation period, a coverglass was lowered and observed the preparation under the Zeiss Axio Imager epifluorescence microscope with HPWB (High Performance Wide Band) filter. For calculating pollen viability, total number of viable and non-viable pollen grains was counted from 10 microscopic fields based on the emittence of yellowish green colour.

3.6 Pollen germination and Pollen tube growth

Freshly collected pollen grains just after anthesis were inoculated for germination test. The basal medium for germinating pollen invitro was followed after Brewbaker and Kwack (1963).The Brewbaker and Kwack's medium was modified by incorporating the optimal requirements of *G. fragrantissima* pollen. Pollen were incubated for 19 hours and then fixed by putting a drop of FAA on the incubated pollen. Data on pollen germination and pollen tube length were recorded by scoring 10 microscopic fields chosen randomly per slide. Statistical analysis of mean data (ANOVA) was

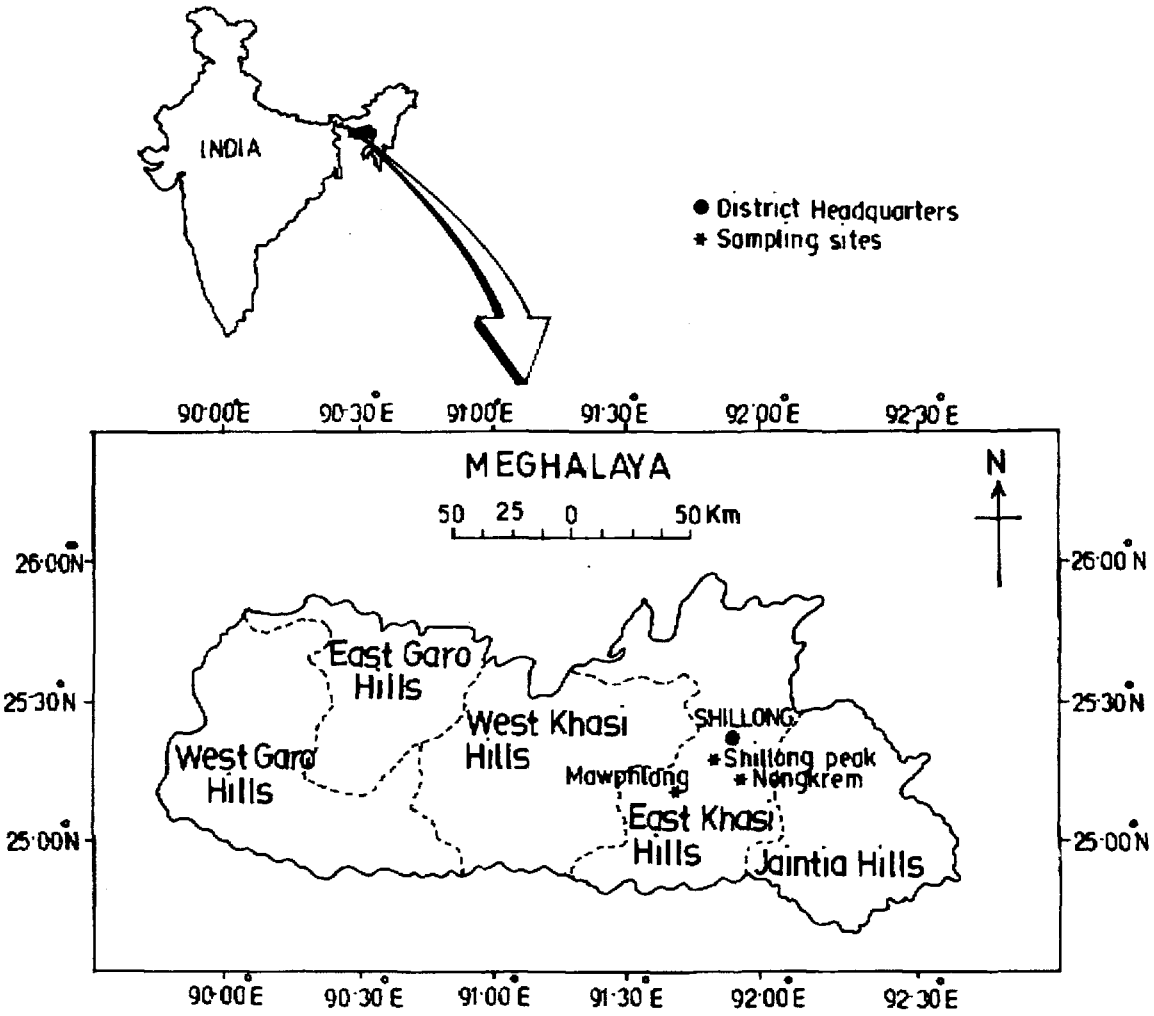
done by using origin 7; Turkey's post hoc test was done to interpret significant difference between mean values.

3.7 Seed germination

1. The presoaked seeds of both male sterile and hermaphrodite plants were surface sterilized using 1.05% (w/v) sodium hypochlorite containing few drops of Tween 20 for 20 minutes. 400 seeds soaked overnight in three different concentrations (100ppm; 200ppm; 500ppm) of gibberellic acid (GA_3) over a period of 24 hours and then washed with distilled water.
2. 200-300 seeds were placed on damp filter paper and *Sphagnum* mosses in six petri dishes and germinated under laboratory conditions at 25 °C with 18 hrs light.
3. Visible emergence of radicle was taken as the criterion for germination (Kaliamoorthy and Rao 1994).
4. The data on the percentage of seed germination in response to varying concentrations of GA_3 treatment was recorded. Statistical analysis of mean data (ANOVA) was done by using origin 7; Turkey's post hoc test was done to interpret significant difference between mean values.

Plate 3.1

Map of Meghalaya state showing the collection sites of samples.



Map of Meghalaya in India showing sampling site

CHAPTER - 4

Floral Biology of Hermaphrodite and Male sterile plant

4.1 Introduction

There are several important aspects, vital for a clear understanding of floral biology, such as morphology, phenology, the reproductive system, pollination and fertilization. The flowering plants display a wide variety of sexual systems ranging from obligate selfing in association with self compatibility to obligate outcrossing in conjunction with self-incompatibility (Solbrig 1976; Navarro 2001, 2007). Superimposed upon these genetic systems are such temporal and morphological mechanisms as protandry, protogyny, heterostyly, monoecism, andromonoecism, gynomonoecism, dioecism, gynodioecism, and androdioecism that are also presumed to regulate the level of outcrossing (Lloyd 1975; Charlesworth and Charlesworth 1978). Darwin (1877) was the first to comprehensively document and explains the diversity of sexual systems in plants. Many authors have dealt with male-sterility and gynodioecy since it was discussed by Darwin in 1877. Gynodioecy, as defined by Darwin, is the occurrence of two kinds of individuals in natural populations: bisexual or hermaphrodite plants, and females or male sterile plants. Darwin (1877) and Valdeyron *et al.* (1973) and others have expressed the belief that this breeding system serves to promote outcrossing, particularly in those species that show a strong tendency toward

inbreeding. The origin of separate sexes (dioecy) from combined sexes (cosexuality) via the gynodioecy pathway has occurred repeatedly during the evolutionary history of the flowering plants (Webb 1999; Weiblen *et al.* 2000) and consists of two successive stages. The first involves the invasion of hermaphrodite populations by female plants, resulting in the dimorphic condition known as gynodioecy (Darwin 1877). The second step occurs as hermaphrodites in gynodioecious populations (hereafter 'males') increasingly favour pollen over seed production (Lloyd 1976) resulting in their replacement by pure males and the establishment of dioecy.

Gynodioecy has long been of considerable interest to plant biologists despite its relative rarity among flowering plants (5–10%) for several reasons mainly based on the following three aspects : (i) theoretical challenges posed by the spread and persistence of female individuals in populations despite their gametic disadvantage relative to hermaphrodites (Lloyd 1976; Charlesworth and Charlesworth 1978; Bailey *et al.* 2003); (ii) its significance as an intermediate step in the evolution of dioecy from hermaphroditism (Barrett and Harder 1996); and (iii) the genetic and evolutionary implications of the inherent nucleocytoplasmic genomic conflict when male sterility is determined by cytoplasmic genes (Budar *et al.* 2003; Burt and Trivers 2006). One of the most common pathways to gynodioecy involves a male-sterile (female) mutant arising within a population of hermaphroditic individuals and increasing in its frequency, resulting in a population that is dimorphic for gender (Frankel 1940; Webb 1999).

In Ericaceae, the flowers are usually hermaphroditic and autogamy is very common (Stevens 2004). The pendulous, urn-shaped, cylindrical, or bell-shaped flowers typically produce nectar and are visited by bees and wasps in temperate and subtropical latitudes. In the montane tropics many species have tubular red flowers and pollinated by hummingbirds (Luteyn 2002; Freitas and Sazima 2006). Flowers of most Neotropical montane Ericaceae species are showy, and there is much variation among species, suggesting that adaptation to a variety of pollinators has been important in the adaptive radiation of the family. However, in spite of the interesting characteristics of this family, published information on floral biology and pollination ecology is still unavailable for most Neotropical species (Melampy 1987; Murcia and Feinsinger 1996; Busby 2000).

The order Ericales has been described as having only bisexual flowers (Yampolsky and Yampolsky 1922; McLean and Ivimcy Cook 1956) or as having mostly bisexual flowers with male and female flowers appearing very rarely (Hutchinson 1964). Most researchers consider the family Ericaceae, which is classified within the order Ericales, to be hermaphroditic (Bentham and Hooker 1876; McLean and Ivimcy Cook 1956; Hitchcock and Cronquist 1973). However a few consider it a unisexual (Franklin 1962; Moore 1983) or functionally dioecious or dioecious (Sleumer 1966; Anderson *et al.* 2000). *G. fragrantissima* has a distinct gynodioecious sex expression where hermaphrodite and male sterile individual co exist in the same population (Venugopal and Langstang 2010).

4.2 Floral morphology of hermaphrodite flower in *G. fragrantissima*

Flowers regular, 1.35x 4.1 cm; Pedicillate 5.5 mm, creamy white, translucent arrange on the axillary racemose and sometimes panicle inflorescence, 7-10 cm long (Plate 4.1A). Calyx pale green, 3 mm, deeply 5 lobed, lobes ovate, lanceolate, acute, ciliate. Corolla 4.80 x 4.10mm creamy white, appear pinkish because of translucent, urceolate, teeth very small, reflexed (Plate 4.1B). Stamen 10, 2mm, epipetalous, height up to the middle of the corolla tube; anther 4 lobes, dorsifixed, pinkish red when young and become dark brown during the dehiscence and release of pollen grains. With two distinct apical setaceous horns or awns, dehiscence porous, extrose (Plate 4.1C). Filament, pale white, pilose on the dorsal side, base flattened. Ovary 5 locular, 4 x 2 mm, globose, pubescent, ovules numerous, axile placentation. Style 1.9 x 0.9 mm, cylindrical, slightly furrowed, erect, as long as the stamen up to the height of the anther awns or horns, stigma 5 lobed (Plate 4.1C) (Table 4.1 & 4.2).

4.3 Floral morphology of male sterile flower in *G. fragrantissima*

Flowers irregular, 7.90 x 3.5 mm; Pedicillate 3.45 mm, creamy white, translucent, arranged on the axillary racemose and sometimes panicle inflorescence, 7-10 cm long. Calyx pale green, 3 mm, deeply 5 lobed, lobes

ovate, lanceolate, acute, ciliate. Corolla 4.4 x 3.5 mm creamy white, narrow tubular to urceolate, teeth very small, reflexed (Plate 4.2A). Anther lobes release degenerated sporogenous tissues and formed a white unorganized mass of tissues with a tuft of hairy outgrowth at the tip therefore viable pollen are absent, awn or horns are absent anther lobes have tuft of pilose hairs in the middle (Plate 4.2B). Filament were recurved to the base of the style,2 mm, pale white, pilose on the dorsal side, base flattened. Ovary 5 locular, 4 x 2 mm, globose, pubescent, ovules numerous, axile placentation. Style 2.4 x 0.9 mm, cylindrical, slightly furrowed, style very long reaching the tip of the corolla tube, stigma 5 lobed (Plate 4.2A& C) (Table 4.1& 4.2).

4.4 Sex ratio and Female frequency

There is a high variance in the sex ratio (Hermaphrodite: Female) of the two populations where it is 8:2 in Lum Shyllong and 6:4 in Nongkrem sacred forest (Table 4.3). Percentage of females varied in both the population where female frequencies is relatively low in Lum Shillong (10%) and more in Nongkrem sacred forest (20%) (Table 4.3).

4.5 Significant difference (P≤ 0.05) between hermaphrodite and male sterile flowers of *G. fragrantissima* in Nongkrem and Lum Shyllong populations.

- The length of pedicel is longer in the hermaphrodite than that of male sterile flowers in both populations (Figs. 4.1a & 4.2a)

- In Lum Shyllong population the length of bract is shorter in hermaphrodite flower than male sterile flower (Fig.4.2b) and there is no significant different in the length of bract in Nongkrem population.
- The length and breadth of corolla in hermaphrodite and male sterile flowers showed significant different in Nongkrem population however in Lum Shyllong population only breadth of corolla showed significant different. In both populations, the corolla of hermaphrodite flowers are larger and urceolate in shape where as in male sterile the corolla are smaller and narrow tubular in shape (Figs 4.1b,c & 4.2 c) (Plate 4.1 B& 4.2A).
- The length of style is also longer in male sterile flowers than that of hermaphrodite flowers in both populations (Fig 4.1d& 4.2 d).
- The lengths of gynoecium is longer in the male sterile flowers of both the populations and the tip of the style reaches up to the rim of the corolla tube whereas in the hermaphrodite flowers the length of the style is limited only up to the middle of the corolla tube. (Figs 4.1e & 4.2e)(Plate 4.1A& 4.2C).
- Sex ratio is 8:2 in Lum Shylllong while in Nongkrem sacred grove is 6:4 (Table 4.3).
- Fruit set was high in both hermaphrodite and male sterile ranging from a low of 77.70 % to as high as 83.17% in both the population studied. There are no significant differences in fruit set and fruit weight of hermaphrodite and male sterile in Nongkrem population where as in

Lum Shyllong population fruit weight showed significantly different (Fig 4.2 f).

4.6 Discussion

Species of Ericaceae are primarily hermaphroditic, although several are gynodioecious or dioecious (Cronquist 1981; Middleton 1991a; Anderson *et al.* 2000). The detailed and thorough investigation on floral morphology, sex ratio and female frequency in the two populations of *G. fragrantissima* in Nongkrem and Lum Shyllong confirmed that the plant has two types of individual each has one flower type i.e hermaphrodite and male sterile; thus, species is gynodioecious. Allan (1961) in his footnotes to the Ericaceae found two type of plants in all pure *Gaultheria* species he studied on the South Island, both of which produced fruits only but only one of which produce pollen, which suggests the presence of both hermaphrodites and females and therefore gynodioecy. Unfortunately, the species were not listed by name. Middleton (1991a) also reported the presence of male sterile individual among herbarium specimen of *G. colensoi* and Franklin (1962) noted that male sterile plants are quite common in *G. colensoi* and *G. rupestris var. subcorymbosa.* Analogous situations have also been reported in seven taxa of Newzealand *Gaultheria* species by Delph *et al.* (2006) as well as in other angiosperms (Charlesworth 1985; Anderson and Symon 1989). The evolution of gynodioecy from hermaphrodite has occurred many times in flowering plants (Mc Cusker 1962; Lloyd 1975; Webb 1999). High variable sex ratio are a feature of models in which gynodioecy is controlled

by cytoplasmic male sterility combined with nuclear restoration of pollen fertility (Gouyon *et al.* 1991; Bailey *et al.* 2003).

Sex-ratio variation among populations of gynodioecious species is a widespread phenomenon (Manicacci *et al.* 1998; Webb 1999) and the factors thought to affect this variation range from purely genetic to ecological in nature. Based on theoretical models, sex ratios are expected to vary more when male sterility is cytoplasmic than when it is nuclear because of founder effects and dynamic equilibria (Delannay *et al.* 1981; Ross and Gregorius 1985; Gouyon *et al.* 1991). Concerning the evolution and maintenance of gynodioecy, most theoretical treatments have relied on the advantages of female plants: the avoidance of inbreeding depression and or the compensation of female fecundity by heterosis (Llyod 1975; Charnov *et al.* 1976; Charlesworth 1981; Charnov 1982). The difference in flower traits between hermaphrodite and female *Gaultheria* species flowers is not an unusual phenomenon (Delph *et al.* 2006). In *G. fragrantissima* male sterile have vestigial, whitish, nonfunctional anthers with rudimentary filaments, and degenerated stamens were occurred at lower positions inside the floral tubes as rudimentary organ than the normal stamens of hermaphrodite flowers. Rudimentary male organs are commonly found in females in many sexually polymorphic species suggesting that male sterility (MS) is derived from hermaphroditism (Darwin 1888; Lloyd and Webb 1977; Pimienta Barrios and del Castillo 2002). The degree of reduction in the staminodes, probably indicating the avoidance of self fertilization and inbreeding depression (Charlesworth 1999; Koelewijn and Van Damme 2005; Ramsey *et al.* 2006).

The degeneration of the anther could be considered as the saving of energy of now useless processes such as differentiation of pollen mother cells and meiosis.

According to resource allocation theory, female plants save the energy needed for the male function and have some greater quantity of resources which can be invested in female function and or other vegetative parts than hermaphrodite plant. However, the energy saving on the male sterile is probably not sufficient from the point of view of resource allocation theory (Charnov *et al.* 1976; Charnov 1982), because the breadth of corolla and gynoecium in hermaphrodite plant of *G. fragrantissima* is far bigger than that of male sterile plant.

Fruit set does not differ significantly between the sexes of *G. fragrantissima*, making them appear similar during the fruiting stage as well as the flowering stage, this is corroborate with the observation of Delph *et al.* (2006) in the gynodioecious species of Newzealand *Gaultheria* species. However, there is significant difference in fruit weight in Lum Shyllong. Darwin (1877) proposed that heavier seeds produced by male-sterile plants may represent a reproductive advantage. He also noted that by not producing pollen, females would save resources that they could then invest in seed and called this type of resource reallocation the "law of compensation". A meta-analysis of data from the literature revealed that females typically compensated at the whole-plant level by producing more flowers and setting more flowers to fruit, rather than by increasing seed number per fruit (Shykoff *et al.* 2003). Another way in which females may

obtain higher seed fitness than hermaphrodites is by avoiding self fertilization and the concomitant inbreeding depression that can occur as a result (Lloyd 1976; 1982).

Several breeding systems in plants may favour outcrossing, either by reducing self-fertilization (Stout 1928; Charlesworth 1981) or by avoiding pollen–pistil interference (Bawa and Beach 1981; Lloyd and Webb 1986; Webb and Lloyd 1986; Bertin and Newman 1993). These mechanisms include: gynodioecy – the presence of plants with hermaphrodite flowers and plants with functionally female flowers or individuals with varying degrees of stamen degeneration (Darwin 1896; Vaarama and Jaaskelanien 1967; Dulberger 1984); dichogamy (Lloyd and Webb 1986) – the temporal separation of male and female functions, either through dehiscence of anthers before the stigma is receptive (protandry) vice versa, the stigma being receptive before the dehiscence of anthers (protogyny); and herkogamy (Webb and Lloyd 1986) – the spatial separation of male and female functions. More strictly, Lloyd and Webb (1986) suggested that the separation of pollen and stigmas acts in general to reduce self-interference and often also reduces self-fertilization. They further suggested that mechanisms preventing self fertilization primarily increase maternal fitness, whereas mechanisms avoiding self interference primarily promote paternal fitness.

Hermaphrodite flowers of gynodioecious species have been considered as pollen donors and, as with all hermaphrodite flowers, have a risk of inbreeding depression (Charlesworth and Charlesworth 1987) and

pollen pistil interferences, which increase the loss of parental fitness by reducing the flower's ability to export pollen (Holsinger *et al.* 1984). Flowers reduce those two risks in different ways: the first by means of dichogamy (Lloyd and Webb 1986) and the second through herkogamy (Webb and Lloyd 1986; Fetscher 2001), i.e. by delaying the growth of or moving the female function, which is observed in protandrous species (Lloyd and Webb 1986).

Table 4.1 Comparison of various floral traits, fruit set and fruit weight of Hermaphrodite and Male sterile plants of *G. fragrantissima* in Nongkrem population.

Sex expression	Pedicel(mm)		Bract(mm)		Calyx(mm)		Corolla(mm)		Gynoecium(mm)		Style (mm)		Fruit set (%)	Fruit weight (mg)
	length	breadth	length	breadth	length	breadth	length	breadth	length	breadth	length	breadth		
Herma-phrodite	5.50± 0.17	1.87± 0.13	1.57± 0.09	1.65± 0.13	3.12± 0.08	2.60± 0.08	4.87± 0.11	4.10± 0.10	3.97± 0.12	2.07± 0.06	1.87± 0.13	0.95± 0.02	77.70± 1.71	95.30± 5.51
Male sterile	3.45± 0.11	1.99± 0.02	1.70± 0.16	1.70± 0.10	3.30± 0.10	2.70± 0.06	4.45± 0.13	3.50± 0.11	4.45± 0.11	2.10± 0.06	2.37± 0.15	0.93± 0.02	78.87± 1.91	100.30± 4.02

Note: Values are mean ± standard error.

Table 4.2 Comparison of various floral traits, fruit set and fruit weight of Hermaphrodite and Male sterile plants of *G. fragrantissima* in Lum Shyllong population.

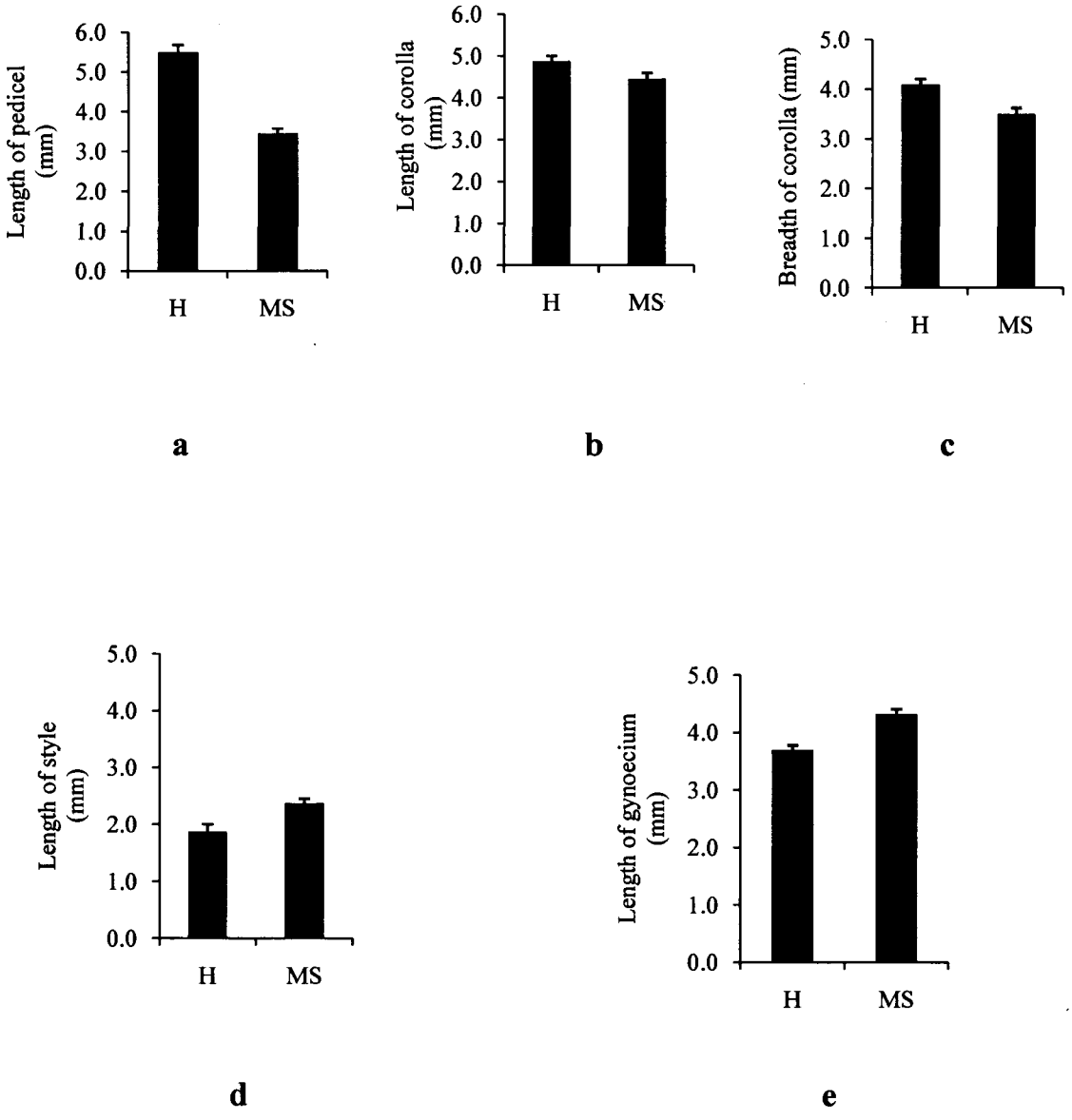
Sex expression	Pedicel (mm)		Bract (mm)		Calyx (mm)		Corolla (mm)		Gynoecium(mm)		Style (mm)		Fruit set (%)	Fruit weight (mg)
	length	breadth	length	breadth	length	breadth	length	breadth	length	breadth	length	breadth		
Herma phrodite	4.80± 0.15	1.82± 0.65	1.40± 0.09	1.45± 0.13	2.95± 0.10	2.20± 0.90	4.40± 0.10	3.77± 0.06	3.70± 0.77	2.10± 0.11	1.67± 0.11	0.93± 0.02	79.12± 1.92	86.90± 3.30
Male sterile	3.40± 0.11	1.70 ± 0.13	1.8 ± 0.67	1.6 ± 0.50	3.45± 0.51	2.70± 0.47	4.50± 0.42	3.07± 0.40	4.32 ± 0.37	2.17 ± 0.33	2.30 ± 0.47	0.95 ± 0.75	83.17± 2.44	99.50± 3.30

Note: Values are mean ± standard error.

Table 4.3 Frequencies of female plant, Sex ratio (Hermaphrodite: Female) of *G. fragrantissima* in two populations investigated.

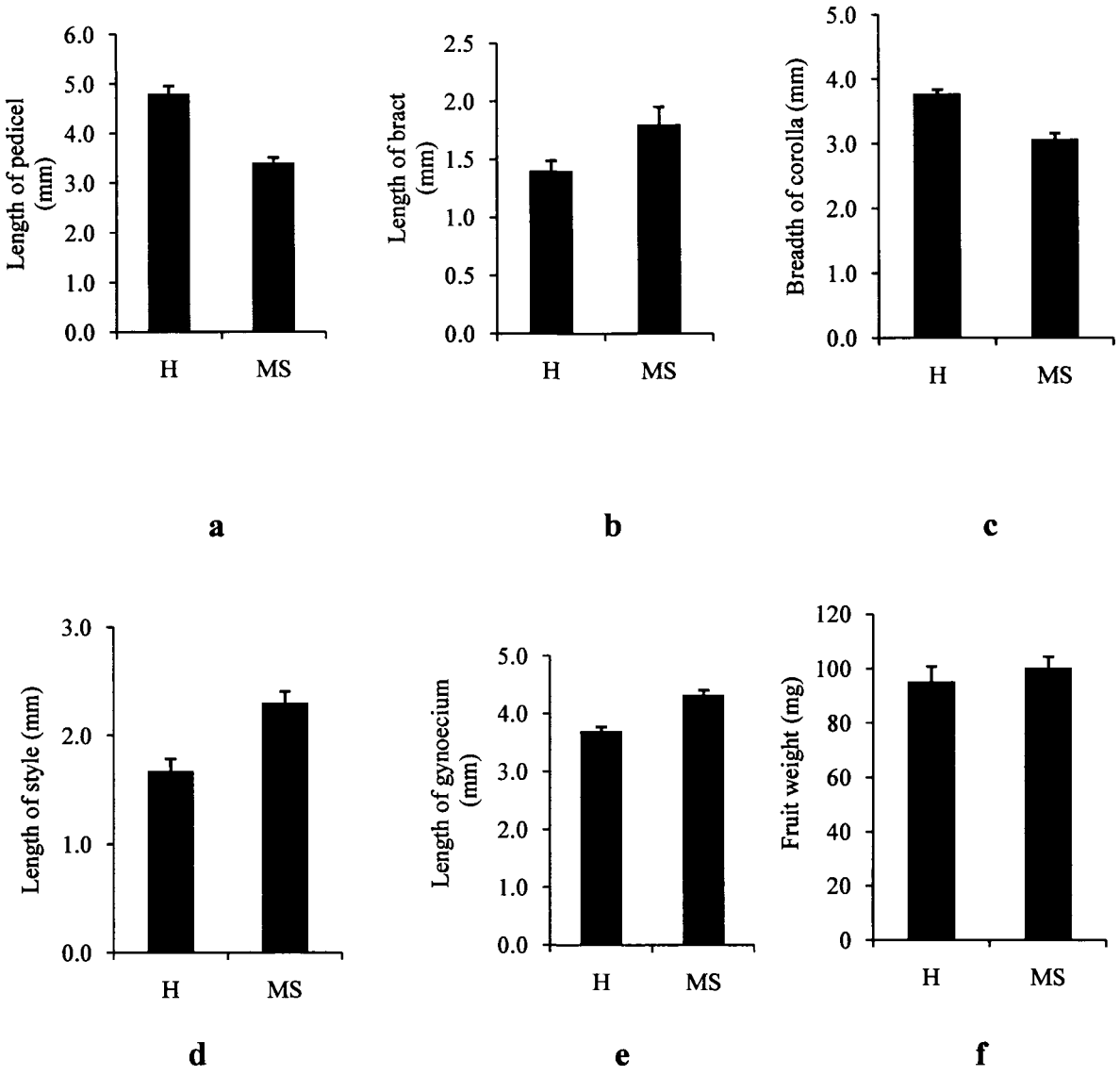
Population	Total no. of plants	No. of hermaphrodite	No. of male sterile(female)	Frequency of females (%)	Sex ratio
Lum Shyllong	125	100	25	10	8:2
Nongkrem	100	60	40	20	6:4

Figure 4.1



Figs a-e: Mean statistical interpretation (ANOVA) of Nongkrem *G. fragrantissima* population is significantly different according to Tukey's test at P≤ 0.05. Note: H = hermaphrodite, MS = male sterile.

Figure 4.2



Figs a-f: Mean statistical interpretation (ANOVA) of Lum Shyllong *G. fragrantissima* population is significantly different according to Tukey's test at $P \leq 0.05$. Note: H= hermaphrodite, MS= male sterile.

Plate - 4.1

A. A twig of *Gaultheria fragrantissima* showing axillary racemose inflorescence with pendulous white flowers. Bar = 0.71 cm.

B. Longitudinal Section (L.S) of hermaphrodite flower showing the fertile anther (fa) with awns, ovary (O) and small style (S). Bar = 0.1 cm.

C. Showing the Scanning Electron Microphotograph (SEM) of hermaphrodite with the fertile anther (fa), Ovary (O) and Style (S). Bar = 500 µm.

Plate - 4.1

***Gaultheria fragrantissima* Wall. Hermaphrodite flower**

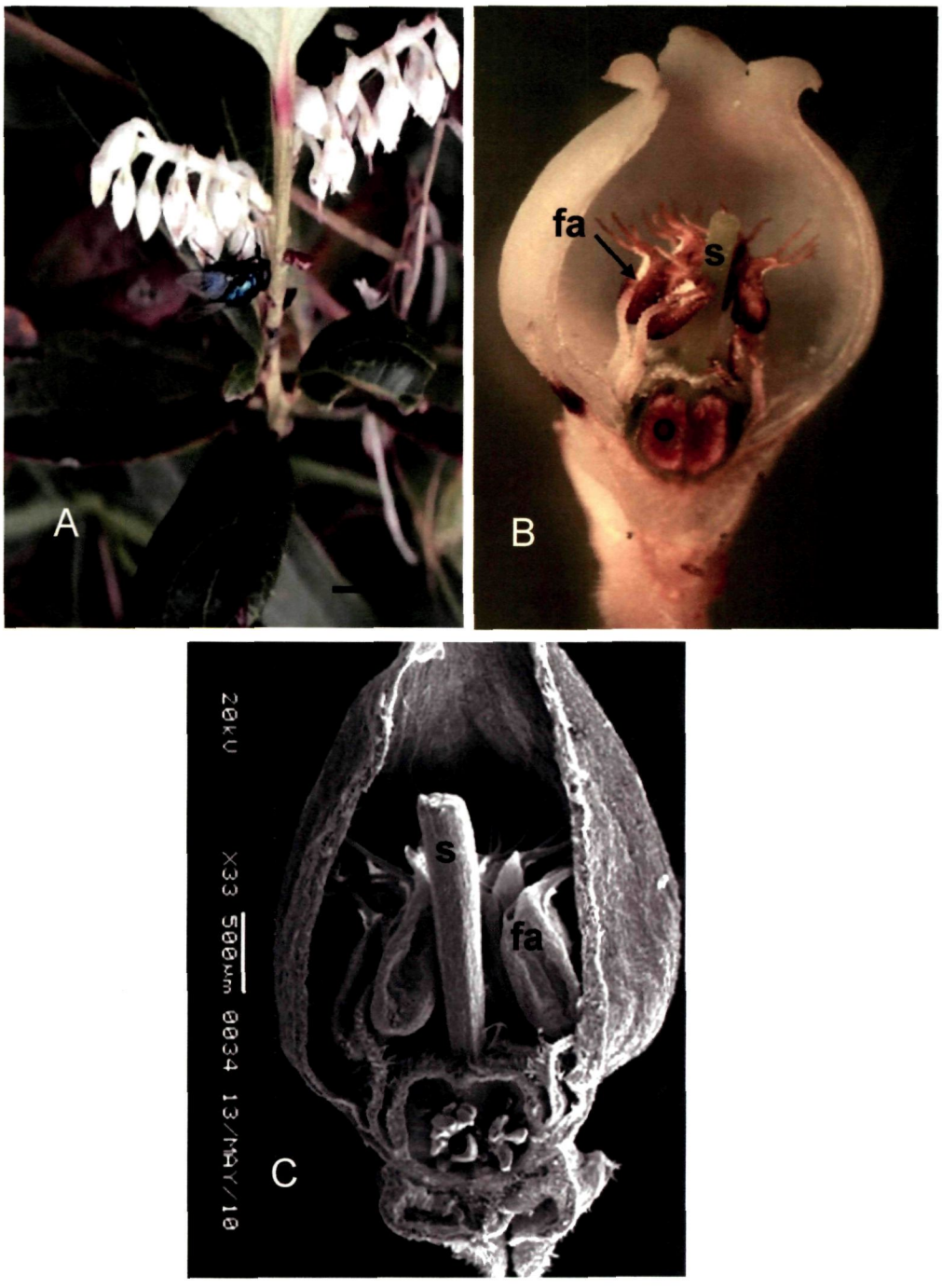


Plate- 4.2

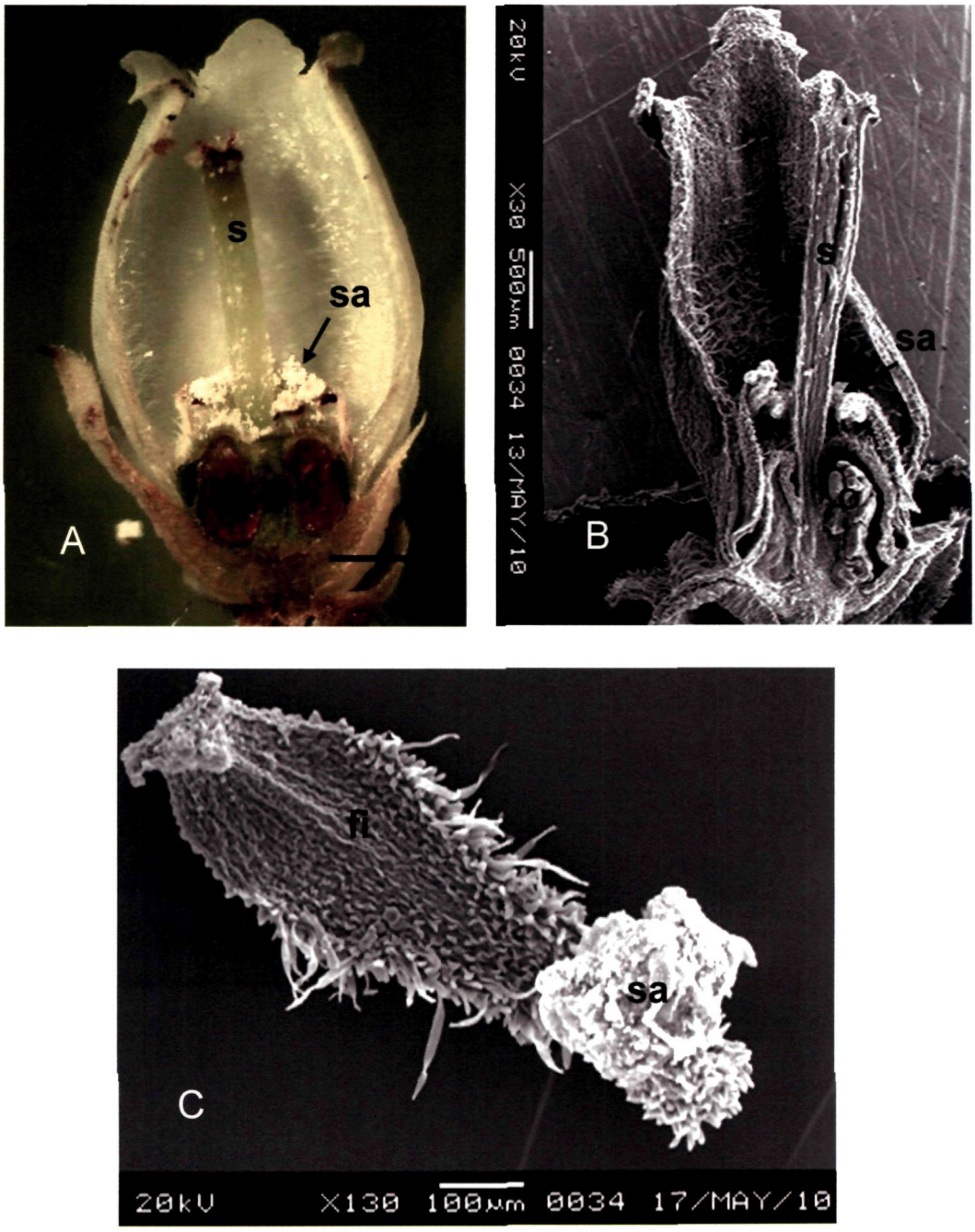
A. Longitudinal Section (L.S) of male sterile flowers showing the sterile anther (sa) with no awn, ovary (O) and long style (S). Note the very long style. Bar = 0.1 cm.

B. Scanning Electron Microphotograph (SEM) of male sterile flowers showing sterile anther (sa), ovary (O) and style (S). Note the difference in the length of style in the hermaphrodite and male sterile flowers. Bar = 500 µm.

C. SEM of single sterile stamen showing the filament (fi) and abnormal of tissue representing the anther (sa). Bar = 100 µm.

Plate - 4.2

Gaultheria fragrantissima Wall. Male sterile flower



CHAPTER - 5

Microsporogenesis in hermaphrodite and male sterile plants

5.1 Introduction

Since Darwin (1877), biologists have been interested in detailing the intricate structure of flowers and their adaptive significance in pollination. Flowering plants have a diverse array of floral mechanisms that reduce the incidence of self pollination and the interference between female and male sexual function (Darwin 1877; Lloyd and Webb 1986; Bertin and Newman 1993; Barrett 2003). Male-sterile mutants have been reported in a large number of plant species (Van Der Veen and Wirtz 1968; Albertsen and Phillips 1981; Kaul 1988). The timing and place of the male sterility has been determined and studied at the light microscope level (Dubey and Singh 1965; Pritchard and Hutton 1972; Kini *et al.* 1994) and later at the ultrastructural level (Overman and Warmke 1972; Horner 1977; Pollak 1992). The tapetum is of considerable physiological significance because it is generally involved in different aspects of pollen development (Maheshwari 1950; Pacini *et al.* 1985). In most of the species, the tapetum differentiation and subsequent disintegration coincides very well with the microspore mother cells post meiotic developmental program, and premature or delayed degradation will contribute to pollen abortion (Li *et al.* 2006; Vizcay-Barrena and Wilson 2006). Warmke and Lee (1977) reported that mitochondrial degeneration in the tapetum of corn was the earliest detectable event in the process of pollen

abortion. Pleomorphic cytoplasmic organelles were also reported in anthers of cytoplasmic male sterile sunflower (Horner 1977), sugar beet (Nakashima 1975), and sorghum (Overman and Warmke 1972). In other plants, mitochondrial degeneration followed the onset of pollen abortion and was, therefore, a result of the abortion process (Lee *et al.* 1980; Bino 1985). In several cases, no disruption of organelles occurred (De Vries and Le 1970; Horner and Rogers 1974).

Studies on the anther development and microsporogenesis have been studied on various families of the order Ericales. Warner and Chinnappa (1986) studied the pollen morphology of 61 species of Ericaceae in Canada, including two species of *Gaultheria* viz *G. procumbens* and *G. shallon.* Sarwar and Takahashi (2006) investigated the pollen morphology of 12 genera of the subfamily Vaccinioideae and nineteen species of *Gaultheria.* There are also descriptions of a few other species of *Gaultheria* included in more general palynological or taxonomic publications (Yang 1952; Luteyn 1995; Hermann and Palser 2000; Lu *et al.* 2009).

Allan (1961) found two types of plants in *Gaultheria* species of New Zealand which suggests the presence of both hermaphrodites and female plants. Franklin (1962) noted that male sterile plants are quite common in *G. colensoi* and *G. rupestris var. subcorymbosa* and Middleton (1991a) reported the presence of male sterile individual among herbarium specimen of *Gaultheria colensoi.* Delph *et al.* (2006) reported some species of *Gaultheria* (*G. depressa var depressa; G. oppositifolia; G. paniculata; G. crassca; G. rupestris var rupestris)* in Newzealand are distinctly gynodioecious. Kanjilal

(1939) reported three species of *Gaultheria* in Khasi hills of Meghalaya viz *G. fragrantissima, G. griffithiana* and *G. mummularioides* of which he marked with a question mark that *G. fragrantissima* anther are sterile in the dimorphic flowers. This feature has also been reported as early as 1882 by Hooker in *G. fragrantissima.* Several gross aspects of stamen morphology has been studied on a large number of species (Peltrisot 1904; Mathew and Knox 1926; Doyel and Goss 1941; Palser 1963; Herman and Palser 2000; Escaravage *et al.* 2001) however, only few attempts particularly at the developmental level has been made to try to arrive at an understanding of several peculiarities of Ericaceous stamens within the order Ericales (Batygina *et al.* 1963; Ganapathy and Palser 1964; Stushnoff and Palser 1969). There are no studies concerning the ultrastructural aspects of microsporogenesis of male sterile line of some of the gynodioecious plant of *Gaultheria* species (Allan 1961; Franklin 1962; Middleton 1991a; Cambi and Herman 1989; Delph *et al.* 2006). Therefore this chapter deals with the general morphology, development of anther wall, sporogenous tissue, pollen grain formation and ultrastructure of pollen grain in both hermaphrodite and male sterile plants of *G. fragrantissima.*

5.2 Hermaphrodite anther

Stamen 10, 2mm, epipetalous, height up to the middle of the corolla tube; anther 4 lobes, dorsifixed, pinkish red when young and become dark brown during the dehiscence and release of pollen grains. With two distinct apical setaceous horns or awns, Filament, pale white, pilose on the dorsal

side, base flattened (Plate 5.1A). In young flower buds during its developmental stages contained ten anther primordia as episepal condition (Plate 5.1B). The anther primordia are roundish or globular in outline with dark reddish in colour because of the anthocyanin pigments of the anther lobes and became more or less rectangular in shape because of accelerated cell division in the four corners. The young anther is four-lobed. In each lobe 2-3 hypodermal cells become enlarged and differentiate into an archesporial initials. Each archesporial cell is radially elongated with densely cytoplasmic content and prominent large nucleus and nucleolus. Archesporial cells divide periclinally into an outer primary parietal cells and an inner primary sporogenous cells. The primary parietal cells undergo further periclinal divisions to form secondary parietal layers of which the inner secondary parietal layer divides and produce the middle layer and tapetum, whereas the outer one forms the endothecium (Plate 5.1C). Therefore, the anther wall development is dicotyledonous type. Finally, the anther wall constitutes epidermis, endothecium, middle layers and tapetum (Plate 5.1D). The outer peripheral portion of tapetum is formed from the primary parietal layer. The tapetal cells that abutting at the connective tissue arise from the cells of the connective tissues, which are much larger in size and radially elongated while the tapetal layer on the protuberant side of the anther are tangentially elongated. All the cells of the tapetum contain two nuclei or polyploidy nucleus which is bigger in size (Plate 5.1E). Thus, the tapetum has a dual origin: (1) the derivatives of the parietal cells (parietal tapetum) and (2) the connective cells (connective tapetum). Both the parietal and connective

tapetum is of secretory type. The cells become binucleate during prophase-1 of meiotic division in the microspore mother cells, whereas the connective tapetal cells become two to three nucleate condition. The parietal tapetum become dark and loses its contents very earlier than the connective tapetum when the microspores are in tetrads. The parietal tapetum disintegrates a little later when microspores attained their maximum size after disappearance of the callose wall. The primary sporogenous tissues (PST) are derived from the primary development sporogenous cells simultaneously along with the anther wall development (Plate 5.1C). Later on, more or less all the cells of sporogenous tissues undergo several divisions and finally formed the microspore mother cell (MMC) (Plate 5.1C). In the sporogenous tissue, the cell have thin cell wall, within which dense cytoplasm, prominent nucleus are the characteristic features.

During the onset of meiosis in the microspore mother cell, a special callose wall layer is secreted around each microspore mother cell (Plate 5.1F). Before entering into meiosis each microspore mother cells is surrounded by special callose wall which is fluorescent when stained with decolorized aniline blue after exposure to UV light at 430 nm observed under fluorescent microscope. The meiotic division in the microspore mother cells are of simultaneous type (Plate 5.2A), the first arrangement of the four daughter nuclei (Plate 5.2C) leads to formation of isobilateral pollen grains, however this is less frequent and the second arrangement of four daughter nuclei (Plate 5.2D) leads to formation of tetrahedral pollen grain (Plate 5.2B). Tetrads are formed due to the simultaneous formation of centripetal furrows

after meiosis II (Plate 5.2A). Tetrads almost filled the entire space of the anther locules. As soon as cytokinesis is completed, each tetrad was surrounded by thick callose envelope. When callose was dissolved, the microspore do not separated from each other and all the tetrads were arranged in tetrahedral and isobilateral manner. Exine formation and deposition of sporopollenin around pollen grains separated the pollen grains. Histochemically, viable pollen grains are rich in starch (Plate 5.2E) and lipids (Plate 5.2F) deposition. Microsporogenesis precedes megasporogenesis. The tetrads of microspores have already been formed in the anther before meiosis of the megaspore mother cell occurs.

5.3 Ultrastructure of Hermaphrodite pollen grains (SEM and TEM)

Scanning electron micrograph of pollen grains of *G. fragrantissima* showed the compact tetrahedral arrangement of pollen grains. The pollen surface is uneven and rugged, primary apocolpial exine sculpture moderate to coarsely rugulate psilate, the rugulae with minute striate surface somewhat flat, without any secondary sculpture surface somewhat flat, colpus membrane from granulate to smooth (Plate 5.3A).

Transmission electron microscopic studies of hermaphrodite pollen grains are tricolpate with thick exine composed of sporopollenin and intine composed of polysaccharides with three distinct germinal pores (Plate 5.3B). The apocolpial exine is composed of ektexine and endexine. Sexine is thick,

endexine is very thin. The septum is thick. Intine is almost evenly thick around the pollen tetrad, but sometimes comparatively thicker near the colpus region and showed low electron density than the endexine at both apocolpial and septal exine. The aperture region is characterized by a thick foot layer, thin endexine and thick intine (Plate 5.3B & C).

The central cores of the cytoplasm are characterized by the presence of stack of endoplasmic reticulum, mitochondria, lipid bodies and small vesicles with abundance of ribosomes. In addition, the central cytoplasmic core is also characterized by the presence of plastids, many of which contain prominent starch grains. The large vegetative nucleus and generative cell are characteristically found stretched in a linear array through the cytoplasm (Plate 5.3 C).

5.4 Male sterile anther

Filament were recurved to the base of the style, 2 mm, pale white, pilose on the dorsal side, base flattened (Plate 5.4A &B). All the ten stamens released degenerated sporogenous tissues and formed a white unorganized mass of tissues with a tuft of hairy outgrowth at the tip therefore viable pollen are absent, awn or horns are absent, anther lobes have tuft of pilose hairs in the middle (Plate 5.4B). Early anther differentiation in male sterile appears normal till formation of archesporial initials, as described for the corresponding stages in hermaphrodite anthers (Plate 5.4C). These sporogenous cells are large, have dense cytoplasm and large nuclei. However at certain developmental stages, the sporogenous tissue divides

abnormally to form central sterile septum (Plate 5.4D). Although there is normal development of part of anther wall, subsequently the septum and the adjacent sporogenous tissue degraded totally which leads to complete degeneration of sporogenous tissues (Plate 5.4E &F). Upon the degeneration of sporogenous tissues and most cells become disorganized resulting in the disappearance of the typical anther lobes as view under scanning electron microscope (Plate 5.5A). The arrest of development and subsequent degeneration of sporogenous tissues occurred at all stages of anther development from the sporogenous cell stage to the pollen stages. In abnormal pollen grains, microspores cytoplasm became less dense and shrunken, became vacuolated and degenerated leaving various sized aborted microspores without cytoplasm (Plate 5.5B).

5.5 Ultrastructure of Male sterile pollen grains (TEM)

Transmission electron microscopic studies of male sterile pollen grains showed that the pollen grains are distorted in shape due to the irregular projection of exine wall. The exine wall which primarily composed of biopolymer sporopollenin dissolved and disorganized due to polymerization of sporopollenin (Plate 5.5C). Topographically, the ektexine and endexine are distinct, however, tectum and baculae of sexine completely fused and lost their integrity and developed a continous layer of radially oriented membranous granular material above the intine. Intine composed primarily of cellulose and pectin. The outermost stratum of the intine was the most dense and compact and showed a defined microfibrillar structure. Below intine, thick

electron dense materials are deposited which prevents the entry of any substance into the pollen. Cytoplasm becomes condensed with disintegration of nuclear and vacuole membranes. Most of the cell cytoplasm was occupied by insoluble starch and autophagocytic vacuoles (Plate 5.5C).

5.6 Discussion

In Davis (1966) summary of embryological characteristics of the Ericaceae, anther wall development is described as being of Reduced type - neither secondary parietal cell divided; this was based on Davis's interpretation of an illustration of *Gaultheria procumbens* by Chou (1952); however in *G. fragrantissima* the outer secondary parietal layer divides to give rise to endothecium and middle layer, while the inner layer directly functions as tapetum which conformed the Dicotyledonous type and the same has been reported by Cambi and Herman (1989) for eight species of *Gaultheria* and Hermann and Palser (2000) for ten species of Ericaceae. An extensive endothecium that occurs in the anther wall of most angiosperm is rare in the Ericales. The presence of distinct, well developed endothecium in anther wall of *G. fragrantissima* is an unusual feature in the order. However there are also several reports where endothecium are present in few species of Ericales such as *Bejeria, Rhodoihamus* by Copeland (1944), *Phyllodoce* of Rhododendroideae by Kavalijian (1952) and *Enkianthus* by Hermann and Palser (2000).

Batygina *et al.* (1963), who did described anther development in *Vaccinium myrtillus* and *V. vitisidaea* in some detail, reported that tapetal

cells originated from sporogenous tissue. Ontogenetically, in *G. fragrantissima* the tapetum has a dual origin and is glandular in nature, the tapetums are binucleate and sometimes polyploidy that is larger in size. Vijayaraghavan and Marwah (1969) reported that in *N. damascena,* there are 2 to 4 nuclei which later fuse to form polyploidy. Similar behavior of tapetal nuclei has also been reported in *Caltha palustris* (Kapil and Jalan 1962), *Actaea spicata* (Jalan 1963) and *Clematis gouriana* (Vijayaraghavan 1963). Oksala and Therman (1977) also observed a similar formation of octaploid tapetal nuclei in *Eremurus.* Batygina *et al.* (1963), who described anther development in *Vaccinium myrtillus* and *V. vitisidaea* in some detail, reported that tapetal cells originated from sporogenous tissue, however in *Vaccinium serratum,* Venkateswarlu and Maheshwari (1973) reported that the tapetum is parietal in origin. According to Periasamy and Swamy (1966) the tapetum is of dual origin in most of the angiosperms e.g. in *Anemone rivularis* (Bhandari 1968), *Alectra thomsoni* (Vijayaraghavan and Ratnaparkhi 1973), *Celsia coromandelina* (Kapoor *et al.* 1978), *Nepenthes khasiana* (Venugopal and Devi 2003) and some species of Acanthaceae, Scrophulariaceae and Labiatae (Bhandari 1968). In *G. ovatifolia* and *G. shallon* the tapetum disintegrate at the time of meiosis, but in *G. procumbens* the tapetum remains intact until the maturation of the microspores, though they become flattened at the time of meiosis (Chou 1952). In *G. fragrantissima* the parietal tapetum become dark and loses its contents very earlier than the connective tapetum when the microspores are in tetrads.

In *G. fragrantissima* the development of sporogenous into microspore mother cells is simultaneous with anther development which is similar to that reported earlier in *Gaultheria procumbens* (Chou 1952), *Vaccinium atrococcum* (Stushnoff and Palser 1969), *V. nummularia, V. retusum,* and *V. serratum* (Venkateswarlu and Maheshwari 1973), several species of the Phyllodoceae (Ganapathy and Palser 1964), eight species of *Gaultheria* and *Pernettya* (Cambi and Herman 1989) and other ericaceous taxa (Hermann and Palser 2000). In *G. fragrantissima* microspore mother cells before entering into meiosis are being surrounded by a distinct refractive wall, special callose wall that composed chiefly of callose- β-1,3-glucans which is fluorescent when stained with decolorized aniline blue after exposure to UV light at 430 nm when observed under fluorescent microscope. The callose wall gives genetic autonomy, so that the influence of neighboring sporophytic tissue is prevented; at the same time it acts as a barrier or molecular filter, it allows only certain macromolecules e.g. entry of ^{14}C thymidine labelled compounds is prevented (Heslop-Harrison and Mackenzie 1967). In *G. fragrantissima* the development of callose at pollen mother cell stages, and its degradation a little after the completion of meiosis suggests that the callose wall layer isolates the sporogenous tissues from somatic tissues which is an important biological event and highly conserved process in angiosperm (Hong 2005; Feng and Dickinson 2007; Dickinson and Grant-Downton 2009). Exine formation and deposition of sporopollenin separated the pollen grains in *G. fragrantissima.* The synthesis of sporopollenin occurs both in the tapetum an in the cytoplasm of young spores. Mepham and Lane

(1970) considered that in *Tradescantia bracteata,* the entire pollen exine sporopollenin is secreted, controlled and polymerized by the microspore itself, and the tapetum makes no contribution. The pollen exine and sporopollenin plays important roles in protecting pollen grains from various environmental stresses and attacks by pathogens as they move from the anthers to the stigmas and during species-specific adhesion of the stigma cells (Piffanelli *et al.* 1998; Scott *et al.* 2004).

All Ericaceous pollen grains so far studied are binucleate (Safijowska 1960; Brewbaker 1967; Hermann and Palser 2000), pollen grains are shed in tetrads with the exception of *Enkianthus* and few Ericoideae (Erdtman 1952; Safijowska 1960; Palser and Murty 1967) in which single grains occur. The meiotic divisions in the microspore mother cells of *G. fragrantissima* are of the simultaneous type resulting in tetrahedral or isobilateral arrangement of tetrads as is found in many other genera of the family, however polyads are also found in *Chimaphila* (Sarwar 2007). Pollen grains are trinucleate in *G. fragrantissima*. Trinucleate pollens are rare in Ericales however it has been also reported in *Enkianthus sp* by Hermann and Palser (2000).

Pollen morphology of *G. fragrantissima* showed similar characters with other members of Ericales (Erdtman 1952; Sarwar and Takahashi 2009; Lu *et al.* 2009). In *G. fragrantissima* the pollen wall is composed of exine; sexine (tectum and columellae) and nexine (foot layer and endexine), and intine. Tectum and foot layer are relatively thicker and commonly constitute the major portion of sexine and nexine, respectively. In *G. fragrantissima* pollen grains showed tricolporate apertures but 4-aperture also found in members

of some genera having monads and/or tetrads viz., *Enkianthus*, *Erica*, *Rhododendron*, *Kalmia*, *Vaccinium*, *Leucothoe*, *Disterigma* etc., or 5-aperture *Enkianthus* (Sarwar 2007).

Male sterile mutants have been reported in a large number of plant species (Kaul 1988). These include mutants defective in anther morphology, microsporogenesis, pollen development, and pollen function (Van Der Veen and Wirtz 1968; Albertsen and Phillips 1981; Kaul 1988; Regan and Moffat 1990). In *G. fragrantissima* anther differentiation in male sterile appears normal till formation of archesporial initials. Hereafter the development of sporogenous tissues became abnormal where sporogenous tissue divides abnormally to form a central sterile septum and later on the entire tissues degenerates which finally form a tuft of sterile masses of tissues in the anther lobes. Similar type of abnormal differentiation of sporogenous tissues was also observed by Damon (1961) in *Sorghum* carrying cytoplasmic genetic male sterility. Shi *et al.* (2009) observed in *Oryza sativa* L. ssp. *Japonica* pollen abortion first occurs before the pollen mother cell stage, and continues during the entire process of pollen development until pollen degradation. Silva *et al.* (2010) also reported similar development of sterile anther until the end of meiosis with that of fertile anther in *Valeriana scandens*, however after this stage, sterile tetrads do not separate as a consequence of exine fusion between adjacent microspores.

Often it has been difficult to pinpoint a specific time when pollen development aborts in these plants because the microspores do not die synchronously (Regan and Moffat 1990). In *G. fragrantissima* the arrest of

development and subsequent degeneration of sporogenous tissues occurred at all stages of anther development from the sporogenous cell stage to the pollen stages. Abnormalities when occurred at tetrad and free microspore stages the male sterile pollen grains of *G. fragrantissima* are distorted in shape due to irregular projection of exine. The irregular projection of exine was due to dissolution and polymerization of sporopollenin. There are several male sterile pollen grains with defective in exine formation, and they have been characterized in details by several authors. Ariizumi *et al.* (2008) observed in *Arabidopsis thaliana* that the primexine synthesis and probacula formation, which are thought to be the initial steps of exine formation, were defective, and that globular sporopollenin aggregation was randomly deposited onto the microspore at the early uninucleate microspore stage. Luo *et al.* (2006) reported development of pollen grain in cytoplasmic male sterile *Capsicum annuum* where no exine layer was observed except for the callose wall, which resulted in a weak cell wall that would be unlikely to be able to resist internal pressure of the swollen tapetal cells. Jewell *et al.* (1988) also reported irregular pollen development in *Hordeum vulgare* due to abnormal behavior of tapetum. Similarly thick exine and absence of intine has also been reported in sterile microspore of *Viccia faba* by Audran and Willemse (1982).

In *G. fragrantissima*, below the intine thick electron dense materials are deposited which prevents the entry of any substance into the pollen. Hu *et al.* (2007) also observed similar phenomena in *Citrus suavissima* mutant that resulted in the production of sterile pollens. Biasi *et al.* (2001) reported in

Actinidia deliciosa, that the degenerated pollen showed a poorly sculptured sexine, an anomalous nexine, and an intine consisting of a single stratum only which was electron opaque and quite uniform in structure. The cytoplasm of sterile pollen grains in *Helianthus annuus* was without nucleus, vacuolated and possessed degentrated organelles with starch and lipid inclusion (Tripathi and Singh 2008). In *G. fragrantissima* the cytoplasm becomes condensed with disintegration of nuclear and vacuole membranes and most of the cell cytoplasm was occupied by insoluble starch and autophagocytic vacuoles. There are also various factors that have been attributed to the breakdown of microsporogenesis in anther of male sterile plants such as shriveled anther with no pollen grains (Chaudhury *et al.* 1994; Strittmatter *et al.* 2006; Li *et al.* 2010), anthers with non functional pollen grains (Anderson and Symon 1989; Caporali *et al.* 2003) or indehiscent anthers (Dawson *et al.* 1993).

Plate 5.1

A. Longitudinal dissected hermaphrodite flower showing the ten fertile anthers (Fa) with distinct awns (An). Bar = 0.1 cm.

B. Cross section of young flower bud showing ten tetrasporangiate anthers (Ta) and style in the centre (S). Bar = 69.71 µm.

C. Cross section of single young anther lobe showing the initials for endothecium (E), middle layers (Ml), tapetum (T) and primary sporogenous initials (Psi). Bar = 23.58 µm.

D. Transverse section of anther lobe showing the tiered arrangement of epidermis (Ep),endothecium (En), middle layers (Ml) and tapetum (T) and central mass of sporogenous tissue (St). Note the sporogenous tissue with dissected nuclei and dense cytoplasm. Bar = 20 µm.

E. Transverse section of anther lobe showing the microspore mother cells (Mmc) and binucleate tapetum (Bt). Arrows indicate the binucleate condition of tapetal cell. Bar = 20 µm.

F. Callose formation during microspore mother cell stage. Arrows showed auto fluorescence emitted by callose around the microspore mother cells. Bar = 268.80 µm.

Plate- 5.1

***Gaultheria fragrantissima* Wall. Hermaphrodite anther**

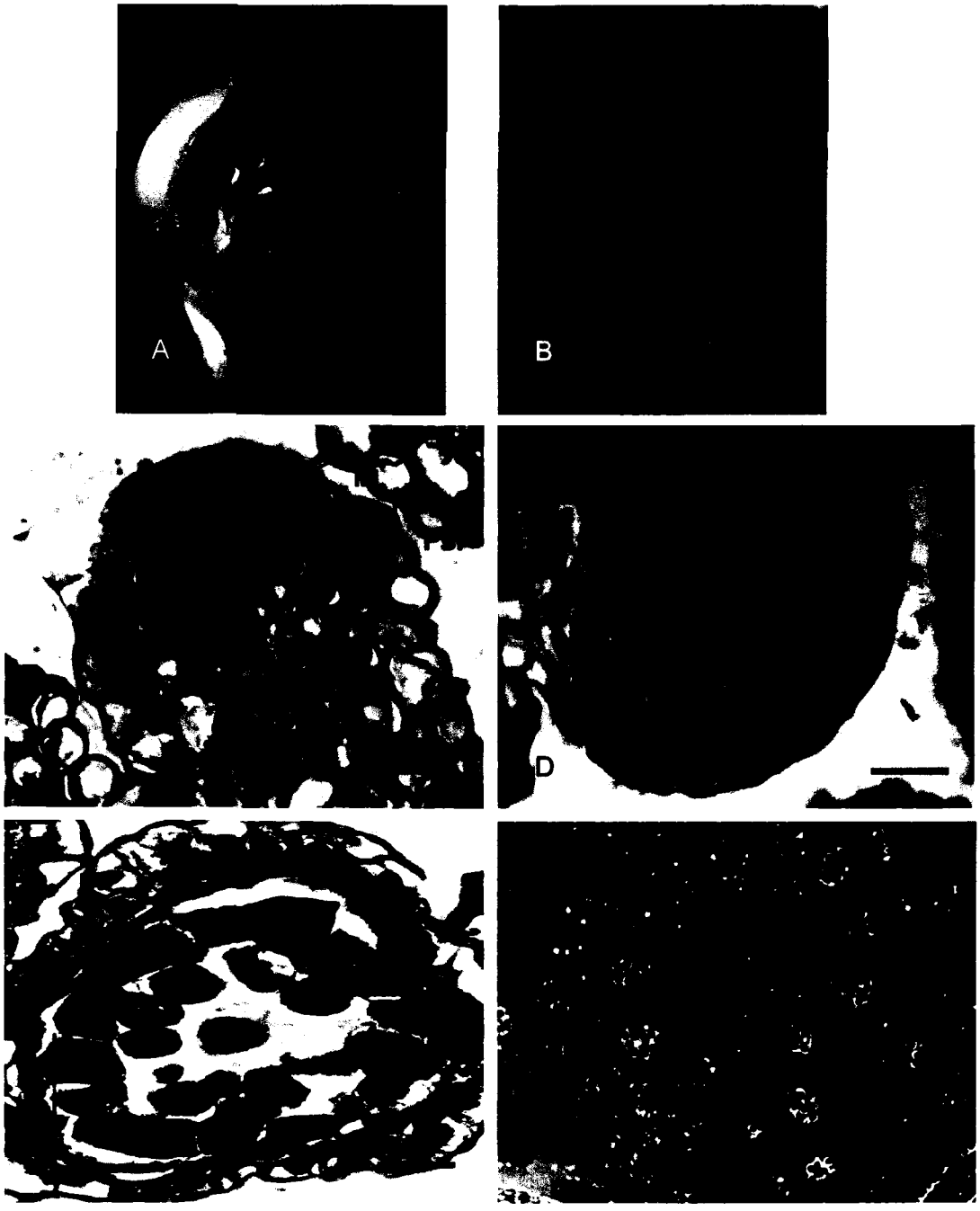


Plate 5.2

A. Cross section of anther lobe showing cell plate formation at the end of meiosis II. Meiosis is simultaneous. Bar =19.23 µm.

B. Emittence of blue fluorescence of tetrahedral pollen tetrad with aniline blue under epifluorescence microscope. Bar = 12.5 µm.

C&D. After meiosis II the four daughter nuclei in pollen mother cells arranged isobilaterally and tetrahedrally results in isobilateral and tetrahedral pollen tetrad respectively. Bar C= 6 µm, D = 6.6 µm.

E. Viable pollen grains (Vp) showing positive reaction with IKI staining. In non viable pollen the starch is absent or very less. Bar = 6.6 µm.

F. Similarly with Sudan III staining the viable pollen (Vp) showing positive reaction. Non viable pollen is with no or less lipid content. Bar = 4.66 µm.

Plate 5.2

***Gaultheria fragrantissima* Wall.**

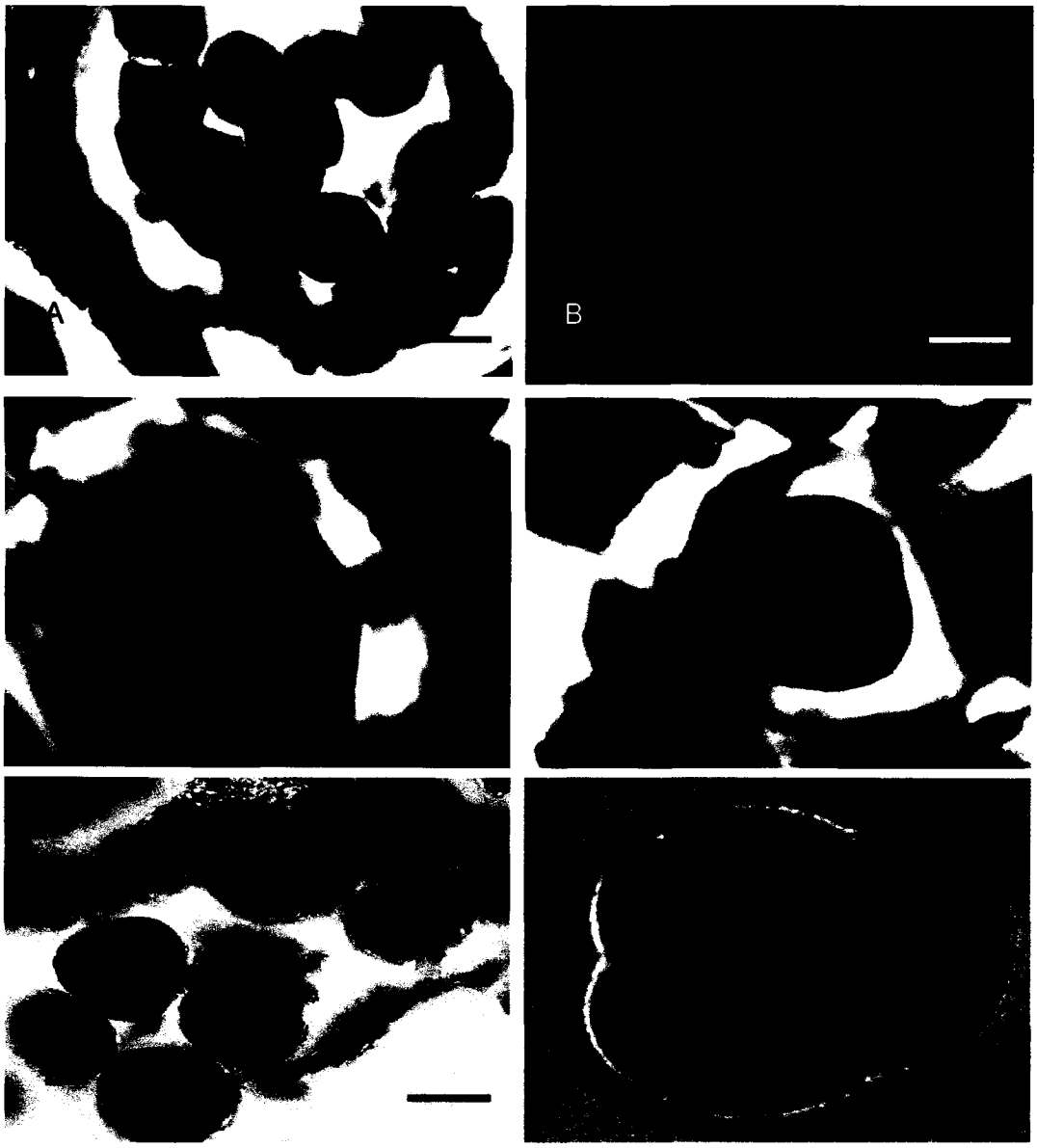


Plate 5.3

A. SEM photograph showing tetrahedral viable pollen tetrads with distinct normal development of exine. Bar =10 µm.

B. TEM photograph of viable pollen tetrads of Hermaphrodite plant with distinct exine and intine. Note abundance of cell organelles like proplastids, ribosomes, mitochondria, and electron translucent lipid droplets and electron dense bodies probably starch, protein etc. Bar 5= µm.

C. An enlarged view (TEM) showing exine organization with tectum, baculum, foot layer and endexine. The tectum is completely coated with pollenkitt and tryphine materials. Note the distinct oblong vegetative nucleus. Bar = 2 µm.

Plate 5.3

Gaultheria fragrantissima Wall.

Plate 5.4

A. Longitudinal section of male sterile flower. Arrow indicates mass of tissue in the male sterile anther (Sa). Note the very long style upto the rim of corolla tube to receive pollen grains. Bar = 0.1 cm.

B. Different views of three dissected stamens showing the filament (Fi) and sterile anther (Sa). Bar = 0.03 cm.

C. Cross section of young undifferentiated anther lobe showing the normal differentiation of hypodermal archesporial initials (Ai). Bar= 23.58 µm.

D. The abnormal behavior of few archesporial initials leads to formation of central septum (S) within the anther lobe. Bar = 20 µm.

E. Degradation of septum as well as anther wall layers and 99% degenerated sporogenous tissue. Bar = 20 µm

F. The entire anther locule is filled with masses of degenerated tissue. Bar = 20 µm.

Plate 5.4

***Gaultheria fragrantissima* Wall. Male sterile anther**

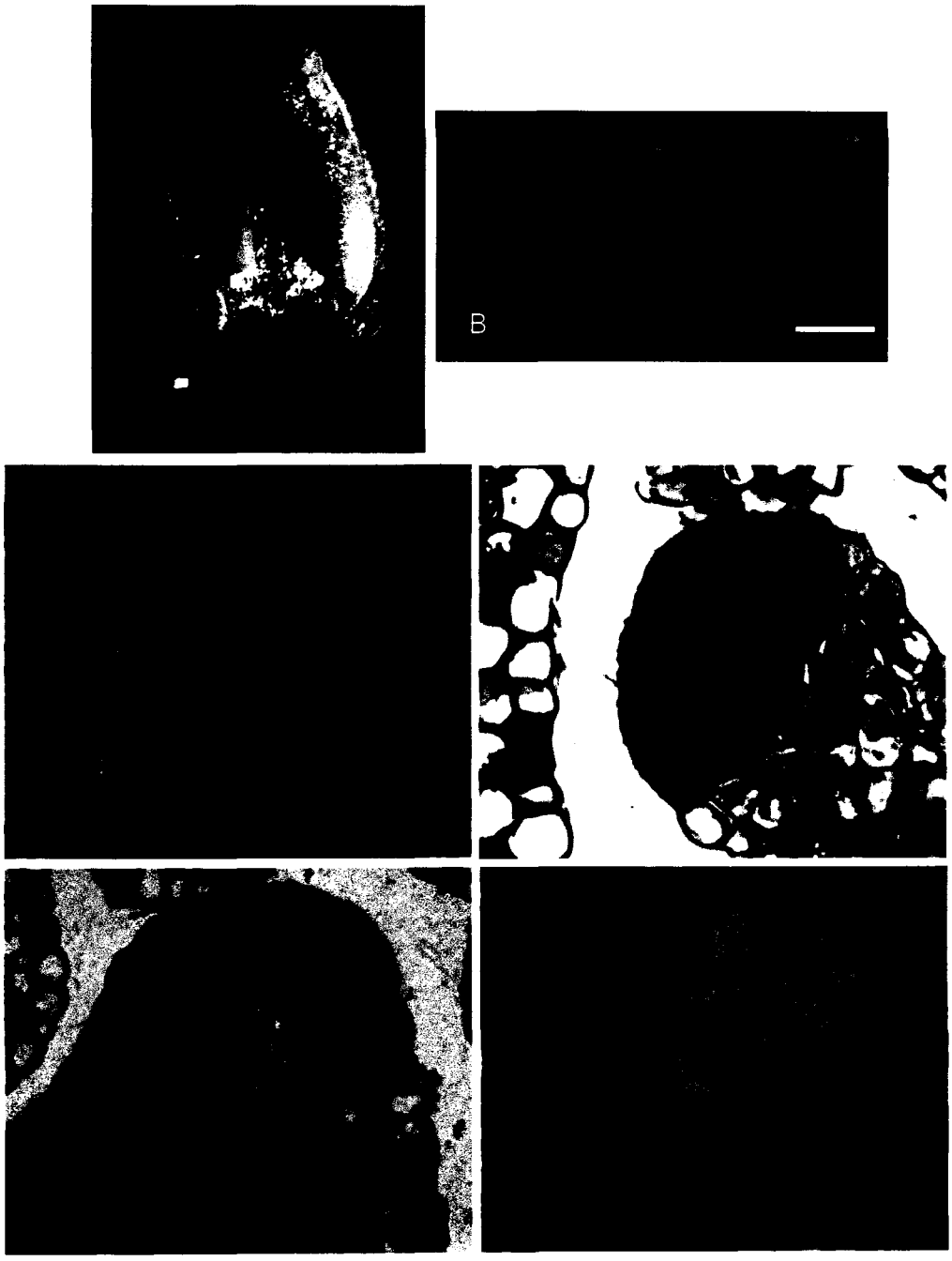


Plate 5.5

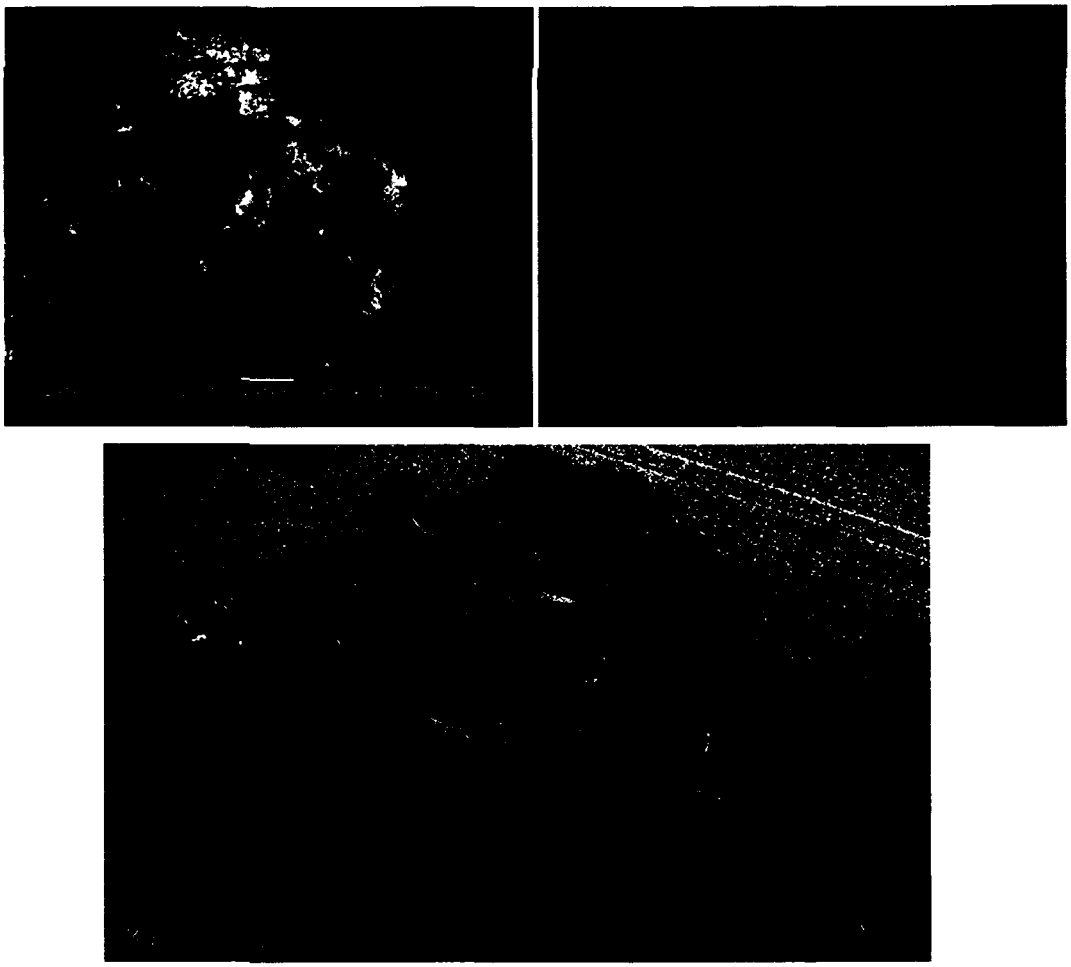
A. Under Scanning Electron Microscope showing a tuft of sterile masses of tissues in male sterile anther. Bar = 20 µm.

B. A few abnormal pollen grains differentiated with irregular projections of exine wall layer under light microscope. Bar = 20 µm.

C. Male sterile pollen grain showing electron dense materials deposited below the intine which prevent entry of any substances into the pollen (TEM); note the presence of starch grains and the extension of exine throughout the surface of the sterile pollen grain. Note the unusual enlargement of intine. Bar = 2 µm.

Plate 5.5

Gaultheria fragrantissima Wall.



CHAPTER – 6

Pollination, pollen viability and pollen germination

6.1 Introduction

Fruit set of most plants depends on successful pollination by wind or animals, but herbivory, nutrient availability, and microclimatic conditions may also be important (Turnbull *et al.* 2000; Eriksson and Ehrlen 1992). Diversity in floral traits, such as color, shape, scent, and size, has developed through the interactions of plants with pollinators (Proctor *et al.* 1996). Other reproductive characteristics, e.g., abundance of pollen and ovule, and nectar production, are also expected to evolve to enhance reproductive success through plant pollinator interactions, producing ecologically varied reproductive systems with two extremes: predominant outcrossing and predominant selfing (Wyatt 1981; Cruden 2000).

Most Ericaceae have two lobed anthers that dehisce by introrse or terminal pores (Curtis 1963; Stephens 2004), an important pre adaptation to buzz pollination in ericads such as *Vaccinium stamineum* L. (Cane *et al.* 1985). Bumble bees (*Bombus* spp.) are important pollinators of wintergreen (Pojar 1974; Reader 1977). Ericaceae in temperate and subtropical latitudes are usually entomophilous, with bees seeking nectar, collecting pollen, or both. In the Neotropics, the flowers of nearly all the superior ovaried taxa are small, usually white (rarely red), urceolate (to rotate), and fragrant, and the plants are found in open, relatively xeric or seasonal habitats. These taxa,e.g., *Arctostaphylos* (Diggs 1995a), *Bejaria* (Clemants 1995),

Comarostaphylis (Diggs 1995b), *Gaultheria* (Luteyn 1995), *Lyonia* (Judd 1981), and *Pernettya* (Heithaus 1983), are pollinated primarily by bees. A small portion of species is also wind pollinated e.g., Empetreae (Kron 1996), *Erica* (Oliver 2000). Interaction between plants and pollinators is postulated as a central mechanism in the diversification of many angiosperm lineages, because evolutionary specialization by plants on their pollinators may drive adaptive divergence in floral traits and may contribute to the origin and maintenance of reproductive isolation (Stebbin 1970; Waser 1998).

The production and the dispersal of pollen have both biological and genetic implications for the quantity and genetic value of the seed produced. Pollen germination and growth of pollen tubes are important research materials for morphological, physiological, biotechnological, ecological, evolutional, biochemical and molecular biological studies (Ottaviano *et al.* 1992). Pollen tube elongation is a dynamic process in which pollen tubes navigate and respond to female tissues to accomplish their mission of delivering the sperm cells for fertilization. Pollen tubes extend exclusively at the cell apex via an extreme form of polar growth, known as tip growth, producing uniformly shaped cylindrical cells (Cheung and Wu 2001). In recent years, pollen germination and pollen tube development are used as materials for determining the importance of cytoskeleton in cell growth and differentiation (Ma *et al.* 2000).

During pollination, pollen grains germinate on the stigmatic surface, each extruding a pollen tube which penetrates the stigmatic tissue and elongates in the extracellular matrix of the transmitting tissue within the style

(Kuboyama *et al.* 1994). Pollen pistil interaction plays a crucial role in the pollen germination and pollen tube entry and probably incompatibility responses (Heslop- Harrison and Shivanna 1977). In species with wet type of stigma, pollen adhesion is mediated principally by the stickiness and surface tension of stigmatic exudates (Swanson *et al.* 2004). Recognition of compatible pollen grains on the stigmatic surface and their adhesion at stigma maturity are conditioned by proteinaceous, lipidic, carbohydrate, and ionic components of pollen coat and stigma surface during the onset of these early events in pollen–stigma interaction. The receptive stigma surface is characterized by the expression lipidic constituents, biomolecules such as peroxidases, esterases, and reactive oxygen species (predominantly H_2O_2) for determining the specificity of pollen (McInnis *et al.* 2006; Shakya and Bhatla 2010; Ismailoglu and Unal 2012). In addition to, biomolecules such as reactive oxygen species, nitric oxide, gamma-aminobutyric acid, and cysteine-rich proteins have been identified to play, yet to be investigated, roles during pollen–stigma interaction process (Palanivelu *et al.* 2003; Prado *et al.* 2004; Hiscock and Allen 2008).

The duration of pollen viability varies greatly between species, and is related to the type of pollination. In general, plants with entomophilous pollination have pollen with longer viability than those with anemophilous pollination (Bassani *et al.* 1993). Pollen viability is related to characteristics such as environmental humidity and temperature. The pollen of a given species is programmed to have a high probability of survival in the environment in which the plant grows (Ottaviano and Mulcahy 1989). For

certain characters of pollen grain morphology, such as the number of germinal pores, affect its physiological properties (Dajoz *et al.* 1991). The most widespread type of dichogamy among the members of the Ericaceae family is protandry, in which the male function precedes the female. Dichogamy is the temporal separation of male and female functions within a flower (Bertin and Newman 1993). Vander Kloet (1988) indicated that *Vaccinium* flowers are generally protandrous, and that the onset of stigmatic receptivity is 'heralded' by the presence of creamy exudates. Wood (1962) investigated flower receptivity of V. *angustifolium* and found that flower receptivity declines with increasing the age of flower. In *Vaccinium ashei* stigma receptivity doesn't correlate with fruit set (Brevis *et al.* 2006).

Thus understanding of various factors influencing pollen germination and tube growth are a prerequisite for the success of hybridization programme (Vasil 1964; Mercy *et al.* 1978). Viable pollen will help in giving successful result for plant breeders in breeding programme which requires huge expenditure of time as well as money. Some of the factors which influence pollen germination and tube growth in plants are carbohydrate (sugar), boron, calcium, enzyme, plant hormones, magnesium, potassium, agar, pH and certain physical factors such as light and temperature. In some species pollen grains germinate in wide ranges of relative humidity and temperature, some pollen germinates in basic medium containing 10% sucrose and 0.01% boric acid *in vitro* (Vasil 1960; Unal 1988; Dane and Olgun 1994). The media used for pollen germination vary according to the plant species (Vasil 1960; Baker and Baker 1979). Pollens of some species

need more complicated media (Cetin *et al.* 2000). The required environment for pollen germination in vitro is related to genetic composition and also the quality and quantity of nutrient reserves of pollen (Baker and Baker 1979).

Gynodioecy is most prevalent in *G. fragrantissima*. In all angiosperms and gymnosperms seed set is depending upon pollen viability, pollen germination and pollination. Thus this chapter specifically meant for pollen germination, pollen viability and pollination.

6.2 Pollination

G. fragrantissima blooms in the month of April to July as does most of the flora in Lum Shyllong. Both hermaphrodite and male sterile plants initiates flowering simultaneously with axillary racemose and sometimes panicle inflorescences. Inflorescence maturation was acropetal and lasted from 20 to 50 days in hermaphrodite and 15 to 45 days in male sterile and open the whole day. Anther dehiscence began before anthesis indicating the proandrous condition in *G. fragrantissima*. The pollen grains of hermaphrodite plants contained lipidic substances on their surfaces and stored starch. Pollen grains are rich in pollenkitt. Flowers of both types have a pleasant smell of methyl salicylate. Based on the number of visits (Table 6.3; Fig 6.3) and time spent on individual flower on sunny weather condition, we considered *Apis* sp. (Honey bees) (Plate 6.1 A) as the primary pollinators (most frequent) and *Bombus* sp. (bumble bee) (Plate 6.1 B), Formicidae (ants) (Plate 6.1C), Diptera (flies) (Plate 6.1D), and Lepidoptera (butterflies) (Plate 6.1E& F) are secondary pollinators (less frequent). These pollinators

collected pollens and nectar and transported the pollen from one flower to another, with activity peaking from 1100 to 1500 h. Bees in pursuit of nectar secreting zone hold the corolla with their legs tightly and shake it, thereby jarring the anther facilitating self pollination. Their movement is intensified by the awns hitting against the corolla which shakes pollen (tetrads) out through the pores. The presence of appendage anther or awns in *G. fragrantissima* facilitates self pollination in hermaphrodite plants. Appendages that occur on the apex of the mature anther are designated as awns.

Bumble bee holds the corolla with their legs and shakes it (Buzz pollination). This vibrates the awns and jars pollen out of the anther onto the abdomen of the bees. Formicidae (Ants) visited both male and female flowers and made contact with the plant reproductive organs when foraging for floral resources, acting as potential pollinators. None of them destroyed floral organs. Some of the visitors foraged more frequently and spent more time in flowers of hermaphrodite plants. Dipterans also used the same posture that the bees employed for collecting nectar but only their frequency of visit is rare. The Lepidoptera species behaved as nectar thieves. They landed on the petals without touching the stigma and collected nectar from the floral tube with their proboscis, remaining on each flower for only a few seconds. Beetles were found moving within and between the same flower of hermaphrodite plant and stays on the floral chamber all the time (Plate 6.1G). They fed on nectar as well as pollen. Enlarged view (SEM) (Plate 6.1H) showed that few pollens are attached in the lower abdomen of the insect thereby facilitate self pollination in hermaphrodite plant. Viscin threads are

also seen attached with lower abdomen of insect with one or two pollen grains attached to the thread. The occurrence of rain and cloudy weather reduces numbers of visits by pollinators. Formicidae, however visit the flowers in all weather conditions. Besides, we have several classes of insect of the class Coloeptera, Orthoptera, Odorata and Hemiptera who come and visit the plant but have no role in pollination and spent most of the time on leaves and stems and hence considered as visitors. Their visits are due to the aromatic methyl salicylate presence in the leaves and flowers.

6.3 Pollen viability

In *G. fragrantissima* pollen grains are united into spheroids or sub spheroidal tetrahedral tetrads (Plate 6.2A). The tetrads range from 27.8-30.0 µm in diameter and the pollen grain are tricellular. In freshly collected flowers at the time of anthesis the pollen grains showed 75 % of the pollen grains are viable under the Fluorochloromatic Reaction (FCR) test. Viable pollen grains emit greenish yellow fluorescence under UV light at 494 nm under fluorescent microscope and decreases rapidly about 10 % the next day (Plate 6.2B).

6.4 Pollen germination

In *G. fragrantissima,* pollen grains began germination after 19 hours in Brewbaker and Kwacks medium. Not every pollen grains of a tetrad forms a pollen tube. Generally 1-2 tubes per tetrad have been observed, but 3 tubes

per tetrad also occurred (Plate 6.2C). Pollen grain show maximum germination after 19 hours at an average length of 50-60 µm with germination percentage of 40.50 ± 3.13%.

In vitro pollen germination percentages varied in different concentration of modified Brewbaker and Kwacks medium. Table 6.1 summarized data on the effect of sucrose (3%,10%,12%,15 %), calcium ($Ca(No_3)_2$ - 120 ppm, 20 ppm, 250 ppm, 300 ppm), boron (H_3BO_3 - 30 ppm, 80 ppm, 120 ppm, 160 ppm), potassium (KNO_3 - 100 ppm, 200 ppm, 250 ppm, 300 ppm), magnesium ($MgSO_4$. 7 H_2O - 50 ppm, 150 ppm, 200 ppm, 250 ppm), pH (5.0; 6.4; 7.0; 7.3) and temperature (15 °C, 20 °C, 25°C, 30 °C) on pollen germination and pollen tube length incubated for 19 hours in dark in BOD incubator at 90% RH. There was no germination of pollen grains in the light condition.

The first factor tested was sucrose concentration in the medium. Out of the four different concentrations of sucrose, individually 3 % sucrose showed 42.60±2.81 % germination. On the other hand 10 % and 12% of sucrose showed relatively high pollen germination of 64.50±3.17 % and 64.40±3.36 % respectively which are not significantly different with one way analysis of variance (ANOVA) at $p \leq 0.05$ using Tukey's pos hoc test. The 15 % was not favorable for pollen germination. Pollen tube elongations in all the different concentrations of sucrose were similar and are not significantly different ($p \leq 0.05$) (Table 6.1; Fig 6.1a&b).

It is observed that if the amount of calcium nitrate is increased in the medium there was gradual increase in pollen germination but subsequently

pollen germination decreased at higher concentration. Pollen germination was higher at 200 and 250 ppm of calcium with 76.40±1.32 % and 72.20±1.15 % respectively and is not significantly different (p≤ 0.05) however they differs significantly (p≤ 0.05) in their pollen tube length where it is 101.31±11.10 µm in 200 ppm and 61.53±4.86 µm in 250 ppm. Thus maximum pollen germination (76.40±1.32 %) and tube elongation (101.31±11.10 µm) was observed at 200 ppm of calcium. At 300 ppm there is a gradual decrease in germination and tube elongation (Table 6.1; Fig 6.1e& f).

There were no significant differences (p≤ 0.05) in germination percentage and tube elongation in the three different concentrations of boron. 120 ppm of boric acid showed higher germination (72.00±3.04 %) where as 30 ppm and 80 ppm showed 63.80±1.29% and 64.30±3.13% germination respectively. At 160 ppm there is no germination at all (Table 6.1; Fig 6.1 c& d).

Pollen germination occurred only in 100 ppm and 200 ppm of potassium and there was no germination in both 250 and 300 ppm. 100 ppm of potassium nitrate account 48.8±3.55% germination, 200 ppm showed 63.5±1.69% germination and are significantly difference (p≤ 0.05), however there was no significant difference (p≤ 0.05) in their pollen tube length (Table 6.1; Fig 6.2 a &b). Of the different concentrations of magnesium, 150 ppm showed 47.50±2.39 % germination with 55.37±6.98 µm pollen tube length. There was no pollen germination in the three different remaining concentrations (Table 6.1; Fig 6.2 c &d).

Variation in pollen germination among the different pH treatments was high. There was no germination of pollen at pH 5.0; pH 6.4 and pH 7.0 showed considerable increase in germination percentage but reduced at pH 7.3. Interestingly, the percentage of germination differ significantly ($p \leq 0.05$) in all the three different pH, however there are no significant difference in pollen tube length. pH 7.0 relatively showed high percentage of germination (Table 6.1; Fig 6.2 e &f). Pollen germination was not at all occurred in the temperature of 15 °C, 20 °C and 30 °C. The pronounced germination of pollen and pollen tube elongation was observed only at 25 °C (Table 6.1; Fig 6.2 g &h).

Based on modified Brewbaker and Kwacks medium, the optimum pollen germination was with the following concentration (i.e 10% sucrose, 120 ppm boron, 200 ppm calcium, 200 ppm potassium, 150 ppm magnesium) at pH 7.0 at 25 °C for 19 hours in dark at 90% RH in BOD incubator.

6.5 Discussion

Bees play an essential role in the pollination of *G. fragrantissima* that promote crossing and selfing as in most Ericaceae (Escaravage and Wagner 2004; Evans and Spivak 2006; Johnson and McQuillan 2011). Bawa (1990) estimated that 89–99% of all flowering plant species in tropical lowland rainforest are pollinated by animals, and bees are the most important pollinators (Roubik 1995; Renner 1998). The fact that staminal appendages, poricidal dehiscence, and introrse orientation are so widespread in the

Ericaceae suggests that they must serve some important function. The most likely possibility would seem to be a role in pollination (Artopoeus 1903; Judd 1979; Dorr 1981). Matthews and Knox (1926) correlated the development of tubules having apical dehiscence with pollen presentation at the mouth of the corolla. Similarly the presence of appendages or awn in the anther of *G. fragrantissima* promotes self pollination as when the bees hold the corolla tube hit the tip of the appendages thereby jarring the anther. Hagerup (1954) observed that not only the appendages help the flowers to predominately self fertilized, but also that the appendages were adaptation for what he called wind autogamy rather than for entomophily.

Buzz pollination or sonication of flowers by bees has been associated with porocidal anthers such as those in the Ericaceae. The introrse and porocidal anthers in *G. fragrantissima* enable bumble bees (*Bombus* sp.) to perform Buzz pollination although the visit is not so frequent. There are also reports of bumble bees as pollinator in wintergreen *Gaultheria procumbens* (Pojar 1974; Reader 1977) and in ericads such as in *Vaccinium stamineum* L. (Cane *et al.* 1985). Buzz pollination occurs when a bee vibrates its thoracic flight muscles over the anthers, vibrating dry pollen onto its body (Harder 1998; Houston and Ladd 2002; Thorp 2000). Many genera of Ericaceae e.g., *Arbutus*, *Arctostaphylos*, *Cassiope*, *Erica*, *Kalmia*, *Vaccinium* etc. are known as buzz pollinated (Buchmann 1983; Knudsen and Olesen 1993; Mahy *et al.* 1998; Houston and Ladd 2002; Escaravage and Wagner 2004).

Naskrecki and Colwell (1998) observed that nectar thieving *Rhinoseius* mites spend virtually their entire life cycle within the flowers of

certain hummingbird pollinated Vaccinieae, with only a single exception known. The mites depend not only upon the flowers as their source of food, shelter, and a place for reproduction, but also upon the birds themselves, because their primary means of dispersal between plants is on the bills and in the nasal cavities of hummingbirds (Colwell 1995; Murcia 2000) .Similar is case of *G. fragrantissima* the presence of minute thrips in each floral chamber of hermaphrodite plant reveals that it is totally depend either on pollen, stigma exudates or nectar for food as it entirely spent its life cycle inside the floral chamber. Beetle in turn facilitate the process of self fertilization probably through it constant movement for harvesting of food that carries little pollen in the lower abdomen through the attachment of viscin thread and their legs.

The existence of a general relationship between pollen morphology and the pollen vectors of anemophilous and entomophilous species has suggested by Wodehouse (1935). The viscin threads are of great importance in the pollination of entomophilous angiosperms (Hesse 1981). Viscin threads, i.e., noodle-like, acetolysis resistant structures consisting of sporopollenin that arise from the exine of a pollen grain usually from the proximal occur only in three distantly related angiosperm families: Onagraceae, Ericaceae, and subfam. Caesalpinioideae of Leguminosae (Skvarla *et al.* 1978; Graham *et al.* 1980; Sarwar *et al.* 2005). In Ericaceae, viscin threads are present on the pollen grains of tribes Bejarieae, Phyllodoceae, and Rhodoreae of subfam. Ericoideae, and the genus *Gaylussacia* of the tribe Vaccinieae, subfam. Vaccinioideae (Waha 1984;

Kron *et al.* 2002). Viscin threads are also seen attached with lower abdomen of insect with one or two pollen grains attached to the thread in *G. fragrantissima* during pollination.

Faegri and Pijl (1979) suggested that in insect pollinated plants, nectar and pollens are the major rewards and are presented only at certain times. In *G. fragrantissima* besides nectar and pollens as major rewards there are also some forms of floral advertisement in the form of floral cues (color and shape) and olfactory cues (scents). The relationship between petal size and flower number (floral display size) and pollinator visitation has been especially well investigated (Dafni *et al.* 1997). Flower of *G. fragrantissima* emits highly detectable odors methyl salicylates which advertise flowers to visiting insects, beetle and flies. This can be witness by several visits of Coloeptera, Orthoptera, Odorata and Hemiptera which are not so important pollinator taxa. Similarly floral scents that are also enhancing pollinator activity have been reported in several taxa and species (Dodson *et al.* 1969; Groth *et al.* 1987; Bergstrorm *et al.* 1992; Dobson *et al.* 1997). There are researchers who have investigated which compounds in floral scents are responsible for pollinator attraction, both ethologically (Haynes *et al.* 1991; Heath *et al.* 1992; von Helversen *et al.* 2000) and physiologically (Thiery *et al.* 1990; Gabel *et al.* 1992; Raguso and Light 1998; Takashi and Yafuso 2003).

Yamashiro *et al.* (2008) have shown that Diptera are significant pollinators within the Tylophora– Vincetoxicum complex in Japan and appear to be responsible for some speciation within the group. While Medeiros *et al.*

(2008) have identified disease carrying black flies (Simuliidae) as pollinators of *Tassadia* spp. in Brazil. The biodiversity of plant pollinator interactions continues to surprise us in its breadth and novelty. Although some studies have shown that ants do act as pollinators of some plant species (Peakall *et al.* 1987; Gomez and Zamora 1992), the overall effectiveness of flower visiting ants has been underestimated due to their low per visit efficiency. Some authors contend that ants cannot carry pollen between different plants, resulting in a high frequency of self-pollination, because they are wingless and very small (Holldobler and Siegel 1984; Schurch *et al.* 2000). The resulting self pollination usually provokes high rates of seed abortion in many plant species, although in some it can lead to the production of progeny with low relative performance (Peakall and Beattie 1991; Ramsey 1995; Puterbaugh 1998). Accordingly, this preliminary studies of ant as a possible pollinator in *G. fragrantissima* can furnished some more evidence highlighting the prominent role that ants can play in some plant pollinator systems (Wyatt 1981; Peakall *et al.*1987; Peakall and Beattie 1991; Gomez and Zamora 1992; 1999; Ramsey 1995; Puterbaugh 1998; Gomez 2000; Ashman and King 2005; Sugiura *et al.* 2006). Floral morphology, nectar characteristics and floral scents could be playing crucial roles in establishing mutualistic pollination interactions in *G. fragrantissima* and may be critical for understanding the evolution of pollination systems in this poorly known genus.

Pollen viability is considered as an important parameter of pollen quality (Dafni and Firmage 2000). *G. fragrantissima* showed high pollen

viability (75%) but remain viable for only a short period that was similarly reported by Nepi and Pacini (1993), Kearns and Inouye (1993) where germination of pollen grain is most successful immediately after anthesis and viability deteriorates rapidly in most species (Nepi and Pacini 1993; Pacini *et al.*1997; Tangmitcharoen and Owen 1997). The decrease in pollen viability over a period of time is due to the loss of integrity of the plasmamembrane of the pollen and reduced activity of esterase capable of hydrolyzing the fluorescein ester (Heslop-Harrison and Heslop- Harrison 1970). Freshly collected pollen grains just after anthesis gave optimal pollen germination and tube elongation in *G. fragrantissima* and decreased 10% the next day. Pollen germination was not occurred in the stored pollen grains of *G. fragrantissima.* This indicated that pollen decreased with time as reported by Pacini *et al.* (1997) where pollen grains of *Cucurbita pepo* was not viable after 48 hours and that of *Mercurialis annua* and *Festuca arundinacea* after 72 hours. In general, plants with entomophilous pollination have pollen grains with longer viability than those with anemophilous pollination (Pacini *et al.* 1997). Trinucleate pollen grains survive for shorter duration than binucleate pollen grains and take longer time for germination *in vitro* (Stanley and Linskens 1974; Mulcahy and Mulcahy 1983). Similarly in *G. fragrantissima* pollination is entomophilous and pollen grains are tricellular, therefore it took 19 hours for pollen grains to germinate.

Sucrose is the best carbohydrate source for pollen germination, having its function in maintaining osmotic pressure of the medium and acting as a substrate for pollen metabolism (Johri and Vasil 1961; Zhang 2000). In

the current work, different gradients of sucrose concentrations exert strong effects on *G. fragrantissima* pollen germination. However, *G. fragrantissima* pollen shows the highest germination rate at a sucrose concentration of 10-12 %. At low sucrose concentration (3%) pollen germination is low and at higher concentration (15%) there is no germination. Thus 10-12% sucrose is the most suitable concentrations for pollen germination of *G. fragrantissima* which does not inhibits or disrupts pollen germination. Similarly Prajapati and Jain (2010) showed in *Luffa aegyptica* 10% sucrose is the optimum concentration of sucrose required for maximum pollen germination. There are also several reports where 10% sucrose concentration showed optimal pollen germination in other species like *Najas marina* (Jain and Shah 1991), *Tradescantia paludosa* (Tanaka 1981), *Corylus avellana* (Daley 1985), *Bambusa vulgaris* (Koshy and Jee 2001), *Cleome gynandra* (Soni *et al.* 2010) and *Bombax ceiba* (Bhattacharya and Mandal 2000). Ghanta and Mondal (2012) reported maximum pollen germination of *Solanum macranthum* in 15% sucrose concentration. At suitable sucrose concentrations, the balance between the internal and external osmotic pressures of pollen can be maintained, thereby preserving the normal vitality of pollen (Johri and Vasil 1961; Zhang 2000; Bhowmik and Datta 2011; Montaner *et al.* 2003).

Ca^{2+} are signaling molecule and are essential requirement of pollen tube growth (Bendnarska 1989). In the present work, the pollen of *G. fragrantissima* exhibited maximum pollen germination and tube growth at a higher concentration of calcium nitrate i.e 200 ppm than that in the original

Brewbaker and Kwacks medium. The results indicate that calcium was the most effective to influence the pollen germination in *G. fragrantissima*. The calcium nitrate concentrations required for optimal germination varies from species to species e.g *Crescentia cujete* require 100 ppm of calcium (Ghanta and Mondal 2013), *Luffa aegyptica* require 350 ppm of calcium (Prajapati and Jain 2010). In *G. fragrantissima* calcium affected the pollen tube growth dramatically but had no influence on rate of pollen germination, this was reflected with the work of Yao and Zhau (2004) on effects of calcium on pollen germination and pollen tube growth of *Torenia fournieri* where calcium play significant role in pollen tube growth. Similarly Chu *et al.* (2005) observed that calcium play an important role in pollen germination and pollen tube growth of *Luffa cylindrica*. Brewbaker and Kwack (1963) and Holdaway-Clarke and Hepler (2003) thought that calcium probably helps in the building of pectic regions in pollen tubes, thus increasing rigidity and stability. This prevents the grains from rupturing and assists in the formation of straight tubes. Absence of calcium in the medium results in an increase in the membrane permeability leading to the loss of internal metabolites (Shivanna 1979).

There was no significant difference in the three different concentrations of boron on pollen germination and pollen tube growth of *G. fragrantissima*. From the comprehensive analysis of the effects of boron on *G. fragrantissima* pollen germination indicated that the optimum requirement for boron is 80-120 ppm as it showed relatively higher rate of germination. Boric acid is known to be crucial for pollen germination and tube growth and

the optimal requirement for most of the species range from 100-120 ppm most of the species e.g in *Solanum melongena* (Vasil 1964), *Lawsonia inermis* (Mondal and Ghanta 2012), *Solanum torvum* (Biswas *et al.* 2013). As in the case of *G. fragrantissima*, 80-120 ppm is probably the optimal requirement of boron for pollen germination and pollen tube growth in most of the species as it has been shown that boron increased the uptake of oxygen during respiration of germinating pollen (O 'Kelley 1957) and also in combination with sugar it form a sugar-borate complex (ionizable) which is translocated with greater facility than are non-borated, non-ionized sugar molecules (Gauch and Duggar 1954). Boron is also said to be involved in the synthesis of pectic materials for the wall of the actively elongating pollen tubes (Stanley and Lichtenberg 1963; Stanley and Loewus 1964). Regarding the effect of potassium on *invitro* pollen germination of *G. fragrantissima* it is observed that optimal requirement for pollen germination was 200 ppm. *G. fragrantissima* pollens geminated only in 150 ppm of magnesium where as Prajapati and Jain (2010) showed that the optimal requirement of magnesium for pollen germination of *Luffa aegyptica* was 200 ppm. Thus optimal requirement of potassium and magnesium for *G. fragrantissima* pollen grains is very essential as the pollen germination and pollen tube growth are significantly regulated by the transport of inorganic ion, such as magnesium and potassium ions, across the plasma membrane of the pollen and pollen tubes.

Pollen grains germinate in a wide range of pH but the best result are obtained at pH 5.5- 6.5 (Johri and Vasil 1961), but in *G. fragrantissima* the

optimal requirement of pH for pollen grains to germinate is 7. Interestingly, a slight change in pH (7.3) reduced the rate of pollen germination, this indicates that *in vitro* germination of pollen of *G. fragrantissima* is very sensitive to pH. At low pH (5) there was also no germination. The present findings corroborate the findings of Cox (1983) where acidity of the substrate always exerts a negative effect on the pollen's germinability. It was also reported by Acar *et al.* (2010) that pollen germination and pollen tube growth of *Pistacia* sp was greatly reduced when grown at lower pH. Pollen germination can be substantially altered by the temperature. Pollen grains of most plant species showed optimum germination and tube growth between 20-30 °C (Johri and Vasil 1961). In case of *G. fragrantissima,* pollen grains germinated only at 25 °C which is similar to that of *Nepenthes khasiana* as reported by Devi (2004). Burke *et al.* (2004) reported an optimum temperature of 28 °C for *in vitro* pollen germination with greenhouse grown cotton cultivar Gregg 65. Suy (1979) and Barrow (1983) have shown that *in vivo* pollen germination and pollen tube penetration was inhibited at 30 °C, suggesting that 25-30 °C is the optimal requirement for proper pollen germination and pollen tube growth. Sexsmith and Fryer (1943), Vasil (1960) and Staudt (1982), have also reported that maximum *invitro* pollen germination and pollen tube growth were obtained between 25-30°C. Beyhan and Serdar (2008) reported that different environmental conditions effect *in vitro* pollen germination of *Castanea sativa.* In *G. fragrantissima* light has least effect on pollen germination, however, humidity plays an important role in germination because there is optimal germination of pollen when relative

humidity of BOD incubator is maintain at 90% RH. This present results correlated with the findings of Dafni *et al.* (2005), Wang *et al.* (2005) and Nyine and Pillay (2007).

Table 6.1 Effect of Modified Brewbaker and Kwack's Medium (MBK) on pollen germination (%) and pollen tube length (μm).

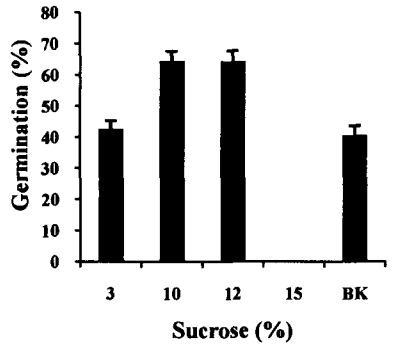
	MBK (ppm)	Germination %	Pollen tube(μm)
BK		24.00-56.00 (40.50±3.13) [a]	47.34-74.44 (60.57±5.19)[a]
Sucrose (%)	3	33.00-63.00 (42.60±2.81) [a]	45.09-65.09 (55.58±3.18)[a]
	10	50.00-80.00 (64.50±3.17) [b]	43.78-65.00 (60.57±3.40)[a]
	12	45.00-82.00 (64.40±3.36) [b]	45.20-76.83[a] (57.39±5.65)
	15	0	0
Calcium nitrate (ppm) $Ca(NO_3)_2$	120	57.00-68.00 (63.10±1.12)[a]	48.35-76.23 (66.60±5.85)[a]
	200	69.00-82.00 (76.40±1.32)[c]	75.30-128.50 (101.31±11.10)[b]
	250	68.00-78.00 (72.20±1.15)[c]	45.23-72.00 (61.53±4.86)[a]
	300	56.00-70.00 (61.30±1.45)[a]	48.00-53.20 (50.45±1.06)[a]
Boric acid (ppm) H_3BO_3	30	58.00-70.00 (63.80±1.29)[a]	45.00-70.02 (59.14±4.31)[a]
	80	56.00-75.00 (64.30±3.13)[a]	47.23—73.20 (60.57±5.19)[a]
	120	57.00-85.00 (72.00±3.04)[a]	60.80-67.00 (64.09±1.09)[a]
	160	0	0
Potassium nitrate (ppm) KNO_3	100	29.00-66.00 (48.8±3.55)[a]	48.64-75.99 (59.70±5.58)[a]
	200	57.00-75.00 (63.5±1.69)[b]	48.67-73.90 (56.31±4.55)[a]
	250	0	0
	300	0	0

Magnesium sulphate (ppm) $MgSO_4.7H_20$	50	0	0
	150	23.00-66.00 (47.50±2.39)[b]	43.78-78.33 (55.37±6.98)[b]
	200	0	0
	250	0	0
pH	5.0	0	0
	6.4	51.00-75.00 (60.4±3.16)[b]	25.46-87.44 (58.87±11.00)[b]
	7.0	45.00-83.00 (72.±1.49)[c]	38.50-74.00 (58.59±8.08)[b]
	7.3	39.00-64.00 (49.4±1.73)[d]	44.23-70.06 (54.31±4.69)[b]
Temperature (°C)	15	0	0
	20	0	0
	25	37.56-51.70 (42.25±2.54)[b]	43.32-69.00 (57.95±5.08)[b]
	30	0	0

Note: Values in the parenthesis are mean ± standard error. One way analysis of variance (ANOVA) showing significant difference at $p \leq 0.05$ using Tukey test. Values with the same letters does not differ significantly.

Figure 6.1

Fig: Graphical representation of pollen germination (%) and pollen tube length (µm) of *G. fragrantissima*.



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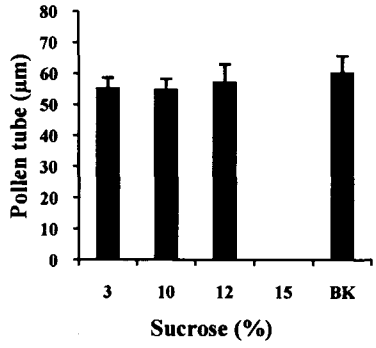

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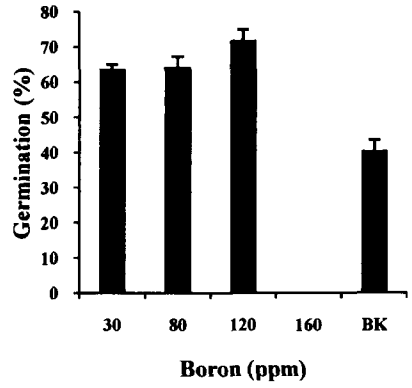
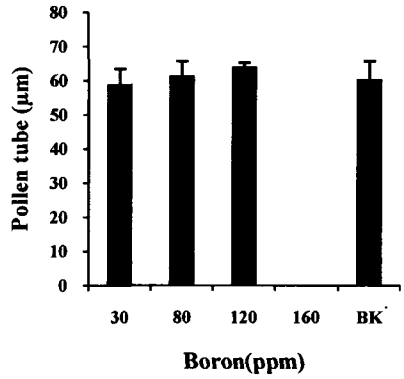


c



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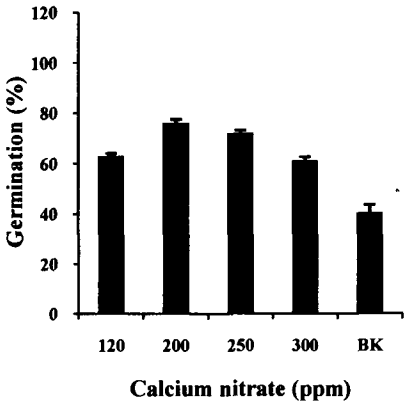

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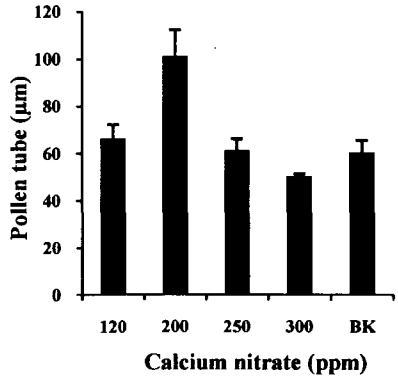

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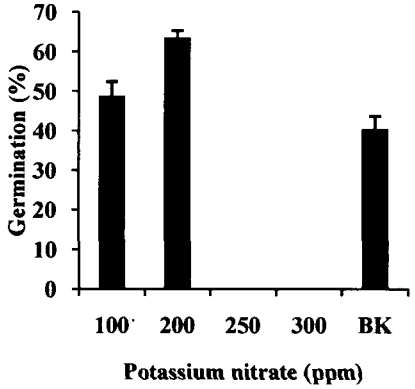
Figure 6.2



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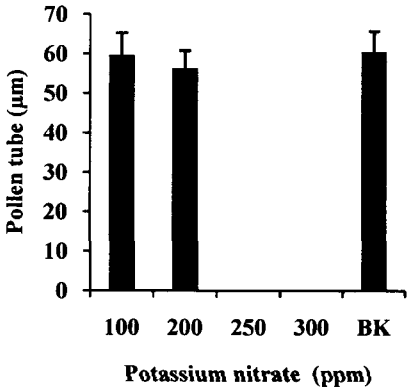

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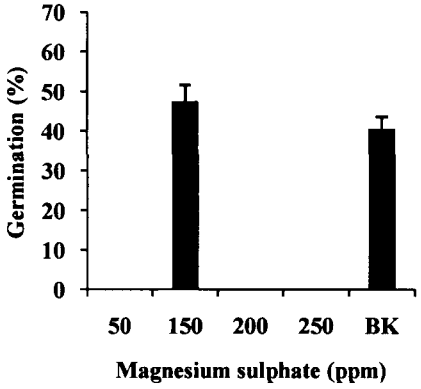

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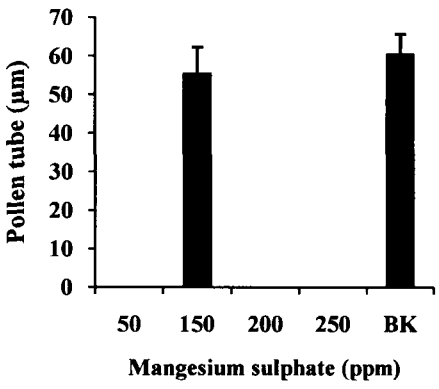

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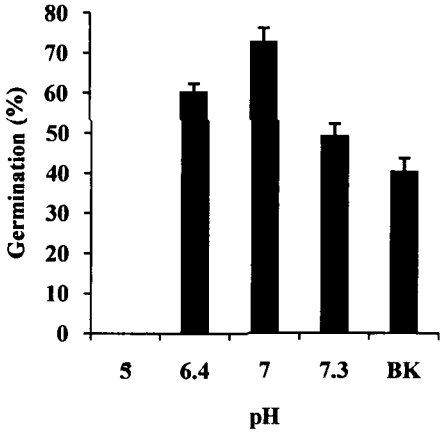
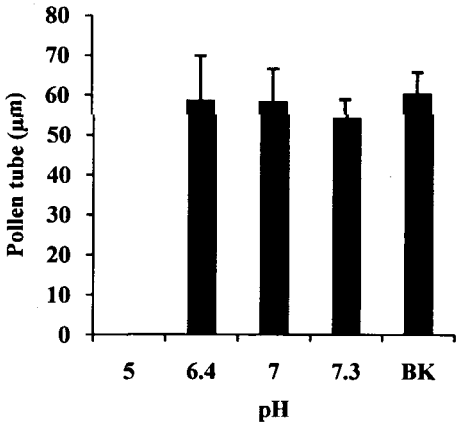

Germination (%)
80
70
60
50
40
30
20
10
0
5
6.4
7
7.3
BK
pH

e


Pollen tube (μm)
80
70
60
50
40
30
20
10
0
5
6.4
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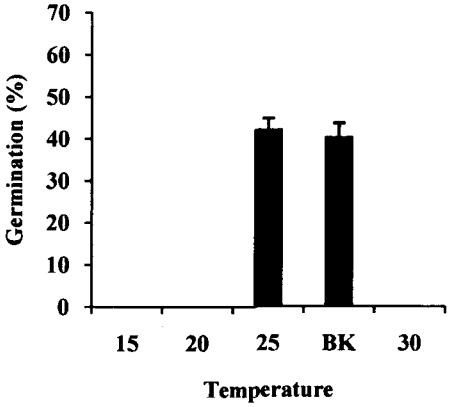
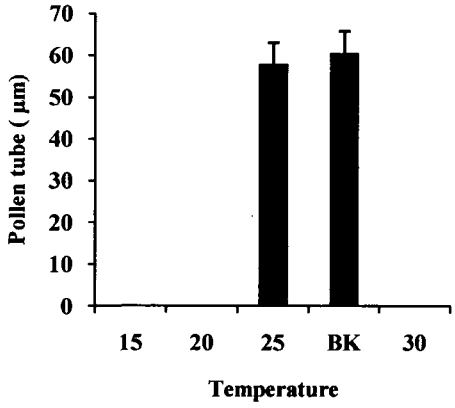
pH

f


Germination (%)
70
60
50
40
30
20
10
0
15
20
25
BK
30
Temperature

g


Pollen tube (μm)
70
60
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Temperature

h

Figure 6.3

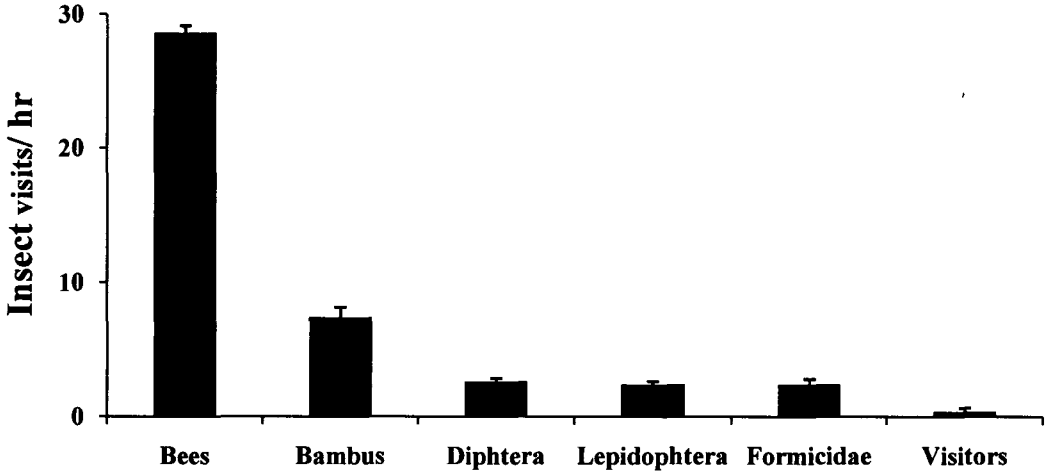


Fig 6.3 Graphical representation of Insect visit frequencies to *G. fragrantissima* flower during flowering peak period.

Table 6.3 Insect visit frequencies to *G. fragrantissima* flower.

Sl. No	Insect visitor	Insect visit/hr
1	Bees (*Apis* sp.)	28.6 ± 0.50
2	Bambus *(Bambus* sp.)	7.40 ± 0.74
3	Diptera	2.60 ± 0.24
4	Lepidophtera	2.40 ± 0.24
5	Formicidae	2.40 ± 0.40
6	Other Visitors	0.40 ± 0.24

Note: Values indicate mean ± standard error.

Plate 6.1

A, B, C, D, E & F. Different insects pollinating the flower of *G. fragrantissima* during flowering peak period. A: *Apis* sp.; B: *Bambus* sp.; C: Formicidae; D: Diptera; E& F: Lepidoptera. Bars: A = 0.83 cm; B = 1.00 cm; C &D = 0.62 cm; E& F= 1 cm.

G. Pollinator inside the corolla tube under SEM view. Bar= 500 µm.

H. An enlarged view (SEM) of pollinator showing pollen tetrads (Arrow) attached in the lower abdomen. Bar = 20 µm.

Plate 6.1

Gaultheria fragrantissima

Pollinators

Plate 6.2

A. Enlarge view of single germinating pollen showing sub spheroidal tetrad with three germinating pollen tubes. Bar = 7µm.

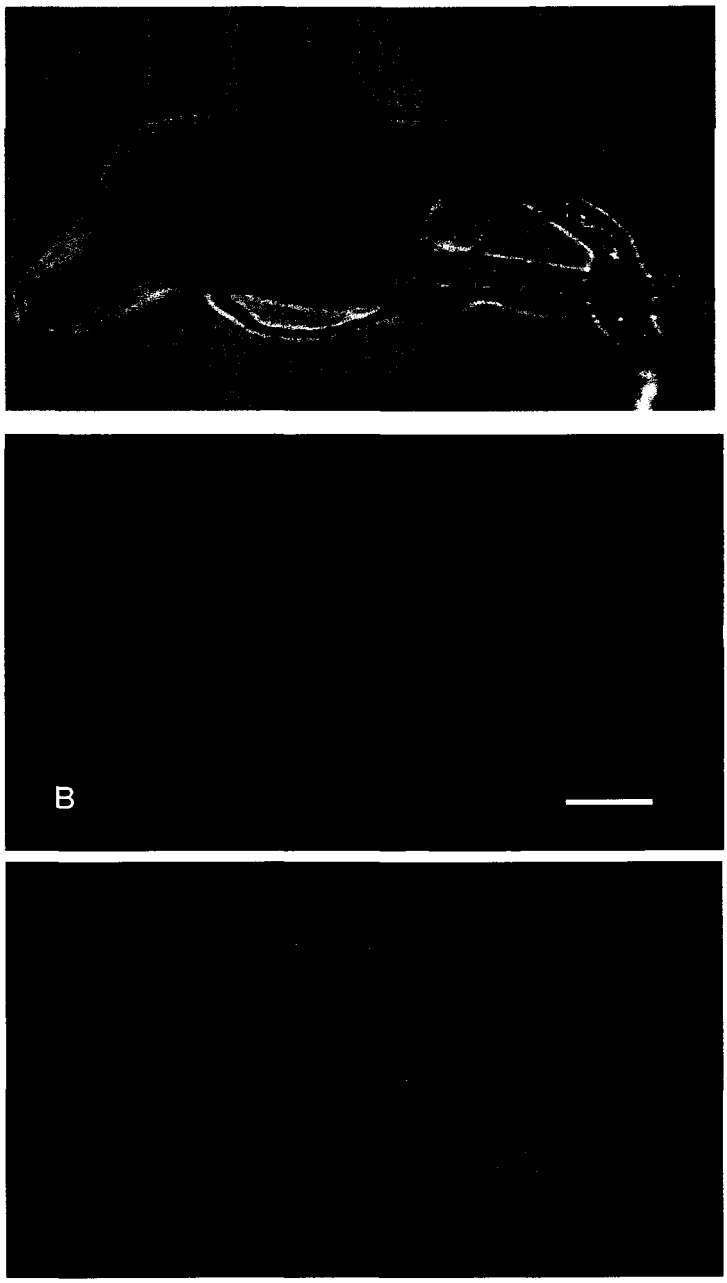
B. Photograph portraying the pollen viability with Fluorochromatic Reaction Test (FCR). Note the viable pollen tetrads fluoresce greenish yellow brightly and non-viable ones did not fluoresce. Bar = 56 µm.

C. Pollen germination in MBK medium stained with TBO Blue. Bar = 56 µm.

Plate 6.2

Gaultheria fragrantissima

Pollen viability and Pollen germination



CHAPTER- 7

Development of female gametophyte and post fertilization changes in embryo sac

7.1 Introduction

The female gametophyte plays a critical role in essentially every step of the reproductive process. During pollen tube growth, the female gametophyte participates in directing the pollen tube to the ovule (Higashiyama 2002; Johnson and Preuss 2002). Cytoskeletal components within the female gametophyte direct the sperm cells to the egg cell and the central cell at the time of fertilization (Russell 1993). Upon fertilization, female gametophyte expressed genes control the initiation of seed development (Chaudhury *et al.* 2001). During seed development, female gametophyte expressed gene products which play a role in controlling development of embryo and endosperm (Ray 1997; Chaudhury and Berger 2001).

In Ericaceous taxa the inflorescence varies among genera and species in its structure and number of flowers. The flowers vary interspecifically with respect to their size, form, anther dehiscence and structure stigma. Flowers aggregated in 'inflorescences' (usually), or solitary; in racemes, in spikes, in heads, in corymbs, and in panicles. Inflorescences terminal, or axillary. Flowers bracteate; usually bracteolate (bracteoles usually 2 or 3); small to large; regular, or somewhat irregular to very irregular (Rhododendroideae). Flower cyclic; nearly always pentacyclic. Free hypanthium absent. Hypogynous disk usually present; intrastaminal. Carpels

isomerous with the perianth, or reduced in number relative to the perianth. Gynoecium stylate. Styles 1; attenuate from the ovary, or from a depression at the top of the ovary; apical. Stigma 1; usually capitate (greatly expanded in *Epigaea*). Placentation axile or apical (Chou 1952; Ganapathy and Palser 1964; Watson *et al.* 1967; Hutchinson 1968; Cronquist 1981; Davis and Cullen 1981; Mirick and Quinn 1981; Mahy and Jacquemart 1998; Saichit and Corlett 2000; Jacquemart 2003; Freitas *et al.* 2006; Radcliffe *et al.* 2010).

In Ericaceae the general ovary characteristics are similar throughout the family. Ovules are unitegmic and anatropous to campylotropous. In the subfamily Andromedeae, the size of the mature ovule varies considerably within, and its condition varies from almost strictly anatropous to campylotropous. In every case, however, the ovule has a single integument, the outer layer of which is made up of cells which, except for those close to the micropyle, are filled with substance probably tannin like in nature (Chou 1952; Palser 1952; Ganapathy and Palser 1964; Palser *et al.*1991; Jacquemart 2003).

7.2 Structure of ovule and embryo sac formation

In *Gaultheria fragrantissima* the ovary is globular, pubescent and has a depression at the top from which the style emerges. The style is cylindrical, slightly open with five furrows and terminates in an unexpanded stigma just below the rim of the corolla opening (Plate 7.1A). A nectariferous gland is located at the base of ovary. The anatropous curvature starts shortly after the

initiation of the ovule due to adaxial intercalary growth of funicle. The curvature takes place on the convex side of the ovular primordium. By this process the nucelli turns over 180° and complete its bending to the anatropous condition (Plate 7.1B).

The single integument arises very early as a small outgrowth around the tenuinucellate ovules (Plate 7.1C). It is initiated by periclinal or oblique divisions in a circular zone around the ovular primordium. The integument is dermal in derivation. Successive longitudinal divisions give rise to an integument of four to five cell layers in thickness, elongates rapidly to completely surround the nucelllus and form the micropyle. The cell of the outer layer of the integument, except for the few around the micropyle, contains a considerable deposit of tannin or calcium oxalate crystals. One of the most remarkable phenomenons in *G. fragrantissima* is the differentiation of an integumentary tapetum or endothelium during histogenesis of integuments (Plate 7.1F). It forms a new limiting layer of the embryo sac. The endothelium originates from the inner layer of the single integument. Typically it surrounds middle portion of the megagametophyte except at the micropylar and chalazal ends. The cells become radially stretched and they contain prominent nuclei, some cells have larger nuclei. A few cells of the integument adjacent to the chalazal end of the gametophyte remain small, possess dense content, and constitute a type of hypostase (Plate 7.1G).

Ovule primordia are initiated by periclinal divisions in the second layer of the placenta. In transverse sections they always exhibit a radial symmetry. The ovule is dizonate as the ovular primordium is initiated by a periclinal

divisions in the second zone of the placental meristem and subsequently become smaller and covered by the anticlinally division dermal layer only. The nucellus develops out of the apex of the ovular primordium as a small protuberance (Plate 7.1D). During early stages one cell, lying directly below the nucellar epidermis differentiates into a primary archesporial cell, which is conspicuous by its larger size, denser cytoplasmic content and more prominent nucleus (Plate 7.1E). The primary sporogenous cell does not undergo further mitotic divisions, it enlarge, elongate and function as megaspore mother cell.

The radially elongated megaspore mother cell or megasporocyte divides meiotically. This process consists of two successive division and give rise to four megaspores. The first or heterotypic division is transverse and results in two dyad cells and the second or homotypic division is also transverse which results in linear tetrads (Plate 7.1F). The three micropylar megaspores degenerate and leaving the chalazal one as the functional megaspore. In *G. fragrantissima* this megaspore is the onset of megagametophyte and is called monosporic type of megasporogenesis. The cellular content of the mature functional megaspore is very active during the meiotic prophase. During enlargement of the functional megaspore a vacuole appears at either end of the cell with the nucleus located in the centre. The nucleus divides mitotically into two which come to lie at the micropylar and chalazal poles respectively with large central vacuole. The cytoplasm is restricted mostly on the periphery of the cell. The resulting two nuclei divide mitotically to form four nucleate conditions and the third mitotic division

produces eight nuclei. All three divisions occur in relatively in close succession. Wall formation, occurring after the last division, leads to the formation of seven cells: the egg cell, two synergids, the central cell, and three antipodal cells. This type of embryo sac is referred to as Normal or Polygonum type (Plate 7.2A). The egg apparatus occurs in the broadened micropylar region. The egg apparatus consists of an oval shaped egg and two synergids. These three cells show a triangular arrangement and share the common surfaces. The egg apparatus is attached at the extreme end of embryo sac wall bordering only and the major portion of the cells is surrounded by the central cell.

The egg cell is relatively large and projects chalazally beyond the synergids (Plate 7.2B). The egg cell wall is thickest at the micropylar region of the cell, and gradually becomes thin towards the chalazal region. The chalazal end of the egg cell is filled by dense cytoplasm with cell organelles with distinct nucleus and nucleolus while the micropylar end is occupied by one or two small vacuoles with a thin layer of cytoplasm at the peripheral side. The development of this highly specialized structure is characterized by a clear polarity from micropyle to the chalazal end in the distribution of nucleus, cytoplasm and vacuoles. Both synergids share the same cell wall in their contact region, beside this cell wall shows considerable variation in thickness. Lateral view of embryo sac shows at the extreme micropylar pole the wall is strongly thickened, forming a lunar or sickle shaped structure known as "filiform apparatus" (FA) (Plate 7.2D). The filiform apparatus increases the surface area of the plasma membrane. From the filiform

apparatus towards the base of the synergids the wall gradually thin. The chalazal half of the synergid is surrounded by the plasma membrane only. Because of the polar distribution of the cytoplasm, a single large vacuole occupies the chalazal pole of the synergid while most of the cytoplasm, cell organelles and nucleus restricted just below the filiform apparatus in the micropylar region. The cytoplasm of the synergids shows a complex organization, and is rich in organelles. In *G. fragrantissima* degeneration of one of the synergids prior to the arrival of the pollen tube has been observed (Plate 7.2E). The second synergid retains its original constitution. The degeneration of the synergid results in a decrease volume of the synergid, disappearance of the vacuole, and an increased stainability and density of the cytoplasm.

In *G. fragrantissima* the central cell is elongated structure from micropylar pole to the chalazal pole and occupies the largest portion of the embryo sac, bordering and surrounding the major portion of the egg apparatus at the micropylar pole, and reaching to the antipodal cells (Plate 7.2C). The lateral cell wall of the central cell and forms the main part of the embryo sac wall in contact with the surrounding tissue. The cytoplasm is confined to a thin layer along the embryo sac wall and accumulates near the egg apparatus and the antipodal cells. The micropylar accumulation contains the two polar nuclei, both the polar nuclei are similar (Plate 7.2C).

The polar nuclei fuse after the egg and sperm fertilized. The fusion nucleus of the central cell is the largest nucleus observed in the embryo sac. The three antipodal cells are arranged linearly and are large, elongated that

they occupy one third of the chalazal end of the embryo sac (7.2C). The antipodal cells degenerate during the embryogenesis. Antipodals have distinct cell wall of uniform thickness and the cytoplasm contains abundant organelles. Antipodals are mononucleate.

7.3 Zygote

In *G. fragrantissima* the pollen tube enters the ovule through the micropyle and in penetrating the megagametophyte destroys one of the synergids. The fusion of one sperm and the egg, and of the second sperm and the two polar nuclei, occurs rapidly. After fertilization the embryo sac contains a zygote and the remnants degenerating synergids at the micropylar ends. The zygote is an essentially elongated ovoid in structure and located at the micropylar pole of the embryo sac with its basal portion attached to the embryo sac wall, while the apical portion projects into the central cell. The zygote exhibits a characteristic polarized appearance with a large vacuole at the micropylar pole while the chalazal pole contains the zygote a prominent nucleus, with nucleolus, dense cytoplasm with all the cell organelles. The zygote undergoes changes in its size before embarking on division. The volume of the zygote is reduced (Plate 7.3A)

7.4 Embryogenesis

The apical end of the zygote contains the nucleus and much cytoplasm, while the basal portion is slender and vacuolated. The first

division of the zygote is transverse, resulting in a small apical cell, '*ca*' and a larger basal cell, '*cb*' (Plate 7.3B). The apical cell divides transversely to form '*cc*' and '*cd*', simultaneously the basal cell and its derivatives also divide transversely to form '*ci*' and '*m*'. This division results in a four celled filamentous proembryo (Plate 7.3C). The two superposed apical cell '*cc*' and '*cd*' again divides and produced a tetrad of the second order which is a tetrad proembryo. The tetrad stage is followed by another vertical division in a plane usually at right angles to the first vertical division in the distal cell. These four juxtaposed cells constitute the quadrants (four cells) which through another division give rise to octant (eight cells) at the distal end of the proembryo (Plate 7.3D). The derivatives of the basal cell '*cb*' do not take part in the organization of embryo proper, which is mostly derived from the apical cell '*ca*' of the two celled proembryo. The derivatives of the basal cell, immediately next to embryonal mass, contribute to the formation of suspensor (Plate 7.3E). The suspensor is prominent, linear and four to five celled. The growth of suspensor is very rapid between the globular and the filamentous stage of development. Thereafter, further increase in length ceases, and during later stages the suspensor begins to degenerate and is apparently resorbed by the embryo.

The further development of embryo from the octant stage following numerous cell divisions in various planes causes the proembryo to assure a globular configuration (Plate 7.4A &B). The globular embryo passes through a phase before cotyledons and epicotyls become outwardly evident at specified loci and there is transition from radial symmetry to bilateral

symmetry. The embryo is now filamentous shaped with endosperm that is rich in food reserved (Plate 7.4C). The embryo of *G. fragrantissima is* straight, cylindrical, about two-thirds the length of the seed with two inconspicuous cotyledons, and is surrounded by endosperm (7.5D).

7.5 Endosperm formation

The fusion product of two polar nuclei with the second male gamete or sperm cell constitutes the primary endosperm nucleus (PEN) (Plate 7.5A) which is triploid in nature located just below the zygote. The cells derived through mitoses by the primary endosperm nucleus constitute the endosperm. In *G. fragrantissima* the division of the primary endosperm nucleus is followed by two free nuclear divisions, and the eight daughter nuclei are arrange in three groups, two at each pole and four in the centre. These nuclei remain in a cytoplasmic sheath around the central vacuole (Plate 7.5B). Then cell wall formation takes place in such a way that the two terminal cells located at the micropylar and chalazal ends respectively with two nucleate, each which develop into two haustoria. The central four nucleate cells give rise to the cellular endosperm proper. The endosperm is thus initially nuclear and later become cellular in the mature seeds. The micropylar cell enlarges and divides longitudinally and then transversely, forming the micropylar haustorium of the endosperm. The micropylar haustorium has dense cytoplasm with two nuclei. The lower portion elongates gradually and formed a sac like chalazal haustorial structure (Plate 7.5C). The chalazal cell of the endosperm is smaller than the micropylar cell

and extends beyond the antipodals and eventually the chalazal cell also undergoes longitudinal divisions in various planes, forming the chalazal haustorium, the chalazal haustoria of *G. fragrantissima* is composed of densely cytoplasmic cells separated from the endosperm proper to form finger like projection (Plate 7.5D). The haustorium transports metabolites from the circumjacent tissue to the developing embryo and presumably the finger like projection help in enhancing for absorption of nutrients from the surrounding tissues. At maturity, the endosperm become packed with reserve food materials mainly starch (Plate 7.6A &B) and proteins (Plate 7.6C).

The seed coat development is triggered when the ovule is fertilized in *G. fragrantissima.* The seed coat is formed by elongation and differentiation of single layer or testa. The thick outer tangential wall collapse and disappear over the time, therefore the testa becomes reticulate which are responsible for the hardness of the testa. Thickenings of cell walls are particularly important in this case (Plate-7.7A). In cells of the testa only the inner tangential wall is strongly thickened, while the outer parietal walls are then hardly visible. The thickenings are nearly even in all cells of the seed coat, forming a uniform layer. In the thickened cell walls, some pores are easily visible under a light microscope, sometimes referred to as pits (Plate 7.7B &C). Simultaneously the seed coat undergoes lignifications to protect the embryo and endosperm which is also a significant feature in *G. fragrantissima*.

7.6 Discussion

In *Gaultheria fragrantissima* the style arises from the depression in the top of ovary and the placentation is axile in the centre of the ovary while at the junction of style and placental tissue the ovules are in parietal placentation. Paterson (1961) and Palser *et al.* (1989) also reported the same features as observed in the order Ericales. In Ericaceae, the ovule is anatropous or slightly campylotropous, unitegmic, tenuinucellate, with a narrow micropyle, and devoid of vasculature, except in *Enkianthus* (Palser *et al.* 1971; Villamil and Palser 1980). In *G. fragrantissima,* ovules are unitegmic, tenuinucellate and anatropous. These features were also reported by Chou (1952) in the other species of *Gaultheria G. procumbens, G. shallon* and *G. ovatifolia.* Similarly Samuelson (1913) reported that anatropous ovules occur in *G. microphylla, Pernettya mucroxata* and *P. pumila* which are members of Ericales. In *G. fragrantissima* the early appearance of integument at the base during the development of ovules and considerable deposition of tannin or tannin like substance in the integument also showed similarity with that of *G. procumbens* as observed by Chou (1952).

Angiospermous ovules may have two, one or no integument(s) and are consequently called bitegmic, unitegmic and ategmic. The inner integument is most often dermal in origin, whereas the outer integument is usually derived from both dermal and subdermal layers (Bouman 1984). The two integuments are considered to have distinct evolutionary origins (Stebbins 1974). Periclinal divisions in the integuments generate an increase in the number of cell layers, whereas anticlinal divisions and cell elongation

are responsible for growth parallel to the nucellus. In *G. fragrantissima* the ovules are unitegmic, the integument becomes four or five cell layers in thickness and elongates rapidly to completely surround the nucellus and forms the micropyle. The number of cell layers or thickness of the integument is highly variable in different members of the order Ericales. Chou (1952) observed four to five layers in *G. procumbens* and when the ovules become completely anatropous, the integument increases in thickness so that it has twelve layers of cells by the time the megagametophyte is mature. In *G. shallon* when the megagametophyte is mature, the single integument consists of nine layers of cells. Peltrisot (1904) and Copeland (1947) reported with two or three layers of cells in Pyroleae and Monotropoideae. In *Daboecia*, Copeland (1944) observed four to six layers of cell with thicker integuments. The number of integuments is a very important embryological feature (Sporne 1969; Philipson 1974; Dahlgren 1980). Bitegmic is the more common and primitive condition. The evolutionary changeover from bitegmic to unitegmic must have taken place many times. The unitegmic ovule in *G. fragrantissima* therefore revealed its advanced character.

In *G. fragrantissima* as the embryo sac develops, the integuments continue to enlarge, typically overgrowing the nucellus. The amount of ovule curvature varies with the extent of differential growth of the integuments and funiculus; the degree of curvature forms a basis for classification of ovule morphology. Ovule of *G. fragrantissima* showed extensive curvature such that the long axis of the nucellus is parallel to the axis of funiculus confirms the anatropous type of ovule. Anatropy is of most common occurrence

among angiosperms. According to Davis (1966), 204 families are exclusively anatropous.

A few cells of the integument adjacent to the chalazal end of the female gametophyte remain small, possess dense content, and constitute a type of hypostase. The term hypostase has been proposed by Tilton (1980) and defined as a group of modified cells wit usually lignified walls, generally within the chalazal region of the ovule, but which may surround a portion of the megagametophyte and extend partially in the micropylar half of the ovule. Tanniniferous cell contents present in the chalazal region of the ovule and seed of *G. fragrantissima* are in agreement with the hypostase definition proposed by Tilton (1980). The differentiation of a hypostase by integumentary cell at the chalazal end of the megagametophyte in *G. fragrantissima* is similar to that observed by Anisimova and Shamrov (2000) in *Daboecia,* sub family Clethraceae, *Vaccinium*, *Arbutus* and in *Vaccinium myrtillus*. The hypostase is densely cytoplasmic which was similarly reported by Stushnoff and Palser (1969). Tilton (1980) mentions that the function of the hypostase is not known with certainty, but because of its position around the megagametophyte, it is generally thought to be related to translocation of nutrients into the megagametophyte and embryo.

Endothelium (or integumentary tapetum) is invariably associated with unitegmic, tenuinucellate ovules. The presence of endothelium is recorded in 65 families of dicotyledons (Kapil and Tiwari 1978). In *G. fragrantissima* the inner layer of the endothelium becomes more or less differentiated as an epithelial like layer surrounding at least the chalazal portion of the

megagametophyte. The disintegration and differentiation of endothelium occurs in the tetrad stage in *G. shallon* and in functional megaspore stage in *G. procumbens.* In *G. shallon* the integumentary tapetum degenerate soon after reaching dyad stages (Chou 1952). In *G. fragrantissima* however, the integuments persist till the complete formation of embryo sac or matured megagametophyte. The embryo sac is then in direct contact with the inner integument. The degree of the differentiation of the endothelium varies from none in *Pholisma* (Copeland 1939) to only slightly in many of the Monotropoideae (Copeland 1939; 1941; Palser 1952) and a few of the Pyroleae (Copeland 1947) to a very distinct in many of the Rhododendroideae (Copeland 1944; Palser 1952) and in the species of Clethraceae (Kavaljian 1952), Arbutaeae, Gaultherieae and Vaccinioideae as reported by Palser 1952. Venkateshwarlu and Devi (1973) reported a biseriate endothelium in *V. retusum* and *V. serratum.*

Radial cell expansion, endopolyploidy, and prominent nuclei are observed in the endothelial cells and also the anther tapetum, which is thought to be involved in secretion and nutrition of the pollen (Bhandari 1984; Goldberg *et al.* 1993). Maheshwari (1950) speculated that the cytological features shared between the endothelium and tapetum could indicate a similar function for both tissues. In species in which the nucellus does not degenerate, the inner integument does not differentiate an endothelium, and the embryo sac may receive nutrients from the nucellus directly. Several functions have been ascribed to the endothelium.

According to Kapil and Tiwari (1978) the endothelium has several functions in different developmental stages of the seeds. During the first ontogenetic stages of the megagametophyte and of the endosperm, the endothelial cells coordinate the development between the embryonic sac, the young endosperm and the integument. In the other stages, the endothelium channels nutritious substances to the embryonic sac and to the endosperm. In several species, the endothelium starts to present a protective function of the embryo, during seed development. Similarly, this would be the function of this layer in *G. fragrantissima*. The endothelium can also act as a barrier tissue to resist, or to localize, the aggressive action of the growing embryo sac or endosperm (Godineau 1973).

The ovule primordium in *G. fragrantissima* is dizonate. The widespread occurrence of three zonate ovular primordia in families of a supposedly primitive status and in derived ones suggests that the three zonate ovular primordium is basic within angiosperm (Johri 1984). Generally, trizonate ovular primordia give rise to larger ovules, with well developed funicles, raphes and nucelli, than two zonate primordia. The relatively small ovule and dizonate ovule primodium in *G. fragrantissima* is also another advanced characteristic feature of the species.

In *G. fragrantissima* a single hypodermal cell of the nucellus differentiates to form the archesporial cell and develops directly into the megaspore mother cell which corresponds to those that have been described by others for various members of the Ericales. Chou (1952) also reported similar development of megaspore mother cells in both *G. procumbens* and

G. shallon. Ganapathy and Palser (1964) however observed a second archesporial cells to occur in some ovule: in *Daboecia*, *Vaccinium angustifolium*, *G. hispidula* and *Andromeda glaucophylla.* Similar situation were also observed by Palser *et al* (1971) in *Rhododendron yunnanense* and Reddy *et al* (1966) in *Vaccinium leschnaultii.*

Like several members of Ericales, the megaspore mother cells of *G. fragrantissima* undergoes meiosis I and meiosis II resulted into linear tetrads, but a few species rarely show T tetrad, as do *Kalmia latifolia* and *Phyllodoce empetriformis*. Ganapathy and Palser (1964) reported the triad condition in *P. breweri.*. Throughout the order Ericales, except for *Cassiope*, a single spore of the megaspore tetrad, normally the chalazal one functions. Occasionally, some spore other than the chalazal may give rise to the gametophyte as in *K. latifolia* and *P. empetriformis* (Ganapathy and Palser 1964). In *G. fragrantissima* the chalazal one developed into the megagametophyte and remaining three micropylar are eventually crushed and degenerated, which was similarly reported by Davis (1966) in majority of angiosperm.

In Ericales the Normal (Polygonum) type of development of the megagametophyte from a single archesporial cell, which functions directly as the megaspore mother cell has been reported in most of the genera (Maheshwari 1950; Palser 1952; Venkateshwarlu and Maheshwari Devi 1973; Vilamil and Palser 1980; Palser 1991). Palser (1951) however reported in *Cassiope mertensiana* an important variation from the typical Polygonum pattern where an Allium type of development has been reported. A Polygonum embryo sac with a broad micropylar and narrower chalazal

region is seen in *G. fragrantissima*. The Polygonum type female gametophyte is exhibited by more than 70% of flowering plants and is the pattern found in many economically and biologically important groups, including Brassicaceae, Gramineae, Malvaceae, Leguminoseae and Solanaceae. (Maheshwari 1950; Willemse and van Went 1984; Haig 1989; Huang and Russell 1992). During cell differentiation, the nuclei at the micropylar end become specified to develop into the egg cell, the micropylar polar nucleus, and the synergid cells; the chalazal nuclei develop into the three antipodal cells and the chalazal polar nucleus. Furthermore, all of the cells within the female gametophyte differentiate into polar structures. For example, in many species, the egg cell's nucleus is located toward the chalazal end and its vacuole occupies the micropylar end; by contrast, the synergid and central cells have the opposite polarity (Willemse and van Went 1984; Huang and Russell 1992).

The egg cell of *G. fragrantissima* is relatively large, oval in shape and projects chalazally beyond the synergids. The distribution of cytoplasm within the egg cell is highly polarized, due to the presence of a large vacuole at the micropylar end that restricts the nucleus and most of the cytoplasm to the chalazal end as was similarly reported by Schulz and Jensen (1968), Cass *et al.* (1985), Sumner and Van Caeseele (1989). The two synergids cells are specialized cells that lie adjacent to the egg cell play an essential role in pollen tube guidance and function. In *G. fragrantissima*, the synergid cell wall forms a highly thickened structure called the filiform apparatus at the micropylar end, consisting of numerous fingers like projections into the

synergid cytoplasm. This structure greatly increases the surface area of the plasma membrane in this region, which is also associated with an elaborated endoplasmic reticulum. The apparatus arises by the transformation of the foam like cytoplasm, its growth continues by apposition, and the upper portion often swells considerably before fertilization. The membrane of the embryo sac is resorbed over the apex of the synergids, which then quite frequently protrude. In *G. fragrantissima*, both synergids are elongated in shape. The extremely elongated condition of the synergids found in *Epigaea* appears to be characteristics of many members of the Rhododendroideae (Copeland 1944). The morphology of the filiform apparatus is highly variable among species. In *G. fragrantissima* the filiform apparatus is sickle shaped like structure. It is spherical in *Torenia fourieri* as reported by Plijm van der (1964). It is wedge shaped and located between the tips of the synergids in *Petunia* as reported by Went van (1970). Similarly Newcomb and Steeves (1971) observed wedge shape of filiform apparatus in *Helianthus annuus* while in most other species it forms a broad layer of long, irregular cell wall projections on top of the synergids. There are also reports where filiform apparatus are conspicuous in nature as in *Acca sellowania* (Pescador *et al.* 2009). It is thought that the filiform apparatus mediates the transport of molecules into and out of the synergid cells (Willemse and van Went 1984; Huang and Russell 1992).

The synergid cells have been shown to play a direct role in pollen tube attraction and guidance in *Arabidopsis*, *Torenia*, and Maize ovules (Higashiyama 2002; Punwani and Drews 2008) and its role in pollen tube

reception has been highlighted by recent research showing that proteins required for sperm cell release are localized to the filiform apparatus (Kessler and Grossniklaus 2011; Leshem *et al.* 2013). The synergids are also essential for the cessation of pollen tube growth and release of the sperm cells. Kasahara *et al.* (2005) previously showed that, within the female gametophyte, MYB98 is expressed exclusively in the synergid cells and that myb98 mutants show defects in pollen tube guidance and development of the filiform apparatus. Punwani *et al.*(2008) extend these observations by showing that MYB98 is localized to the nucleus and binds DNA and that it is required for the expression of at least 16 genes previously identified (Steffen *et al.* 2007) as being expressed predominantly in synergid cells. Interestingly, many of these genes (11 of 16) encode small, Cys-rich proteins, and further investigation of five of these showed that the encoded proteins are secreted into the filiform apparatus. These data show that MYB98 is a key regulator of transcriptional events in the synergid cells, and it points to small Cys-rich proteins as playing a role in pollen tube guidance and successful fertilization.

The central cell of *G. fragrantissima* is positioned in the center and is the largest cell of the embryo sac; this cell contains two nuclei, a large vacuole, and many cytoplasmic organelles. The polar nuclei originate at both the micropylar and chalazal ends of the coenocytic megagametophyte and migrate to the center after cellularization. The time of fusion of the polar nuclei varies from species to species. In *Capsella* (Schulz and Jensen 1968) and *Heliathus* (Newcomb 1973) the fusion completed before fertilization. In *Gossypium* (Jensen 1964) and *Spinacia* (Wilms 1981) fusion starts in the

embryo sac and completed after the arrival of sperm nucleus. In *G. fragrantissima* polar nuclei fuses only after the egg and sperm fertilized. However, Palser (1952) reported in *G. procumbens* and *G. shallon* that polar nuclei fused prior to fertilization. The central cell plays a critical role for the development of endosperm after triple fusion (Willemse and Went 1984; Lopes and Larkin 1993).

The antipodal cells showed much variation among angiosperms. In many taxa of the dicotyledons they degenerate before or during the maturation of the embryo sac. In other species they persist even during embryo and endosperm formation, although their condition can be highly variable. The great variability makes it impossible to draw general conclusions on the structure and functions of antipodal cells, but they are thought to be involved in the import of nutrients to the embryo sac (Diboll 1968). These cells also produce and secrete growth controlling substances which regulate the development of the endosperm (Willemse and Went 1984). The development and persistence of the antipodals vary considerably in members of the Ericales. They are large, though they may disintegrate early, in several species of *Erica* (Beijerinck 1940), in *Calluna* (Beijerinck 1940; Peltrisot 1904), *Clethra* (Kavaljian 1952), *Bruckenthalia* (Peltrisot 1904) etc., while in others, such as *Newberrya* (Copeland 1934), *Pholisma* (Copeland 1935), *Kalmia, Menziesia* (Copeland 1934; Peltrisot 1904) and *Styphelia* (Brough 1924), they are very small or have essentially completely disappeared before fertilization. In *G. fragrantissima* the three antipodal cells are arranged linearly and are large that they occupy one third of the chalazal

end of the embryo sac and persist till embryogenesis. Samuelsson (1913) has reported similar enlarged antipodals in *G. shallon, G. microphylla* and *Pernettya mucronata.*

The endosperm is essentially a triploid tissue, but can be diploid as in *Butomopsis lanceolata* (Johri 1936) and even pentaploid as in *Fritillaria* (Maheswari 1950). In *G. fragrantissima* the division of the primary endosperm nucleus precedes that of the zygote and endosperm is initially nuclear and become cellular in the mature seed. In Ericaceae, the development of endosperm is either of the Nuclear or Cellular type. Nuclear type occurs in *Vaccinium augustifolium* (Bell 1957), *V. leschenaultia* (Reddy *et al.* 1966), *V. mummularia, V. retusum* and *V. serratum* (Venkateswarlu and Maheshwari Devi 1973). Cellular type of endosperm occur in *Daboecia, Kalmia, Keiophyllum, Loiseleuria, Phyllodoce* and *Rhodothamnus* (Ganapathy and Palser 1964), *Rhododendron ferrugineum, R. yunnanense* (Palser *et al.* 1971) and *Vaccinium* sp (Stushnoff and Palser 1969). Samuelsson (1913) has stated that the endosperm in the mature endosperm derived entirely from the middle two of the four cells and that the two terminal ones give rise to haustoria. Chou (1952) also observed similar pattern of endosperm development in *G. procumbens* and *G. ovatifolia.*

All the members of the Ericales, except the Diapensiaceae (Samuelsson 1913), are characterized by the development, to a greater or lesser extent, of micropylar and chalazal endosperm haustoria. In *G. fragrantissima* the micropylar haustorium is frequently larger and contains more cells than the chalazal. A characteristic feature has been observed in

the structure of both micropylar and chalazal haustoria in *G. fragrantissima*. It is sac like structure in micropylar and finger like projection in chalazal haustoria, thus increase the absorbtive surface. Venkata (1962) observed similar fingers like projection of haustorium in *Lomatia polymorpha*. Swamy and Ganapathy (1957) also observed in *Nothapodytes foetida* that the chalazal chamber has dense cytoplasm, hypertrophied nucleus, and functions as a haustorium and give rise to many, small, sac like processes. In *G. fragrantissima* both haustoria are well developed with multicellular structure; Chou (1952) however reported in *G. ovatifolia* both are rather poorly developed. In the sub family Pyroleae both are extremely small as reported by Camp (1944). Palser (1951) observed in another member of Ericales, *Cassiope hypnoides* that the micropylar one is well developed though with few cells, while the chalazal one is poorly developed. Copeland (1939), observed reduced seed without haustoria in *Monotropsis odorata.* In *G. fragrantissima* chalazal haustoria is already formed before the formation of primary endosperm nucleus. Chou (1952) also reported in *G. procumbens* and *G. ovatifolia* that the region of the micropylar haustorium may be apparent even before fertilization and the chalazal only later.

After fertilization the angiosperm zygote must begin a new programme of sporophytic development based on the diploid genotype established at the time of gamete fusion. In close proximity to maternal and endosperm cells of different genotypes, this cell must initiate expression of a new genotype in a new developmental sequence. In Ericaceae the embryogeny conforms to the Solanad Type (Ganapathy and Palser 1964; Stushnoff and Palser 1969). In

G. fragrantissima the first division is transverse, resulting in a small terminal apical cell and an elongated basal cell. Apical cells divides transversely to form four celled filamentous embryo. Derivatives of the terminal cell formed the octant proembryo and basal cell formed the suspensor which is of the series C as per the recognition of embryogeny type by Schnarf (1929) and Johansen (1950). Embryogeny thus follows the Solanad type. Chou (1952) also reported a Solanad type in *G. ovatifolia*, as the basal cells does not seem to take part in formation of the embryo.

Little work has been done on embryogeny of Ericales. This is reflected in Johansen (1950) listing of only a few members. He classified the pattern of embryo development in *Pyrola rotundifolia* as a *Myriophyllum* variation of the Caryophyllad Type, in *Slyphelia longifolia* as a *Vaccaria* variation of the same type, in *Monotropa hypopitys* as probably of the Onagrad type, in *Sarcodes sanguine* as probably as the Caryophyllad Type, and in *Diapensia lapponica* as probably of the Solanad type. In *G. fragrantissima* during early development the zygote is located anterior to the constriction, and the primary endosperm nucleus posterior to it. Similar pattern of development was also observed by Chou (1952) in *G. procumbens.*

In *G. fragrantissima* the suspensor is linear; four to five celled thus pushing the embryo toward the middle of endosperm. Similarly, Doyel and Goss (1941) reported that the seeds of *Sarcodes sanguinea* Torr. were found to contain small undifferentiated globular embryos which are projected by suspensors into the endosperm. Chou (1952) reported in *G. ovatifolia* the two celled embryo divides to four and then eight cells, but the suspensor shows

no further divisions. Copeland (1938) described seeds of *Pleuricospora fimbriolata* and *Allotropa virgata* were found to contain reduced suspensor less embryos embedded in endosperm. The suspensor plays a very dynamic role in nourishing the embryo proper at specific developmental stages by synthesizing essential growth factors and transport nutrients to the embryo proper to stimulate its growth and development (Bohdanowicz 1987; Picciarelli *et al.* 1991). In *Sedum* the suspensor is involved mainly in absorption and transport of metabolites from the ovular tissues to the developing embryo proper (Kozieradzka- Kiszkurno and Bohdanowicz 2006).

Major angiospermous embryos are generally differentiated into distinct hypocotyl, epicotyl, and cotyledonary regions (Maheshwari 1950). Studies by Ganapathy and Palser (1964) and Stushnoff and Palser (1969) indicate that ericaceous embryos are differentiated and develop cotyledons except in the subfamilies Pyroloideae and Monotropoideae where embryos remain undifferentiated (Copeland 1947; Pyykko 1968). Copeland (1933; 1947) studied seeds and embryos from certain members of the Pyroloideae and found that the embryos had failed to differentiate distinct cotyledons. The mature embryos remain undifferentiated and embedded in endosperm (Pyykko1968). In *G. fragrantissima* the mature embryo is straight, cylindrical, about two thirds the length of the seed with two inconspicuous cotyledons, and is imbedded in endosperm. Martin (1946) described the mature embryo of *Gaultheria* as small, straight, with the two cotyledons inconspicuously developed. Similarly Fagundez (2009) reported in *Calluna, Daboecia* and *Erica* of Ericaceae, that the embryo is axial and straight with two short

cotyledons. Seed food reserved in angiosperm can be located throughout the various tissues and usually consist of proteins, carbohydrates, and lipids. These reserves undergo hydrolysis and are mobilized to provide the needed substrates for the developing seedling upon germination (Raghavan 1986; Bewley and Black 1994). Endosperm of certain members of the Pyroloideae has been reported to contain starch (Pyykko 1968). Endosperm of *G. fragrantissima* contain starch, protein bodies etc as stored reserve material. The protein bodies occupied most of the endosperm and are degraded during germination.

The seed coat often plays an essential role in various processes such as nutrition of the growing embryo, mechanical and chemical protection, dehydration, imbibition, and maintenance of seed dormancy (Boesewinkel and Bouman 1995). Periclinal and anticlinal cell divisions, combined with cell enlargement, are responsible for the growth of the seed coat. At seed maturity, however, much of the integumental tissue may be degenerated and absorbed by other developing tissues (Fahn 1990). The seed coat in Ericaceae is commonly a single cell layer (Takhtajan 1992; Szkudlarz 2001; 2008; Stevens *et al.* 2004; Fagundez *et al.* 2009), although two to three cell layers have been recorded in *Andromeda* and *Oxycoccus* (Szkudlarz 2001). In *G. fragrantissima* the seed coat is single cell layer, the thick outer wall collapse over the thin inner periclinal wall. This was also reported in *Calluna, Daboecia and Erica* by Fagundez *et al.* (2009). Seed coat of *G. fragrantissima* shows a characteristic features in which the seed coat is with thickening wall like which subsequently lead to formation of pores that would

facilitate exchange of water molecule and gases during seed germination. Wall thickening of seed coat in other Ericaceae species has also been reported by Takhtajan (1992) and Szkudlarz (2001; 2006).

The observed features of wall thickenening in the seed coat of *G. fragrantissima* seems to be taxonomically valuable, but to use them for diagnostic purposes it is necessary to collect more detailed on its structural and developmental aspects. Seed coat of *Cercis canadensis* was observed with discontinuous area between macrosclereid cells in the palisade layer of the seed coat which formed a hilar slit. A cap was formed during germination as the seed coat separated along the hilar slit and was hinged by the macrosclereids in the area of the seed coat opposite to the hilar slit that facilitated seed germination (Jones and Geneve 1995). In *Vicia sativa*, the walls of the macrosclereid cells were thickened, and the cell vacuoles were filled with tannin (Buyukkartal *et al.* 2013). Indeed, seed coats may retain ingenuous indications of phyletic commonality (Martin 1946; Esau 1977; Barthlout 1981), and many studies in various, unrelated groups have confirmed the general utility of seed coat characters for assessing systematic relationships (Chuang and Heckard 1972; Clark and Jernstedt 1978; Canne 1979). Also recent studies have shown that seed coat ornamentation can furnish important information for determining taxonomic relationships in many plant groups (Koul *et al.* 2000; Munoz-Centeno *et al.* 2006; Khalik and Osman 2007; Akcin 2009; Lu *et al.* 2010; Baretto *et al.* 2013; Khalik 2013).

Plate 7.1

A. Longitudinal section of hermaphrodite flower showing globular ovary with numerous dark brown ovules, fertile anther with awns *and a short style below the rim of the corolla tube. Bar = 0.71 cm.*

B. SEM view of anatropous ovules. Bar = 50 µm.

C &E. Longitudinal Section of tenuinucellate ovule showing initiation of the hypodermal archesporial initials (Ai) and the initial of integument (I) on its lateral side with distinct nucleus and dense cytoplasm respectively. Bar = 0.71 µm.

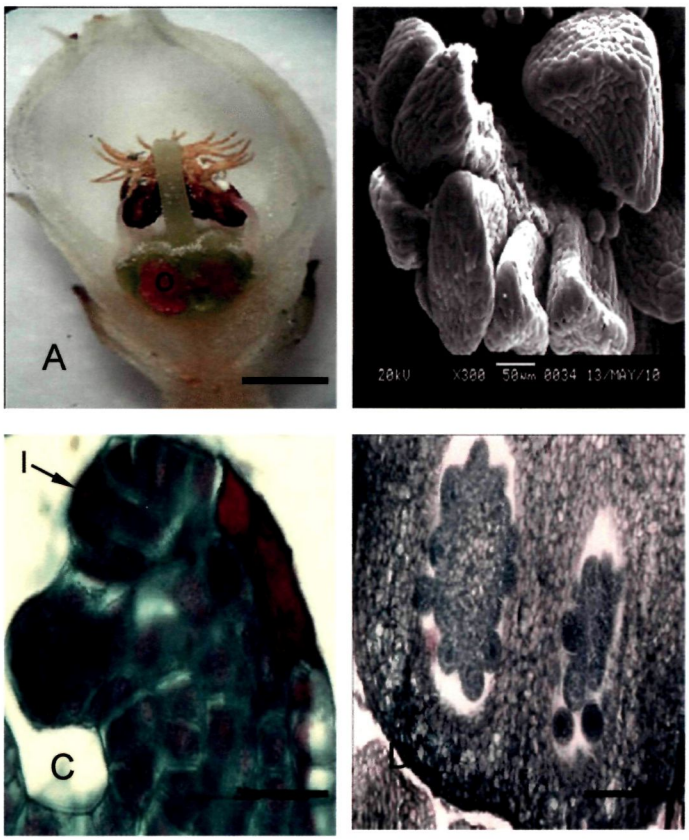
D. Transverse Section of young ovary showing ovule primordia on the placental tissue. Bar = 20 µm.

F. Resultant four megaspore (Arrow) after meiosis. Note the presence of distinct endothelium (E). Bar = 20 µm.

G. Chalazal end of the female gametophyte showing hypostase (Hy). Bar = 20 µm.

Plate 7.1

Gaultheria fragrantissima Wall.



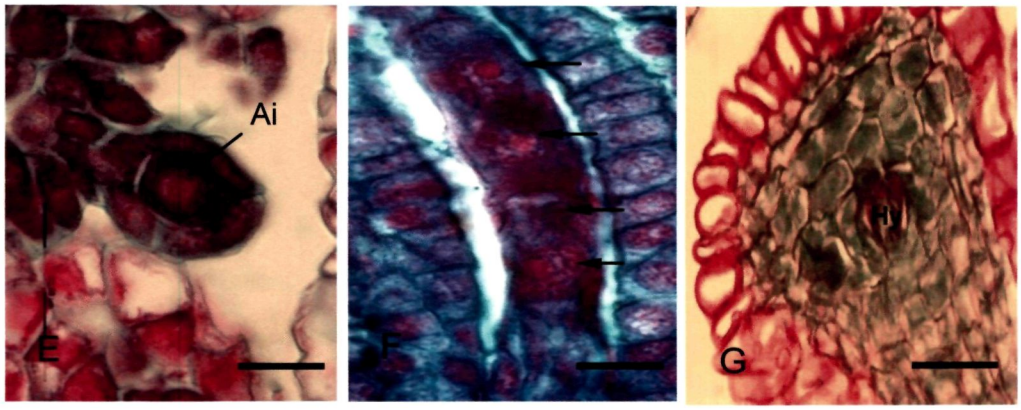


Plate 7.2

A. Longitudinal Section (L.S) in the middle of the ovule showing the organization of embryosac. Note the Egg cell, Synergids, Central cell nucleus, Antipodals as well as Endothelium in the middle of embryosac. Bar = 5 µm.

B. An enlarged view of Egg apparatus showing apical end of an egg cell and two synergids; note the position of vacuoles, nucleus and cytoplasm. Bar = 17.5 µm.

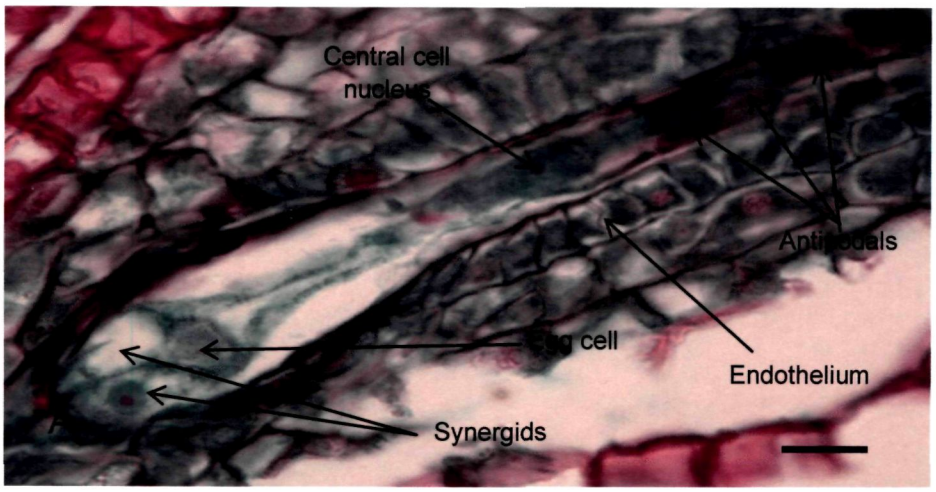
C. An enlarged view of middle portion of the embryo sac showing two polar nuclei and Antipodals. Bar = 8.75 µm.

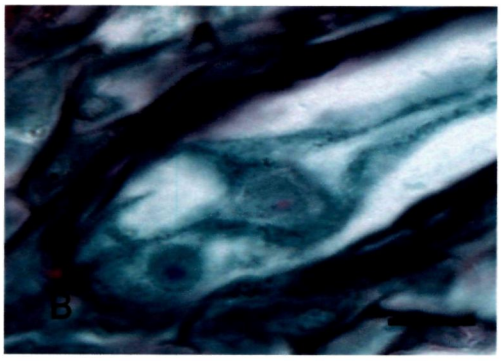
D. Lateral view of embryo sac showing egg cell, synergids with filiform apparatus (FA). Bar = 10 µm.

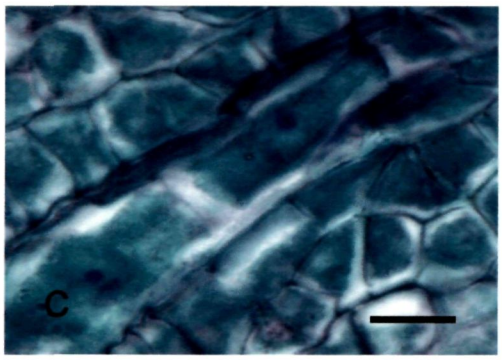
E. Degenerating synergid (Ds) prior to arrival of pollen tube while another synergid is normal. Bar = 10 µm.

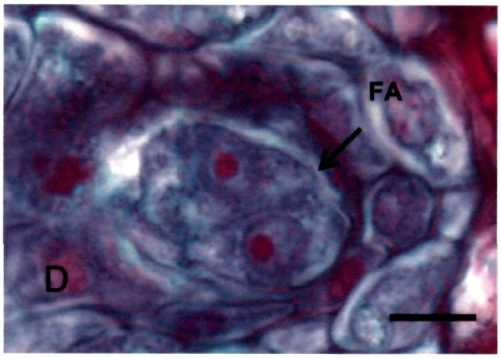
Plate 7.2

Organization of embryo sac (Polygonum Type)









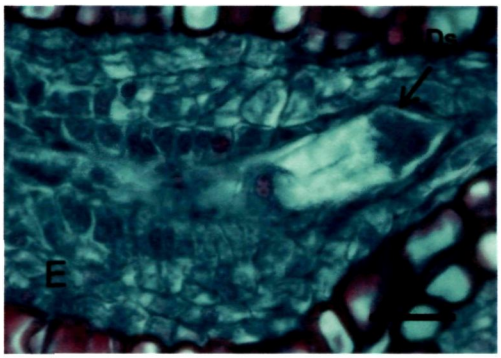


Plate 7.3

A. Longitudinal Section of ovule showing zygote with two nuclei of endosperm. Bar = 0.4 µm.

B. First division of zygote form apical cell 'C*a*', basal cell 'C*b*' and division of primary endosperm nucleus leads to two nucleate endosperm. Bar = 0.87 µm.

C. Four celled filamentous proembryo arranged in a linear patterns viz. *cc,cd, ci* & *m.* Bar = 0.87 µm.

D. 8-10 celled filamentous proembryo. Bar = 0.87 µm.

E. The terminal tier (derivatives of '*ca*') forms the octant proembryo (o) and the sub terminal tier (derivatives of '*cb*') form the suspensor (s). Bar = 0.80 µm.

Plate 7.3

Embryogeny

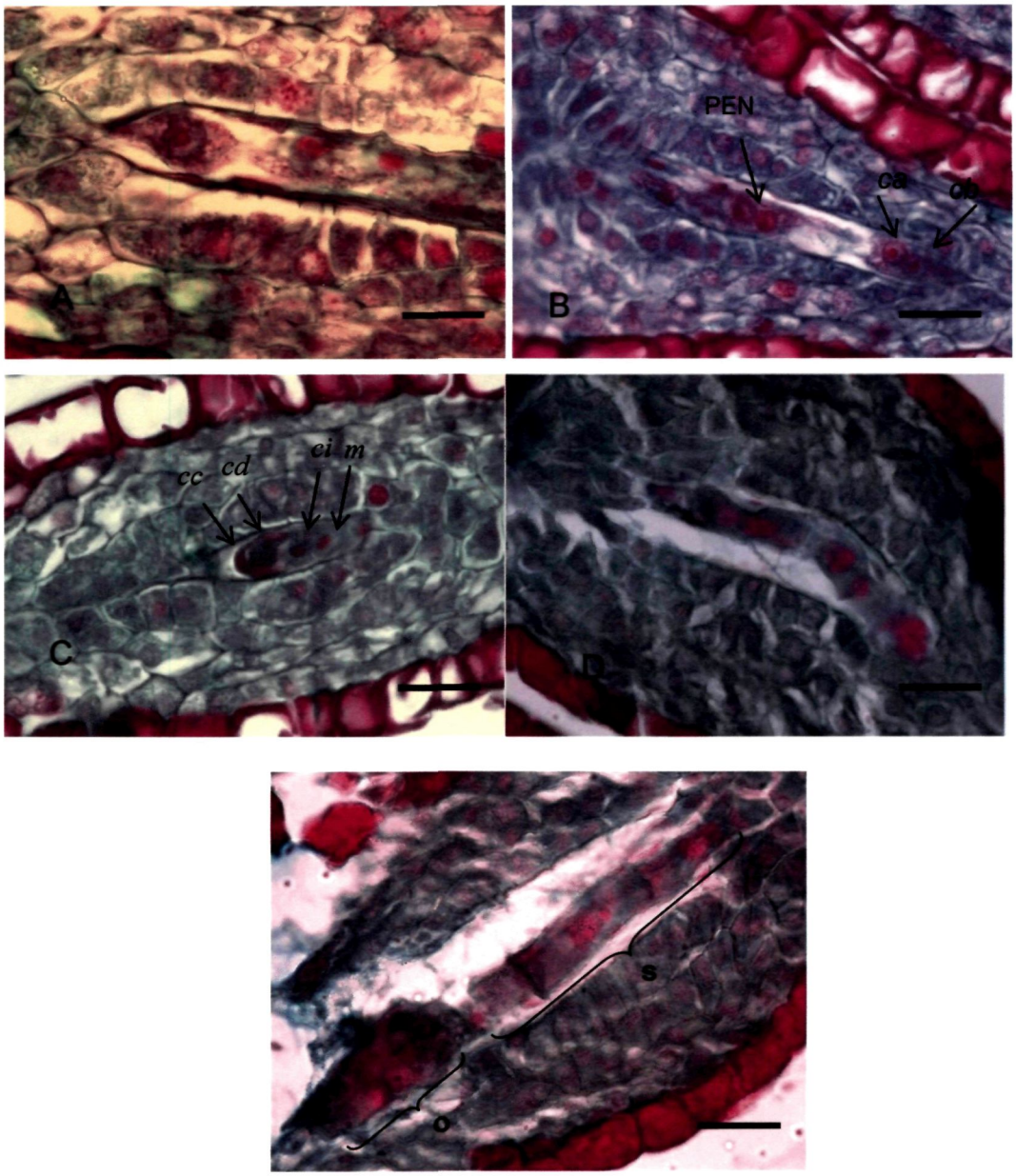


Plate 7.4

A. L.S of seed showing the globular embryo (Ge) with suspensor (s). Bar =100 µm.

B. An enlarged view of globular embryo (Ge) with suspensor (s). Bar =14 µm.

C. L.S of seed showing embryo (Em) and endosperm (En) with rich reserved materials. Bar =14 µm.

D. Whole mount of seeds showing endosperm (En) and embryo (Em). Bar =140 µm.

Plate 7.4

Embryogeny

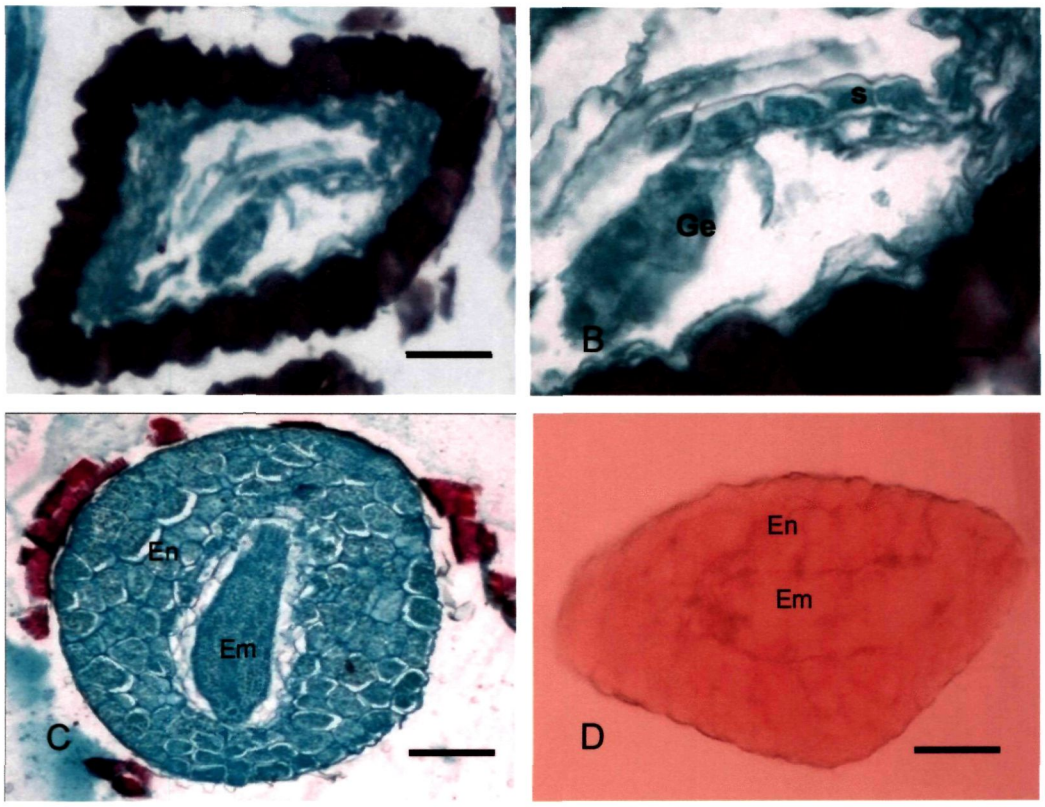


Plate 7.5

A. Embryo sac with primary endosperm nucleus (PEN) and the degenerated synergid of egg apparatus. Bar = 17.5 µm.

B. Enlargement of central cell and micropylar chalazal end respectively. Bar = 17.5 µm.

C. Micropylar haustorium of endosperm; note sac like structure with two nuclei. Bar =10 µm.

D. Chalazal endosperm haustoria with finger like projection towards the chalazal end. Bar =10 µm.

Plate 7.5

Endosperm formation

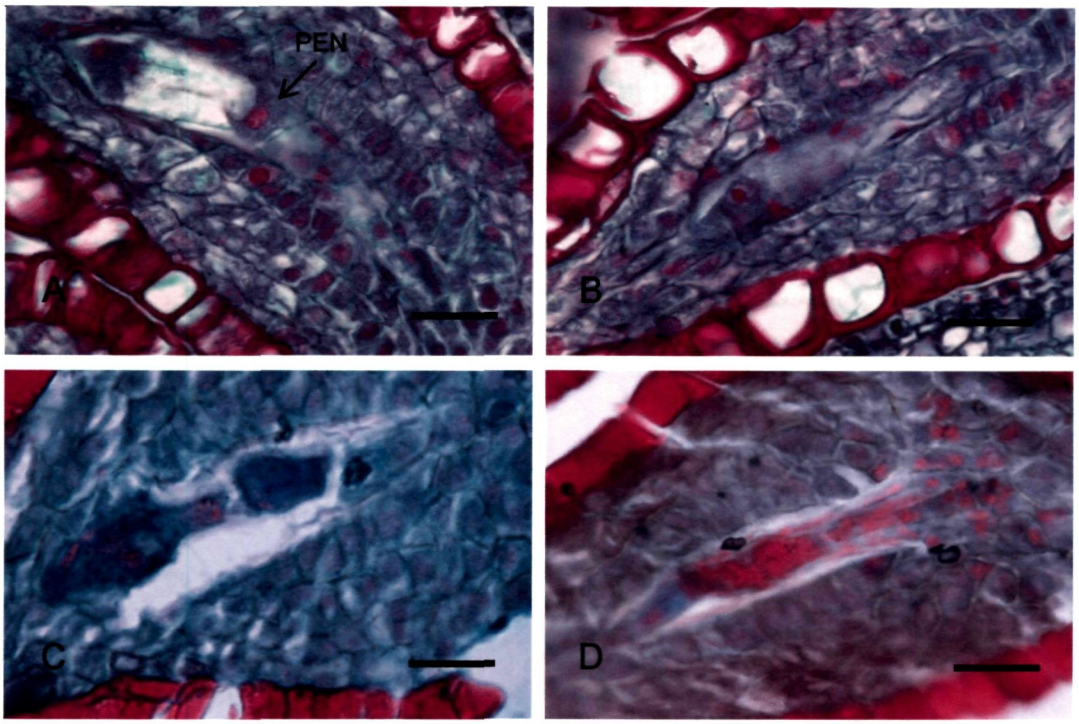


Plate 7.6

A. L.S of seed showing the endosperm and embryo in the seed.

Bar = 2.8 µm.

B. Showing an enlarge view of endosperm cells rich in starch grains.

Bar = 0.56 µm.

C. L.S of seeds stained with Bromophenol blue indicates the protein bodies in the endosperm and embryo respectively. Bar = 0.56 µm.

Plate 7.6

Endosperm formation

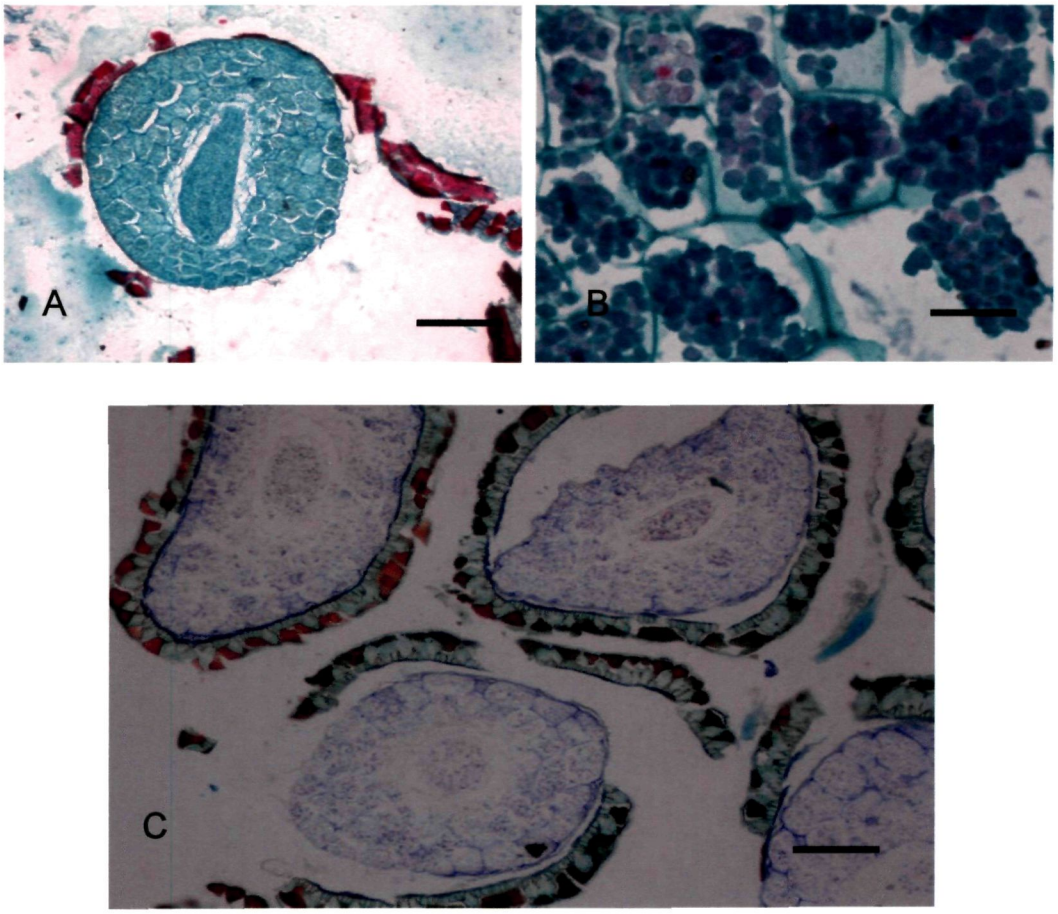


Plate 7.7

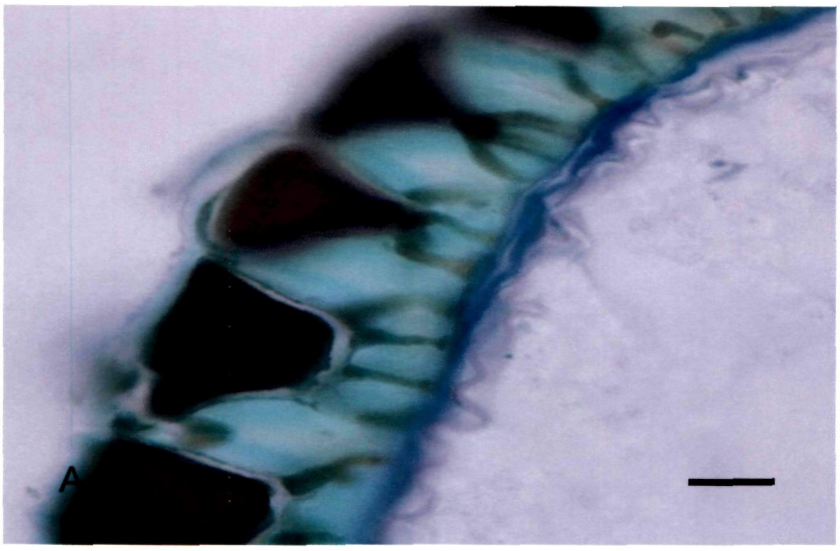
A. L.S of seed coat showing the deposition of phenolic content and wall thickenings in the seed coat. Bar = 0. 51µm.

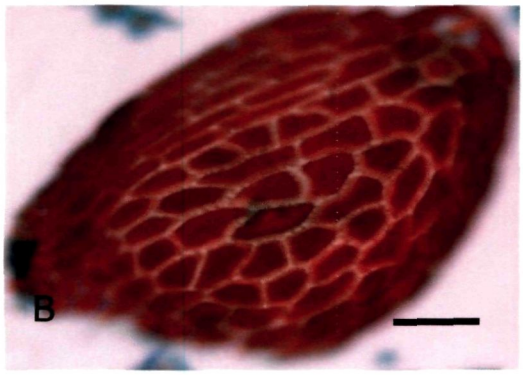
B. Surface view of seed coat with numerous pores due to the lignifications of cell wall as well as pillar like structure. Bar = 94.11 µm.

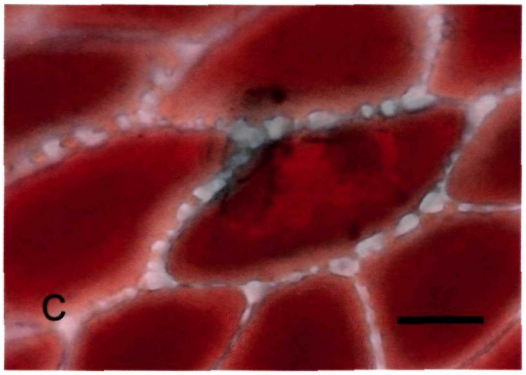
C. An enlarged view of seed coat showing the pores between adjacent cells. Bar = 23.52 µm.

Plate 7.7

Wall thickening in the seed coat







CHAPTER - 8

Seed germination

8.1 Introduction

The seed containing the embryo as the new plant in miniature is structurally and physiologically equipped for its role as a dispersal unit and is well provided with food reserves to sustain the growth of seedlings until it establishe itself as a self-sufficient, autotrophic organism (Bewley 1997). The first transition phase in the life cycle of a higher plant is seed germination. Germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis (Bewley and Black 1982). Subsequent events, including the mobilization of the major storage reserves, are associated with growth of the seedling. .

Seed germination is blocked by seed dormancy, which is sometimes considered as an adaptive trait that optimizes the distribution of germination over time (Graeber 2012). The transition phase from dormancy to germination is coordinated by both exogenous and endogenous cues (Koornneef *et al.* 2002). The exogenous cues, which include light, temperature, osmotic potential, pH and nutrients availability, in the control of seed dormancy and germination are well documented (Bewley and Black 1982). Earlier research on the control of seed dormancy and germination was mainly focused on the environmental factors, including temperature,

water, light, oxygen, etc (Ungar 1995; El-Keblawy and Al-Rawai 2006; Gorai and Neffati 2007). It was found that certain environmental condition may favor the germination of seeds in some species, but not other species. Different seeds have different temperature ranges within which they germinate (Mayer and Poljakoff-Mayber 1982). The optimum germination temperature for most seeds lies between 13°C and 40°C (Copeland 1976; Gorai *et al.* 2011). Therefore, no generalizations can be made as to which environmental factor plays a constant role in the control of seed dormancy and germination over a variety of species.

Endogenous cues, especially abscisic acid, gibberellin, brassinosteriods, ethylene, auxin, and cytokinin, interact with each other to form complicated signaling networks that regulate several processes in seed development (Kucera *et al.* 2005; Tamura *et al.* 2006). Plant hormones or phytohormones, including abscisic acid, gibberellins, brassinosteroids, ethylene, auxins, and cytokinins , are signaling molecules synthesized within the plant (Kucera *et al.* 2005). They exert profound effects on many fundamental processes during plant growth and development even at extremely low concentrations. Gibberellin plays a vital role in releasing dormancy and promoting germination and thereby counteracts the inhibitory effect of abscisic acid on seed germination (Xie *et al.* 2006; Beaudoin *et al.* 2000). Other phytohormones are not as crucial as abscisic acid and gibberellins in the seed germination process, while they act synergistically with or antagonistically to abscisic acid and/or gibberellins in the control of seed dormancy maintenance and alleviation (Bewley and Black 1982).

Gibberellins are a group of tetracyclic diterpene acids that are well known for their capability to promote plant growth. The gibberellins are named as GA_1 to GA_n according to the sequence of their discovery. Gibberellic acid (GA_3) is most frequently used in laboratory to trigger seed germination (Bewley 1979; Young *et al.* 1978; Debeaujon and Koornneef 2000; El-Barghathi and El-Bakosh 2005). In the process of seed development, gibberellins levels are usually high during the embryo morphogenesis phase and decreased during the embryo maturation phase (Hedden and Kamiya 1997). Active gibberellins may help promote the growth of embryo and later gibberellins are deactivated to avoid precocious germination (vivipary) (Hays *et al.* 2002). During the germination process, gibberellic acid is activated in the embryo and triggers the specific genes for α-amylase mRNA transcription (Chandler *et al.* 1984; Taiz and Zeiger 1998).

Seeds which do not germinate under favorable conditions, but may be induced to germinate, are considered dormant (Mayer and Poljakoff-Mayber 1982). Immature embryos, impermeability of seed coat to water or gases, mechanical causes, light and temperature requirements, or the presence of germination inhibitors are factors which may cause seed dormancy. A hard seed-coat may be impermeable to water, gases, or may constrain the embryo. Chemical and mechanical scarification, stratification, subjecting seeds to temperature extremes, soaking seeds in various concentrations of thiourea solutions, gibberellic acid, and combinations of one or more treatments have been used to break seed dormancy (Mc Connell 1960; Everett and Meeuwing 1975; Mc Henry and Jensen 1967).

In the Ericales, seeds are unitegmic, tenuinucellate, albuminous, and form both micropylar and chalazal haustoria (Peltrisot 1904; Maheshwari 1950; Palser 1961). Many common species from temperate understorey vegetation, including several members of the Ericaceae, Rutaceae and Dilleniaceae, have unknown dormancy mechanisms (Fox *et al.*1987; Dixon *et al.* 1995; Allan *et al.* 2004), although it is likely that many of these species have some form of physiological dormancy. Some progress has been made in unravelling dormancy cues of individual species from these groups, including *Leucopogon conostephioides* and *L. melaleucoides* (Tieu *et al.* 2001; O'Brien and Johnston 2004) and several *Hibbertia* species (Schatral *et al.* 1997; Allan *et al.* 2004).

There are scanty literatures on seed germination of *Gaultheria* species except for *G. antipoda, G. depressa* (Bannister 1990) and *G. procumbens* (Ruchala 2002). The information regarding the germination characteristics and requirements of the seeds of *G. fragrantissima,* growing in Meghalaya is not available. Therefore, this study deals with germination of medicinal plant *G. fragrantissima* which may be helpful in seed quality programme that enhance the percentage of seed germination and restoration programme for conservation and sustainable utilization.

8.2 Seed germination of *Gaultheria fragrantissima*

In natural condition seed germination of *G. fragrantissima* is low. Seed germination is hypogeal. Under laboratory condition, it took 19 days for the seed to germinate in control germination (water soaks only). During its

germination, a seed that contains the embryo imbibes water and testa is ruptured at the micropylar end (Plate 8.1A). After 5 days the growing axis has initiated and radicle became visible (Plate 8.1B). Subsequently it took 9 days for complete elongation of radicle till the formation of leaf primordia (Plate 8.1C-F). At this stage the radicle are about 5 mm stage. Radicles are smooth, shiny and greenish. After 18 days of growth, two leaflets became visible (Plate 8.1G). At about 34 days the embryo has completely reached the seedling stage. Primary root are well established at about 10-12 mm stage, however the number of rootlets are less of only two or three per seedling (Plate 8.1H). Seed germination in both petri plate (Plate 8.2A& B) and *Sphagnum* moss germination (Plate 8.2C &D) showed similar result, however in *Sphagnum* moss the seed can be left till it attains root formation that would be easy for its hardening and establishment when transplant in the soil. Seedling when transplanted in the soil established very well in hermaphrodite and male sterile plants but seedling survival is comparatively low in hermaphrodite plants (Plate 8.2E).

8.3 Seed morphology and seed germination of hermaphrodite plants

Seeds in hermaphrodite plants have both obliquely pyramidal and trapezoidal seeds in the same fruits (Plate 8.3A). This is due to the fact that large numbers of seeds are produced in the capsule, so they are crowded and assumed the shape depending upon the availability of space during its development. Seeds are glossy and are either yellow or light brown in colour

(Plate 8.3A). The epidermal cells of the seed coats are short, narrow and elongated. Outer periclinal cell walls of the seed coat rather broadly elevated and form concave structure so as to mark the margins around the cells. Seed coat ornamentation is reticulate (Plate 8.3C& D). Hilum is located at the narrow part of the seed (Plate 8.3C). In hermaphrodites, length of the seed ranges from 675.8 – 911.6 µm with a mean of 891.5 µm and breadth ranges from 287.6 – 355.00 µm with a mean of 322.0 µm. The number of fruits produced in *G. fragrantissima* ranged from 5–22 per inflorescence. A single fruit contains 68-250 seeds.

Gibberellic acid increased the seed germination and rate of germination in hermaphrodite plants (Table 8.1; Fig 8.1A). The increase in seed germination percentage is directly proportional to the increase in gibberellic acid concentrations within the range used (100, 200, 500 ppm). Seed germination percentage in control was 74.11 ± 4.66 %. 100 ppm gibberellic acid showed 80.58 ± 2.57 % seed germination. The highest percentage of seed germination occurred in 200 ppm with 87.39 ± 2.50 % and in 500 ppm with 86.23 ± 2.16 % (Table 8.1 and Fig 8.1A). One way analysis of variance (ANOVA) at $p \leq 0.05$ using Tukey's pos hoc test showed that there are significantly different between the control and gibberellic acid treatments, and also there were significant differences among three different gibberellic treatments in seed germination (Table 8.1). In control (water soak only), it took nineteen days for the seed to germinate where as in the three gibberellic acid treatments it only took twelve days for the seed to germinate (Table 8.1 and Fig 8.1B). Thus gibberellic acid treatments were effective for

increasing the speed of seed germination. There are significant difference ($p \leq 0.05$) between the control and gibberellic acid treatment, however there were no significant differences among all three different gibberellic treatments in time taken for germination.

8.4 Seed morphology and seed germination of male sterile plants

Seeds in male sterile plants of *G. fragrantissima* have both obliquely pyramidal and trapezoidal seeds in the same fruit which is similar to that of hermaphrodite plants (Plate 8.3B). This is due to the fact that large numbers of seeds are produced in the capsule, so they are crowded and deform one another during development. Like hermaphrodite seed the seeds of male sterile plants are also glossy and are either yellow or light brown in colour (Plate 8.3B). The epidermal cells of the seed coats are narrowly elongated cells. Outer periclinal cell walls of the seed coat slightly sunken. Seed coat ornamentation is reticulate (Plate 8.3E &F). The length of the seed ranges from 745.4 –941.2 µm with a mean of 901.1 µm and breadth ranges from 281.7- 375.2 µm with a mean of 342.8 µm. The number of fruits produced ranged from 5–22 per inflorescence and a single fruit contains 78-349 seeds.

Gibberellic acid also had a significant effect on seed germination of male sterile plant of *G. fragrantissima*. Gibberellic acid increased seed germination and the rate of germination in male sterile plants (Table 8.2; Fig 8.2A). Similarly as in hermaphrodite seed germination, the increase in seed

germination is directly proportional to the increase in gibberellic acid concentrations within the range used (100, 200, 500 ppm). Seed germination in control was 73.89 ± 2.31 %. 100 ppm gibberellic acid showed 84.12 ± 0.56 % seed germination. The highest percentage of seed germination occurred in 200 ppm with 89.23 ± 2.84 % and in 500 ppm with 84.80 ± 2.3 %. (Table 8.2 and Fig 8.2A). One way analysis of variance (ANOVA) at $p \leq 0.05$ using Tukey's pos hoc test showed that there are significant difference between the control and gibberellic acid treatments, and there were no significant differences among three different gibberellic treatments in seed germination (Table 8.2). In control (water soak only), it took nineteen days for the seed to germinate where as in the three gibberellic acid treatments it only took twelve days for the seed to germinate (Table 8.2 and Fig 8.2B).. Thus gibberellic acid treatments were effective for increasing the speed of seed germination in male sterile plants. There are significantly different ($p \leq 0.05$) between the control and gibberellic acid treatment, however there were no significant differences among all three different gibberellic treatments in time taken for germination.

Thus 200 ppm gibberellic acid treatment showed highest percentage of seed germination in both hermaphrodite (87.39 ± 2.50 %) and male sterile plants (89.23 ± 2.84 %) and significantly reduced the time taken for germination from 19 days to 11-12 days in the gibberellic acid treatment.

8.5 Discussion

When a quiescent, nondormant seed is supplied with water and oxygen at favorable temperatures the embryo rapidly takes up water and continues its temporarily suspended development. After building up a certain threshold hydrostatic pressure the seed coat is ruptured and visible protrusion of the elongating radicle indicates the onset of elongation of the embryonic axis (Baskin and Baskin 1998). The developmental period from the increase of metabolic activity after imbibition up to this point of no return, logically separating the embryo from the seedling stage, can be referred to as germination (Jann and Amen 1977; Baskin and Baskin 1998).

The seeds of Ericaceae are derived from anatropous, unitegmic, tenuinucellate ovules (Peltrisot 1904; Netolitzky 1926; Stevens 1971; Corner 1976; Stevens *et al.* 2004). Seed sizes range from 0.2 to 3.5 mm (Szkudlarz 2001; Stevens *et al.* 2004), their shape is variable from globose to strongly flattened seeds, and they are winged in genera such as *Rhododendron* or *Ledum*, in which wings develop at both of the polar ends and continue the seed coat. The seed in hermaphrodite plant of *G. fragrantissima* are oblique pyramidal and trapezoidal in nature, 0.89 mm long and 0.32 mm wide. Male sterile seed are also oblique pyramidal and trapezoidal in nature, 0.91 mm long and 0.34 mm wide. Thus morphologically seed shape and seed size are more or less same in hermaphrodite and male sterile plants. Pianka (1970) observed that seeds of most species of Gaultherieae are minute (only 0.37 ± 0.26 to 1.98 ± 0.84 mm in size), dry and numerous in each fruit. Seed coat ornamentation is reticulate in *G. fragrantissima*. Lu *et al.* (2010) reported in

G. ovatifolia and *G. humifusa* that the seeds of both species have a light brown colour, an indistinctly protuberant hilum region, a nubiform epidermal cell shape and granulate periclinal wall ornamentation. But in hermaphrodite plants of *G. fragrantissima* the outer periclinal cell walls of the seed coat rather broadly elevated and form concave structure so as to mark the margins around the cells where as in male sterile plant the outer periclinal cell walls of the seed coat are slightly sunken.

There are significant different in germination rate between control and three gibberellic acid treatment in both hermaphrodite and male sterile plants of *G. fragrantissima*. However, germination rate across all treatments was not significantly different ($p \leq 0.05$). 200 ppm gibberellic acid treatment showed highest percentage of seed germination in both hermaphrodite (87.39 ± 2.50 %) and male sterile plants (89.23 ± 2.84 %) of *G. fragrantissima* and significantly reduced the time taken for germination from 19 days to 11-12 days. It was also similarly reported by Ruchala (2002) in *G. procumbens* where 200 ppm of gibberellic acid treatment recorded highest percentage of seed germination. Gibberellic acid can improve seed germination as well as substitute for the cold stratification that many seeds require (Taiz and Zeiger 1998). Bannister (1990) germinated two *Gaultheria* sp viz *G. antipoda* and *G. depressa* under 26 days cold stratification and took almost 120-121 days to germinate. However, in *G. fragrantissima*, with gibberellic acid treatment it took only 11-12 days to germinate there by enhanced the rate of germination. Thus gibberellic acid treatment may substitute for the cold stratification requirement of other *Gaultheria* sp. also

that may reduce the time taken for germination. Some researchers also suggested that gibberellic acid treatment may substitute for cold stratification and reported that gibberellic acid increased the germination percentage of *Arbustus andrachne* and *Arbustus unedo* (members of Ericaceae) (Tilki 2004). Karam and Al-Salem (2001) also indicated that treatment of *Arbustus andrachne* seeds with 250 or 500 ppm gibberellic acid was successful in breaking dormancy and resulted in 83–86% germination.

It is well known that hormones are a controlling factor in seed dormancy and germination. Exogenous gibberellic acid in dormant seeds results in dormancy breaking and germination promotion (Roychowdhury *et al.* 2012). Gibberellins also promote germination in non dormant seeds. The effect of gibberellins depends on the germination conditions and mainly on temperature, seed coat and balance between the endogenous hormones (Webb & Wareing 1972). Active gibberellins may help promote the growth of embryo and later gibberellins are deactivated to avoid precocious germination (vivipary) (Hays *et al.* 2002). Accumulation of gibberellin is accompanied with reduction of abscisic acid during seed imbibition, suggesting that gibberellin and abscisic acid play antagonistic roles in germination process (Olszewski *et al.* 2002). Gibberellin counteracts the effect of abscisic acid by promoting the embryo growth potential and the weakening of tissues covering the embryo (Bentsink and Koornneef 2008; Holdsworth *et al.* 2008).

Similarly, there are several reports by different authors on seeds treatments with similar concentrations of gibberellic acid that have been

shown to effectively increase seed germination for a wide variety of seeds of different groups of plants such as *Verbena bonariensis* (Kornegay and Doubrava 2006), *Sesamum indicum* (Kyauk *et al.* 1995), *Trichocereus terscheckii* (Ortega-Bae and Rojas-Arechig 2007), *Sesleria varia* (Castiglioni *et al.* 2004), *Ferula gummosa* and *Teucrium polium* (Nadjafi *et al.* 2005), black mulberry (Koyuncu 2005), *Cyclocarya paliurus* (Fang *et al.* 2006), *Prunus avium* (Cetinbas and Koyuncu 2006), and *Galeopsis speciosa* (Karlsson *et al.* 2006). Rossini *et al.* (2009) observed the same enhancement effects of gibberellic acid application in *Erica andevalensis* seed, but with higher gibberellic acid concentration of 400 ppm. In *Arbutus unedo* the increased in seed emergence percentage was with 1,200 ppm of gibberellic acid that was reported by Demirsoy *et al.* (2010).

Environment has a profound influence on seeds ranging from acquisition of dormancy to initiation of germination. Earlier research on the control of seed dormancy and germination was mainly focused on the environmental factors, including temperature, water, light, oxygen, etc. It was found that certain environmental condition may favor the germination of seeds in some species, but not other species. Therefore, no generalizations can be made as to which environmental factor plays a constant role in the control of seed dormancy and germination over a variety of species (Finch-savage and Leubner-Metzer 2006). Light also a significant effect on seed germination.

In general, small seeds respond to light during germination (Atwater 1980). In the case of *G. fragrantissima,* eighteen hour light is sufficient to

elicit greater germination percentages under laboratory conditions. There are several reports in Ericaceous taxa where light plays important role in seed germination as in *Calluna vulgaris* (Gimingham 1960), *Erica cinerea* (Bannister 1964), *E. tetralix* (Pons 1989), and *Vaccinium vitis-idaea* (Grime *et al.*1981). Light characteristics can be composed of intensity, wavelength, duration and direction, plants sense these different parameters of light to adapt themselves to the environment and to control various aspects of growth and development, such regulation is usually via the phytochrome family of photoreceptors (Quail *et al.* 1995). In seeds with coat dormancy, it is thought that light and gibberellins can both release (seed coat) dormancy and promote germination (Casal and Sanchez 1998; Leubner-Metzger 2001; Sanchez and Mella 2004).

Temperature plays a major role in determining the periodicity of seed germination and the distribution of species (Guan *et al.* 2009). In *G. fragrantissima* temperature ranges from 22- 25 °C seem to be favourable for the germination of this species. This is consistent with previous reports of seed germination of other members of the Ericaceae as reported by Malek *et al* (1989) in *Kalmia latifolia*, Arocha *et al.* (1999) in *Rhododendron chapmanii*, Blazich *et al.* (1991) in *Leucothoe fontanesiana,* and by Blazich and Rowe (2008) in *Rhododendron* and azalea species. In nature, the changes in seasonal temperature result in different germination timing of different plants. A well known example is annual plants, which are principally classified into two categories, summer-annuals and winter-annuals. Summer-annuals overwinter as seeds and complete their life cycle during the same summer

season; while winter-annuals germinate in the autumn, overwinter as seedlings before flowering in the spring. In other words, these two types of annual plants adopt different germination strategies in response to ambient temperature changes.

Germination commences with the uptake of water by the dry seed, imbibitions and is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it. Dry seed of *G. fragrantissima* must first absorb water to initiate subsequent physiological and metabolic processes. Water can soften the seed coat and cause the endosperm to swell. Meanwhile, nutrients in the endosperm are dissolved for embryo growth. But a seed with hard seed coat which is impermeable to water remains quiescent until the seed coat is forced to open by weathering or scarification. In *G. fragrantissima* treatment of seeds for 15 minutes with sodium hypochlorite essentially promote permeability of seed coat to water. Besides, the ability of a seed to uptake water also determines the efficiency of seed germination. In *G. fragrantissima* the present of numerous pores due to wall thickening of seed coat (Chapter 7) enhanced the rate of water uptake and exchange of gases which are also important factors that determine the efficiency of seed germination. The mucilage on the seed coat plays an indispensable role in enhancing water uptake during germination (Rautengarten *et al.* 2008). Water uptake during seed imbibition may also be controlled by aquaporins (a class of major intrinsic proteins), among which some members in the plasmamembrane intrinsic proteins (PIP) subgroup and the tonoplast intrinsic proteins (TIP) subgroup have been suggested to

be involved in the regulation seed germination (Gao *et al.* 2007; Vander Willigen *et al.* 2006; Liu *et al.* 2007).

Apart from suitable temperature and moisture status, the presence of oxygen is also of great importance to ensure the success of seed germination. Oxygen is an atmospheric gas, which means that it is deprived in deep soil or waterlogged environment. Therefore, seeds buried too deeply in the soil or immersed in water can be oxygen starved, and most of them will eventually die although some can survive by going into dormancy. Meanwhile, oxygen is consumed by seed respiration and energy (ATP) is produced in order to decompose the storage materials in the seed (Hourmant and Pradet 1981). The correlation between a high ATP level and oxygen availability means that oxidative phosphorylation should occur during the beginning of seed germination, which is the case in lettuce seeds (Hourmant and Pradet 1981).

In *G. fragrantissima* the fruit is berry like, usually bright blue or occasionally black, with a large fleshy calyx inclosing a loculicidal capsule. Immediately after fertilization the five sepals become fleshy and fuse with one another from the base upward; however, no fusion occurs between the calyx and the ovary wall. In both hermaphrodite and male sterile plants of *G. fragrantissima* seeds are small and numerous. However in both populations of Nongkrem and Lum Shyllong forest of hermaphrodite and male sterile plants of *G. fragrantissima* there are no seedlings observed in and around where the mother plant grows despite many fruits were observed to be old and shrivelled near the plants. Reyes (2002) found seeds of *G. procumbens*

to be abundant in the soil profile of the open habitat and young recovering forest sites, however Moola and Vasseur (2009) observed no seedlings in any of the population survey plots nor did they found any polycormons of recent sexual origin. Matlack and Good (1990) similarly failed to observe any evidence of seedling establishment in *G. procumbens*, despite a much greater sampling effort and surveys over a much larger range of soil types. *Monotropa uniflora* L., like others in the Monotrojoideae, produces numerous seeds per capsule but, presumably, produces very few seedlings (Wallace 1975). With few exceptions, radicle extension through the structures surrounding the embryo is the event that terminates germination and marks the commencement of seedling growth.

Variation in the shape of seeds is most often related to differing modes of their dispersal (Harper *et al.* 1970;) and thus variation in seed shape (Harper *et al* 1970; Szkudlarz 2009; Lu *et al.* 2010) observed suggests that there are different adaptations for seed dispersal among the members of the sub family Gaultherieae. Seed dispersal may be facilitating more efficient as the seeds are minute in members of Gaultherieae and, thereby, assure that every habitat can be colonized (Salisbury 1942). Species of *Leucothoe* have winged seeds and a dry fruit, a probable adaptation for wind dispersal (Middleton 1991b). Species of Gaultherieae with fleshy fruit are generally thought to be dispersed by birds, insects or mammals (Airy Shaw 1941; French 1992; Duthie *et al.* 2006). Nonetheless, Middleton (1991c) had a contrary opinion that animals might not disperse *Gaultheria* because many fruits were observed to be old and shrivelled on the plants. Similarly in *G.*

fragrantissima fruits were observed near the mother plant suggesting that they might not be dispersed by birds or mammals. *G. macrostigma* were found to possess a dorsiventral wing, suggesting that these species may be dispersed at least in part by wind. Lu *et al* (2010) observed the interweaving threads that appear to emanate from the seed surface in *G. hookeri* and *G. pyrolifolia*. The function of these threads is unknown, but could be related to seed dispersal. In *G. fragrantissima* however, the structure of seeds do not possess any adaptation like wings, arils, caudicles etc for dispersal mechanism.

Seedling establishment is a critical phase in its re-establishment to become autotrophic. In *G. fragrantissima* seeds are small and minutes. A major disadvantage in smaller seeds is the limited amount of food available for the new sporophyte, suggesting the possibility of specialized germination requirements (Salisbury 1942; Kuijt 1969). Francke (1934) found that seeds of *Monotropa hypopithys* L. of Ericaceae would not germinate unless a mycorrhizal fungus was present, indicating possible derivation of some factor from the fungus. This could represent a mechanism to circumvent the limited amount of endosperm by permitting germination only when mycorrhizal establishment is imminent (Olson 1980). Therefore three factors are considered to be very important for seedling establishment of *G. fragrantissima*; the maintenance of a very moist atmosphere around the seedling for much of the time, watering them with a garlic infusion or fungicides can also help to prevent damping off especially during initial stage of seed establishment and the introducing of ericoid mycorrhizal inoculants

that will enhance mycorrhizal association to acquire soil nutrients. The members of the Ericaceae often show association with ericoid mycorrhizal fungi; some have arbutoid mycorrhizas and ectomycorrhizal and few have arbuscular mycorrhizal fungi (Urcelay 2002). Bagyalakshmi *et al.* (2010) reported in the root of *G. fragrantissima* was found to be colonized by ericoid mycorrhizal fungi. Das and Kayang (2012) also observed multiple mycorrhizal structural occupancy in the roots of *G. fragrantissima.* Therefore, the survival of seedling of *G. fragrantissima* in the soil does not just depend on the ability of seeds to germinate; the ability of seedlings to become established is also critical which may be achieved through the mycorrhizal association in its habitat. For example the ericoid mycorrhizal fungi *Hytnenoscyphus ericae* is usually associated with *Calluna vulgaris,* and assist nutrient uptake in the limited nutrient environment of upland moorland as shown by Allsopp and Stock (1992). Also Strandberg and Johansson (1999) observed that growth rate has been shown to increase where *C. vulgaris* is inoculated with ericaceous mycorrhizal fungi.

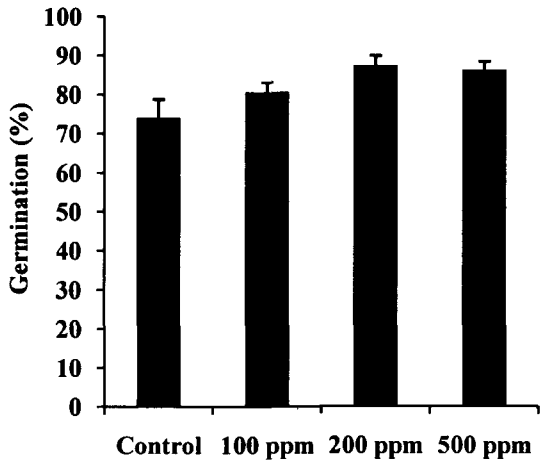
Thus these studies of seed germination of *G. fragrantissima* would enhance our understanding of the seed biology of these important medicinal plant species and contribute to the knowledge on the world biogeography of seed dormancy and germination (Baskin and Baskin 1998).

Table 8.1 Effect of Gibberellic Acid treatment on Germination (%) and Time of germination (Days) in hermaphrodite plants.

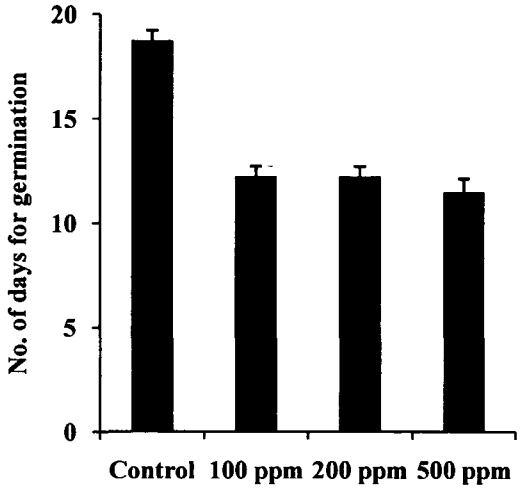
Sl. No	Treatment	Seed germination (%)	Time of germination(Days)
1	Control	55.55-80.09 $(74.11 \pm 4.66)^{a}$	18.00-20.00 $(18.75 \pm 0.47)^{a}$
2	100 ppm	74.88-89.68 $(80.58 \pm 2.57)^{ac}$	10.00-13.00 $(12.25 \pm 0.47)^{b}$
3	200 ppm	79.16-92.00 $(87.39 \pm 2.50)^{bc}$	11.00-13.00 $(12.25 \pm 0.47)^{b}$
4	500 ppm	80.08-91.16 $(86.23 \pm 2.16)^{ac}$	10.00-13.00 $(11.50 \pm 0.64)^{b}$

Note:(Values in the parenthesis are mean ± standard error. One way analysis of variance (ANOVA) showing significant difference at $p \leq 0.05$ using Tukey test. Values with the same letters does not differ significantly.)

Figure 8.1



A. Effect of different treatment of Gibberellic acid on percentage of germination of Hermaphrodite plants.



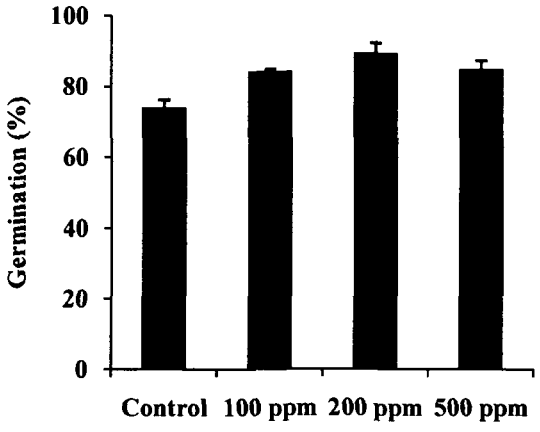
B. Effect of different treatment of Gibberellic acid on number of days of germination of Hermaphrodite plants.

Table 8.2: Effect of different Gibberellic Acid treatment on Germination (%) and Time of germination (Days) in male sterile plants.

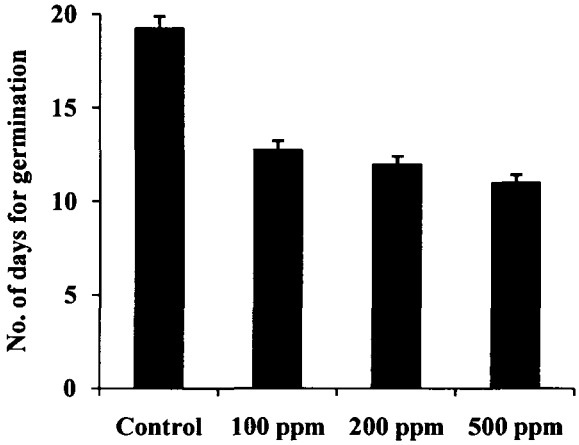
Sl. No	Treatment	Seed germination (%)	Time of germination(Days)
1	Control	67.56-81.86 (73.89 ± 2.31)^a	18.00-21.00 (19.25 ± 0.62)^a
2	100 ppm	83.33-86.38 (84.12 ± 0.56)^b	12.00-14.00 (12.75 ± 0.47)^b
3	200 ppm	82.30-97.23 (89.23 ± 2.84)^b	11.00-13.00 (12.00 ± 0.40)^b
4	500 ppm	78.83-91.24 (84.80 ± 2.3)^b	10.00-12.00 (11.00 ± 0.40)^b

Note: Values in the parenthesis are mean ± standard error. One way analysis of variance (ANOVA) showing significant difference at $p \leq 0.05$ using Tukey test. Values with the same letters does not differ significantly.)

Figure 8.2



A. Effect of different treatment of Gibberellic acid on percentage of germination of Male sterile plants.



B. Effect of different treatment of Gibberellic acid on number of days of germination of Male sterile plants.

Plate 8.1

A. Presoaked seed in water after 24 hours showing rupture of seed coat by imbibition of water. Bar = 400 µm.

B. Emergence of radicle after 5 days. Bar = 200 µm.

C- F. Elongation of radicle with two young cotyledons after 5 days to 13 days. Bar C = 266.66 µm; D = 400 µm; E = 320 µm; F = 177.77 µm.

G. An enlarged view of a seedling showing two leaf stage seedlings developed from the seeds still with no roots observed after 18 days. Bar = 177.77 µm.

H. A group of seedling showing the formation of primary and secondary root from the radicle as well as plumule in between the cotyledonary leaves after 34 days. Bar = 177.77 µm.

Plate 8.1

Stages of seed germination

***Gaultheria fragrantissima* Wall.**

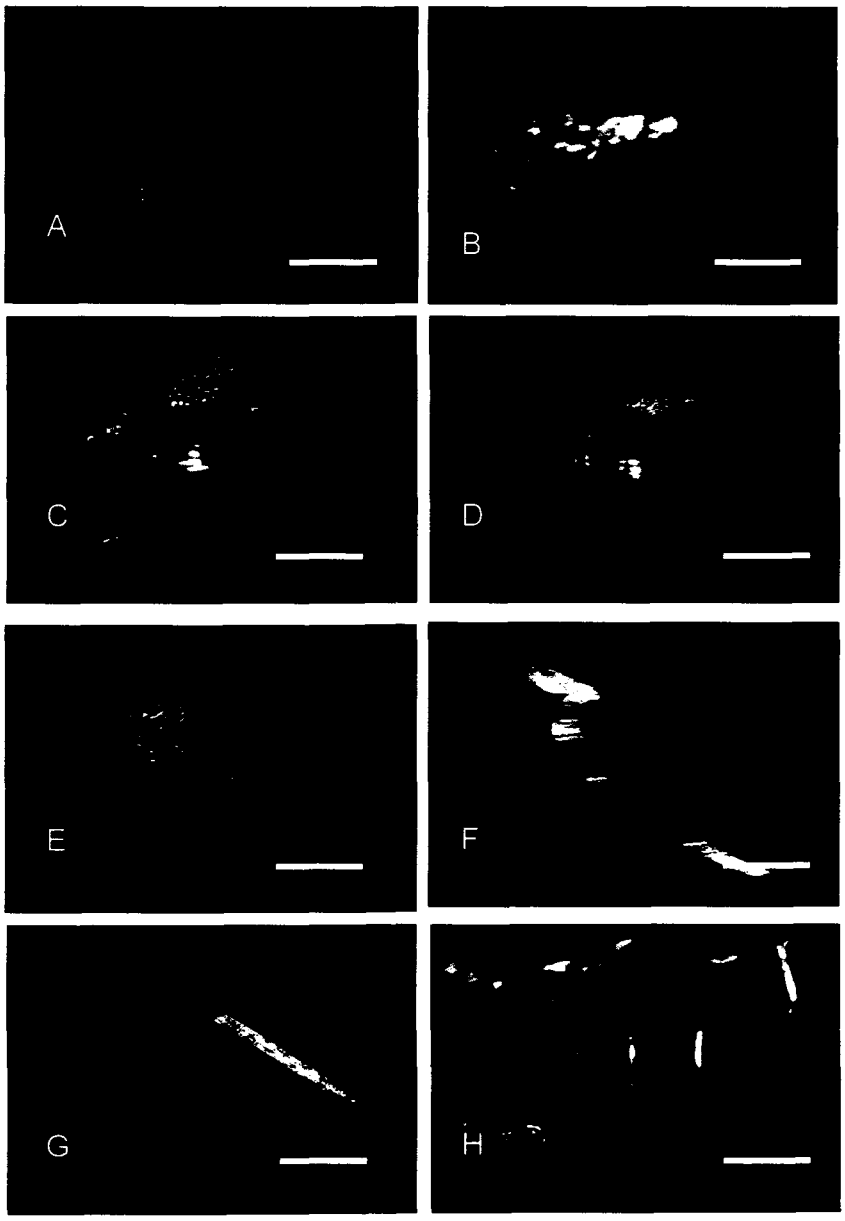


Plate 8.2

A & B. Seed germination in petri plate on the soaked filter paper. Bar A = 1.38 cm; B = 400 µm.

C & D. Seed germination in *Sphagnum* mosses after 34 days. . Bar C = 1.33 cm; B = 333 µm.

E. Establishment of seedling for transplantation on the garden soil after 57 days. Bar = 400µm.

Plate 8.2

Seedlings establishment

***Gaultheria fragrantissima* Wall.**

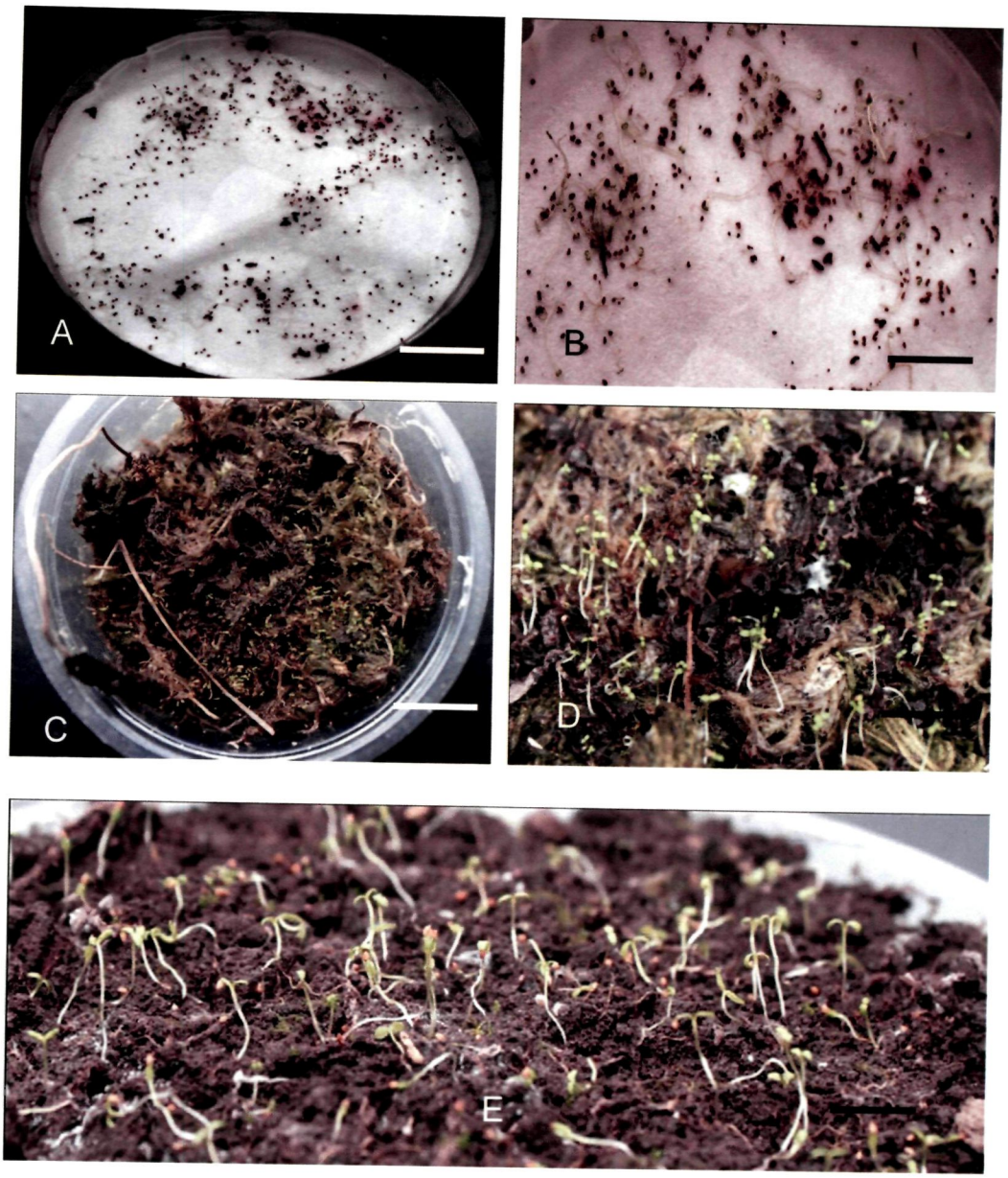


Plate 8.3

Seed Morphology *Gaultheria fragrantissima* Wall.

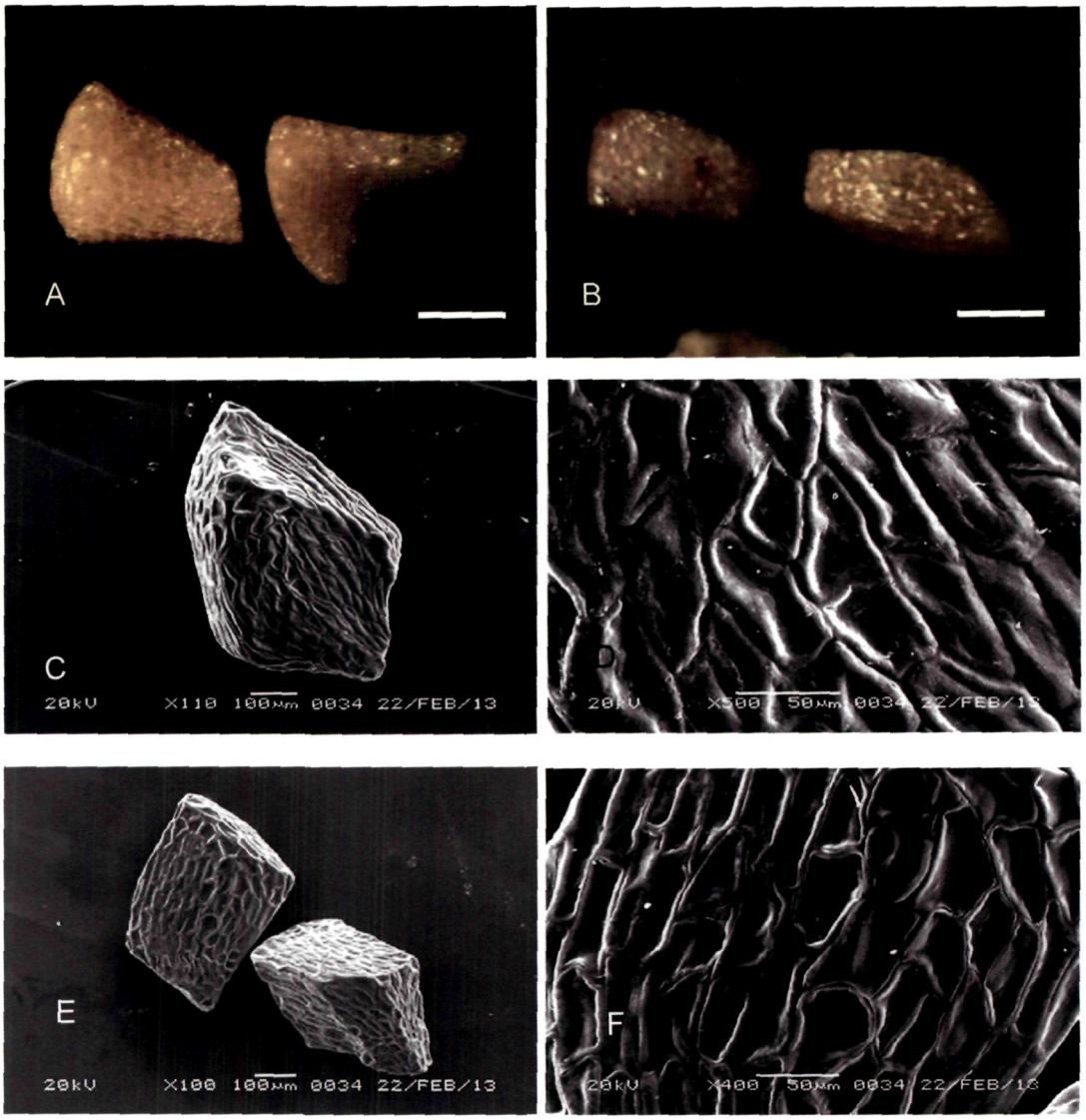


Plate 8.3

A & B. Obliquely pyramidal and trapezoidal seeds in the same fruits of hermaphrodite and male sterile seeds respectively. Note seed are glossy and are either yellow or light brown in colour. Bar A &B = 400 µm.

C & D. SEM view of hermaphrodite seed and seed coat showing reticulate ornamentation of the seed coats. Bar C = 100 µm; D= 50 µm.

E & F. SEM view of male sterile seed and seed coat showing reticulate ornamentation of the seed coats however seed coats are narrowly elongated cells. Bar C = 100 µm; D= 50 µm.

Chapter- 9

Summary and Conclusion

Plant sexual diversity presents many intriguing challenges and opportunities for the study of evolution and adaptation (Falk and Holsinger 1991). This interest arises because transitions affecting modes of reproduction have profound ecological and evolutionary consequences influencing genetic diversity within populations, phenotypic evolution and patterns of diversification (Bawa and Beach 1981; Bawa *et al.* 1985).

G. fragrantissima Wall (Indian wintergreen oil) belongs to family Ericaceae also known as Heath family is an evergreen ericaceous shrubs found in Indo-Malaya, North-East India; common at higher elevation in Shillong about 1500m a.s.l., particularly under pine forests and open places. (Haridasan and Rao 1985; Meher - Homji 1972).

G. fragrantissima Wall. is medicinally and economically important plant because of the presence of an essential oil in its leaves. Oil extracted from the leaves of *G. fragrantissima* Wall. is similar in its physical and chemical properties to the oil of Wintergreen obtained from *G. procumbens* Linn. and *Betula lenta* Linn, both natives of North America and thus it is commonly known as Indian Wintergreen Oil (Anonymous 1956). Therefore extraction of oil from *G. fragrantissima* would be a significant contribution to many small scale enterprises, farmers and local people as new income generating opportunities and earned a name of its own in the world market.

G. fragrantissima are found only in wild habitat, such as primary forest, and do not lend themselves to domestication. In *G. fragrantissima* the percentage of seed germination is very low in natural condition. Seed germination is hypogeal. Seed germination in both petri plate and *Sphagnum* moss significantly increased under laboratory condition. Seedlings of both hermaphrodite and male sterile plants when transplanted in the soil established very well but rate of seedling survival is comparatively low in hermaphrodite plants. Therefore, establishing plant nurseries with locally collected germplasm of male sterile plants for possible cultivation and domestication of *G. fragrantissima* by small scale farmers should be encouraged for optimize used and sustainable utilization.

Therefore, in this thesis, embryology of *Gaultheria fragrantissima* Wall. growing in North-East region of Meghalaya was studied with reference to the following aspects:

- Floral biology – morphometric details of flower and inflorescence, sex ratio in both hermaphrodite and male sterile plants.
- Microsporogenesis, megasporogenesis and formation of embryo sac in both in hermaphrodite and male sterile plants.
- Pollen viability and pollen germination of hermaphrodite plants.
- Pollination – pollination mechanism, pollinators and rewards to the pollinators.
- Post-fertilization changes in the embryo sac.
- Seed biology – Seed morphology, seed germination and viability

- *G. fragrantissima* has a distinct gynodioecious sex expression where hermaphrodite and male sterile individual co exist in the same population.
- Hermaphrodite flower are regular, 1.35x 4.1 cm, with axillary racemose and sometimes panicle inflorescence. Stamen 10, 2mm, epipetalous, height up to the middle of the corolla tube; with two distinct apical setaceous horns or awns, dehiscence porous, extrose.
- Ovary 5 locular, globose, pubescent, ovules numerous, axile placentation. Style cylindrical, slightly furrowed, erect, as long as the stamen up to the height of the anther awns or horns.
- Male sterile flower are irregular with axillary racemose and sometimes panicle inflorescence. Anther lobes release degenerated sporogenous tissues and formed a white unorganized mass of tissues.
- There is a high variance in the sex ratio of the two populations. Percentage of females varied in both the population where female frequencies is relatively low in Lum Shillong (10%) and more in Nongkrem sacred forest.
- There were significant differences ($P \leq 0.05$) between hermaphrodite and male sterile flowers of the two populations studied.
- Pollination is entomophilous where *Apis sp.* are primary pollinators.

- Pollen tetrads of hermaphrodite flower showed 75 % of the pollen grains are viable under the Fluorochloromatic Reaction (FCR) test.
- Based on modified Brewbaker and Kwacks medium, the optimum pollen germination was with the following concentration (i.e 10% sucrose, 120 ppm boric acid, 200 ppm calcium nitrate, 200 ppm potassium nitrate, 150 ppm magnesium sulphate) at pH 7.0 at 25 ^{o}C for 19 hours in dark at 90% RH in BOD incubator.
- The development of anther wall in hermaphrodite flowers of *G. fragrantissima* is of dicotyledonous type. Tapetum are binucleate sometimes polyploidy with dense cytoplasm and prominent nucleus and nucleolus. The tapetum is of secretory type.
- A special callose wall layer is secreted around each microspore mother cell, meiosis is of simultaneous type.
- Under SEM, the pollen tetrad surface is uneven and rugged, primary apocolpial exine sculpture moderate to coarsely rugulate psilate, the rugulae with minute striate surface somewhat flat, without any secondary sculpture surface somewhat flat, colpus membrane from granulate to smooth.
- Transmission electron microscopic studies of hermaphrodite pollen grains are tricolpate with thick exine composed of sporopollenin and intine composed of polysaccharides with three distinct germinal pores.
- Pollen grains in male sterile plants are with irregular projection of exine and presence of thick electron dense layer below the intine

which prevents the exchange of substances and formation of pollen tube.

- In *G. fragrantissima* the ovary is globular, the ovules are unitegmic, tenuinucellate and anatropous. The ovule is dizonate.
- The primary sporogenous cell does not undergo further mitotic divisions, it enlarge, elongate and function as megaspore mother cell. Endothelium surrounded middle portion of the megagametophyte except at the micropylar and chalazal ends.
- The development of the embryo sac confirms to the Polygonum or Normal type. Lateral view of embryo sac shows at the extreme micropylar pole the wall is strongly thickened, forming a lunar or sickle shaped structure known as "filiform apparatus".
- The zygote is an essentially elongated ovoid in structure and located at the micropylar pole of the embryo sac with its basal portion attached to the embryo sac wall, while the apical portion projects into the central cell.
- Embryogeny follows the Solanad type. The embryo of *G. fragrantissima* is straight, cylindrical, about two-thirds the length of the seed with two inconspicuous cotyledons, and is surrounded by endosperm.
- The endosperm is initially nuclear and later become cellular in the mature seeds.

- ❖ The micropylar haustorium has dense cytoplasm with two nuclei. The lower portion of endosperm elongates gradually and formed a sac like chalazal haustorial structure.
- ❖ The chalazal haustoria of *G. fragrantissima* are composed of densely cytoplasmic cells to form finger like projection.
- ❖ Seeds in hermaphrodite and male sterile plants have both obliquely pyramidal and trapezoidal seeds in the same fruits. SEM view of hermaphrodite and male sterile seeds showed reticulate ornamentation of the seed coats however in male sterile the seed coat are narrowly elongated.
- ❖ Gibberellic acid increased the seed germination and rate of germination in both hermaphrodite and male sterile plants. 200 ppm gibberellic acid treatment showed highest percentage of seed germination in both hermaphrodite (87.39 ± 2.50 %) and male sterile plants (89.23 ± 2.84 %) and significantly reduced the time taken for germination from 19 days to 11-12 days in the gibberellic acid treatment.

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Curriculum - vitae

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M. Sc	*I st*	*64.28*	*Botany*	*2004*	*NEHU*
B.Sc	*I st*	*74.00*	*Botany,Zoology, Chemistry,Environmetal Science, English*	*2002*	*NEHU*
AISSCE	*I st*	*64.00*	*Biology, Chemistry, Physics, English, Hindi*	*1999*	*CBSE*
AISSE	*II nd*	*56.66*	*Maths, Science, Social Science, English, Hindi*	*1997*	*CBSE*

Workshop and Trainings

- Participated in a national workshop on "**Reproductive biology of flowering plants for their conservation and improvement"** organized by Delhi University Botanical Society, Dept. of Botany, *Delhi University. Delhi- 110007 during* 27th -29 *April*, 2012.
- Participated in a workshop on "**Basic principles, preparatory methods and application in Transmission Electron Microscopy**" organized by SAIF, NEHU, Shillong from 22nd -25th March, 2011.

Presentation and Conferences

- Attended and participated in oral presentation entitled "**Anther development and microsporogenesis in hermaphrodite and male sterile plants of *Gaultheria fragrantissima* Wall**" in National Symposium on " Himalayan Biodiversity: Prospects and Challenges" held in the Centre of Advanced Studies in Botany, Department of Botany, North Eastern Hill University, Shillong from March 20-21,2014.
- Attended and participated in poster presentation entitled ***"Gaultheria fragrantissima Wall.* :Medicinal and Wintergreen oil yielding plant growing in Meghalaya, North East India"** during the 96th Indian National Science Congress held in North Eastern Hills University during the 3rd – 7th January 2009.

- Attended in National Roving Seminar on "**Traditional Knowledge**" organized by Ministry of Commerce and Industries, Government of India and the World Intellectual Property Organization in association with Centre for Environmental Studies, NEHU during 07-08 August, 2008 at Shillong.
- Participated in the Regional Workshop on "**Traditional Knowledge Based Biodiversity Conservation Practices in North East India**" jointly organized by the United States Education Foundation in India, New Delhi and Centre for Environmental Studies, North Eastern Hill University, Shillong during 23-24, March, 2006.
- Attended in "Regional Seminar on "**Biodiversity- Herbal Medicine**" organized by the Internal Quality Assurance Cell, Synod College at Dinam Hall Jaiaw, Shillong on 3rd August, 2006.

Publications

- **Floral biology of *Gaultheria fragrantissima* Wall. (Ericaceae) in Meghalaya, Northeast India**. *The International Journal of Plant Reproductive Biology.* 3(1). 23-29. 2011.
- **Anther development and microsporogenesis in hermaphrodite and male sterile plants of *Gaultheria fragrantissima* Wall.** *Grana* (Communicated).