

REPRODUCTIVE BIOLOGY OF
***PANAX WANGIANUS* S. C. SUN**

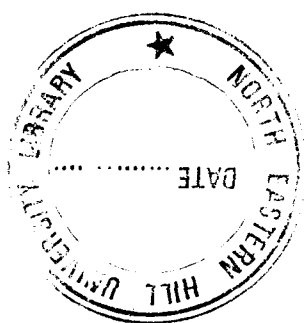
BY

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

DOCTOR OF PHILOSOPHY

IN BOTANY



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2011

DEDICATED

to

MY DEAR BROTHER

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SHILLONG

December, 2011

DECLARATION

I, Preeti Ahuja, hereby declare that the subject matter of this thesis entitled “Reproductive biology of *Panax wangianus* S.C. Sun” is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

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

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CHAPTER-1

Introduction

The Indian subcontinent has been reputed as the treasure house of valuable medicinal plants of the world (Padulosi *et al.* 2002). India has been identified as one of the top sixteen-mega diversity countries of the world. India has two mega-biodiversity hotspots: 1. Western Ghats (Karnataka, Kerala and Tamilnadu) 2. North-East region (Myers *et al.* 2000). An estimated number of about 70,000 such medicinal plants have been reported worldwide (Prajapati *et al.* 2003). Over 17,000 species of higher plants are found in India, of which 7,500 are believed to have therapeutic properties. Modern pharmaceuticals still contain at least 25% drugs derived from plants.

The North-Eastern region of India assumes a special significance in the biogeography of the country due to its unique location and varying eco-climatic conditions. North-East India is indeed a 'store house of biodiversity' because of its rich spectrum of species of flora and fauna. It is 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 1993; Khan *et al.* 1997). The Northeastern region comprises the eight states of India viz. Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Sikkim. It forms a unique biogeography province encompassing major biomes recognized in the world. It has the richest reservoir of plant diversity in India and is one of the 'biodiversity hotspots' of the world supporting about 50% of India's plant biodiversity (Mao *et al.* 2009; Mao and Hynniewta 2000). The undulating topography, high rainfall and varied altitudes are main factors that have contributed to its rich ecosystem and habitat diversity. North East India with its unique floristic diversity is endowed with rich heritage of medicinal plants. In northeastern region of India about 300 species of angiosperm plants having medicinal and aromatic properties are grown in large quantities as well as 400 additional species are also used by the villagers for curing for various ailments (Sharma 2005). Medicinal plants are of enormous economic importance and they are used as raw materials for the extraction of active constitution in pure form, as precursors for synthetic vitamins and steroids, and as preparations for herbal and indigenous medicines (de Padua *et al.* 1999).

Medicinal plants are not only a major resource base for the traditional medicine and herbal industry but also provide livelihood and health security to a larger segment of Indian population. The world markets for herbal medicine including herbal products and herbal material have been estimated to have an annual growth rate from 5% to 15% (<http://www.nmpb.nic.in>). The curative properties of drugs are due to the presence of complex chemical substance of varied composition (secondary plant metabolites) in one or more parts of these plants. These plant metabolites are grouped as alkaloid, glycosides, corticosteroids, essential oils etc.

Medicinal Properties and Uses

Most species of *Panax* are important medicinal herbs widely used as tonic medicine that supports the function of the adrenal glands, in particular the production of corticosteroids and male sex hormones. It also helps to improve blood flow through the coronary arteries, thus finding use as a treatment for arteriosclerosis, high blood pressure and angina. The roots are said to be analgesic, anti-inflammatory, antiphlogistic, antiseptic, astringent, cardiogenic, discutient, diuretic, haemostatic, hypoglycaemic, syptic, tonic and vulnerary. They are used in the treatment of contused wounds, soft tissue injuries and all kinds

of bleeding, both internal and external, like haematuria, nose bleeds, haematemesis, uterine bleeding etc. They are also used in the treatment of coronary heart disease and angina pectoris (Hu 1976; Carlson 1986; Dubrick 1983). The rhizome of *Panax wangianus* S. C. Sun has been used as a blood-regulating medicine and as a tonic and also in cases of asthma and cancer in Meghalaya (Sharma 2005; Wen 2007). Therefore, the therapeutic use of the species is well-established both in modern and traditional system of medicine. The principal reference components, to which pharmacological effects have been attributed, are in its ginsenosides (steroidal glycosides).

Meghalaya is one of the seven north eastern states lying between 25° & 26° 10' N latitude and 89° 45' & 92° 45' E longitude. The state has an area of 22549 sq km, and is surrounded by Assam and Bangladesh. This hilly state is an abode of clouds and thus increased moisture conditions prevail. Meghalaya state is endowed with rich genetic resources of horticultural, medicinal and aromatic plants, because it has a diverse topography, varied and abundant rainfall and different climatic and edaphic conditions (Falk and Holsinger 1991). Jain and Sastri (1980), Khan *et al.* (1997) have reported that more than 200 forest plants are used not only for their food, medicine, dye,

ornamental and constructional purposes but also for cultural and religious practices by the tribal community of Meghalaya.

The rich biodiversity in Meghalaya has been considerably affected by the various anthropogenic activities such as: urbanization, agricultural growth, introduction of exotic species, over exploitation of natural resources, pollution of soil, water and atmosphere due to coal mining and lime stone activities which contribute to the global climatic change (Pushpangadan and Nair 2005). There is a need to conserve the gene pools while utilizing them for sustainable economic development of the country (Jain and Sastri 1980; Roy and Tomar 2000).

The process leading to flower, fruit and seed formation remain poorly known particularly in the medicinal plants. Plants need to develop all parts of the reproductive structures and need to repeat that each time they re-initiate sexual reproduction (Bernardello *et al.* 1999; Moza and Bhatnagar 2007). The propagation, conservation and sustainable utilization can be done through the study of reproductive biology including the study of pollination, pollen vectors, gamete development, endosperm and embryo formation, operational mechanism of sterility factors at various levels like stigma, style, ovary and fertilization, flower architecture, number and age at flowering,

seed dispersal, germination capacity, survival rate of seedlings etc. and this will enhance the better seed set, multiplication, conservation of medicinal plants and revitalization of local health care. The knowledge will also help in the reintroduction of many plant species which are raised by micro propagation techniques (Pandey and Dhakal 2003).

Successful fertilization is dependent on the effective pollination (Shivanna and Sahney 1997). Pollination studies alone can provide a gamut of information about the loss of many species, because pollination is the fundamental step in plant sexual reproduction. Successful pollination is an essential pre-requisite for survival of plants in natural communities and is dependent on many biotic and abiotic factors. Because small populations may be less attractive to pollinators, the reduction in population size results in decrease of fruit set or seed production because of insufficient pollen transfer (Agren 1996).

The reproductive strategies affect its effective population size, the distribution of genetic variation, and the design of sampling and management strategies for conservation (Olfelt *et al.* 1998). Understanding the reproductive biology of species is essential to conservation efforts (Falk and Holsinger 1991; Anderson 1995),

particularly of endangered species where there are only a very few populations with limited number of individuals.

The family Araliaceae consists of approximately 55 genera and 1500 species which include a number of important medicinal and ornamental plants (Wen *et al.* 2001). The members of this family is mostly distributed in the tropics and subtropics especially in southeastern and southern Asia and the Pacific islands, but there are several well-known genera from the temperate zones as well (e.g. *Aralia*, *Hedera*, *Oplopanax* and *Panax*). The family includes a number of important medicinal plants, such as *Panax* (ginseng) and *Eleutherococcus* (Siberian ginseng), and several well-known ornamentals, including *Hedera* (English ivy), *Schefflera* (the umbrella trees), and *Polyscias*. In India, Araliaceae is represented by 16 genera distributed mostly in the northern and north-eastern regions of Himalayas.

The genus *Panax* consists of approximately 18 species out of which 16 are from eastern Asia and two from eastern North America (Reunov *et al.* 2008). The genus name '*Panax*' was derived from Greek. 'Pan' means 'all' and 'axos' means 'cure'. Literally '*Panax*' can be translated as 'cure-all' or panacea (Hu 1977). The herbal root is

named ginseng because it is shaped like a man. Actually the term 'ginseng' represents two Chinese ideograms: 'gin' (pronounced shen) refers to 'essence'. It is believed to embody man's three mythical essence- body, mind and spirit. Thus it is also referred to as the Lord or king of herbs (Yue *et al.* 2007; Anderson *et al.* 2002).

P. wangianus (syn. *P. pseudoginseng* Wall.) is a perennial, critically endangered (Pushpangadan and Nair 2005) herb native to sub-tropical wet forests of North-East Himalayan regions especially in the Law Lyngdoh, Smit sacred grove in Nongkrem (east khasi hills district), Law Lyngdoh (Mawphlang) and Shillong peak in Shillong, Meghalaya. The age old traditions of all three tribes of Meghalaya, namely the Khasis, Jaintias and the Garos, have saved the patches of forests in pristine conditions till date. These sacred groves chiefly fall in the sub-tropical zone represent the tropical broad-leaved hill forests (Champion and Seth 1968). These groves harbor a plant wealth uncomparable with any other forest types of the region. In the above mentioned sacred groves and undisturbed forests anthropogenic activities like cutting, grazing, mining etc. are almost absent because of various religious beliefs of tribal communities of Meghalaya in northeast India. The indigenous people of Meghalaya believe that their

sylvan deities live inside these sacred groves. They also believe that these deities would be offended if any damage is caused to the plants and animals in these sacred groves. Hence, nobody collects anything from the forest, not even fallen branches of trees (Samati and Gogoi 2007). Based on these beliefs, certain patches of forests are designated as sacred grove under customary law and are protected from any product extraction by the community (Tiwari *et al.* 2008). Because of its medicinal value and high price in the market, the species have been over-exploited illegally from all its natural habitats pushing them to the verge of extinction. The collection and export of *P. wangianus* has been banned by the Government of India (www.bsienviis.nic.in). *P.wangianus* was abundantly distributed about a century ago, but now their population is decreasing alarmingly (Nayar and Sastry 2000).

The members of the family Araliaceae are characterized by the relatively conserved floral, but diverse vegetative features (Philipson, 1970a; Eyde and Tseng, 1971). Members of the family possess pentamerous flowers, inconspicuous calyx lobes, solitary ovule in each locule, the ovarian disc, sub-prolate 3-colporate pollen grains, occurrence of abortive ovules, mono-or bisporic type of embryo sac development, nuclear endosperm, multilacunar node, medullary

bundles in the petiole, secretory ducts and rubiaceous stomata (Davis 1966; Elizabeth and Pigott 1914; Engler 1964; Erdtman 1952; Gopinath 1944; Pigott 1927; Cammerloher 1910; Schnarf 1931; Singh 1954). A considerable amount of work has been carried out particularly on the reproductive biology of some members of the Araliaceae, viz., *Panax fruticosum*, *Hedera australiana*, *Polyscias pinnata*, *Heptapleurum venulosum* and *Brassia actinophylla*, *Panax quinquefolium*, *Panax ginseng*. A perusal of literature shows that the reproductive biology has not been investigated thoroughly in *P. wangianus*, so far. Therefore, the present study aims to investigate the reproductive biology of *P. wangianus* an important endangered medicinal plant growing in Meghalaya.

The thesis deals with the following objectives in *P. wangianus*:

- ❖ To determine the interrelationship between the age, size, fecundity and climatic factors.
- ❖ To investigate breeding system and pollination mechanisms (It includes floral rewards, pollinators, pollen viability and pollen germination; stigma receptivity, pollen load on the stigma, ratio between pollen and ovule, number of viable seed in a fruit and seed dispersal).

- ❖ Developmental aspects of reproductive organs.
- ❖ Post-fertilization Development: endosperm and embryo formation.
- ❖ Seed germination.

CHAPTER-2

Review of Literature

Araliaceae are characterized by diverse vegetative but relatively conserved floral features (Philipson 1970a; Eyde and Tseng 1971). Most species of the family Araliaceae are trees, shrubs, or woody climbers; there are also a few herbaceous perennials (e.g. some species of *Aralia* and *Panax*). The leaves vary from simple to variously lobed or divided, to palmately and/or pinnately compound with sheathing leaf base, medullary bundles in the petiole, secretory ducts and rubiaceous stomata (Wen *et al.* 2001). Members of the family possess pentamerous flowers, calyx lobes are inconspicuous, five sepals; petals 5–10 (–12), which are free or form a calyptra (as in *Pentapanax* and *Tupidanthus*). Most araliads produce berries or drupaceous fruits that are bird dispersed (Ridley 1930).

Wen (2001) and Shu (2007) demonstrated the high level of morphological and reproductive plasticity in eleven species of *Panax* with respect to the shape and size of rhizome, number of leaves, leaflet shape, pubescence of leaves and number of flowers in an umbel

inflorescence. Guo *et al.* (2010) reported the morphological variations in stem, leaf, root, flower and fruit in the cultivated populations of *Panax notoginseng*. The retention of ancestral juvenile characters by mature plants was reported in few members of family Araliaceae such as *Schefflera racemifera* and *S. longipetiolata* by Fiasch and Frodin (2006) and Venugopal and Ahuja (2011). The young leaves are linear in *S. veitchii* however; *S. racemifera* and *S. longipetiolata* produce linear leaves during maturity.

The two American species, *Panax trifolius* and *Panax quinquefolius*, were defined on the basis of their morphology, molecular and pollen ultrastructure. Numerous studies on *P. quinquefolium* and *Panax ginseng* examined in relation to morphological features and ecological parameters (Lewis and Zenger 1982; Lewis 1984; Anderson *et al.* 1984; Anderson 1996; Hackney 1999; Shahi 2007; Van der Voort 2005; Stathers and Bailey 1986; Venugopal and Ahuja 2011); reproductive biology (Carpenter and Cottam 1982; Lewis and Zenger 1982; Schlessman 1985, 1987), chemical composition (Shim *et al.* 1983; Tomoda *et al.* 1985; Hansen and Boll 1986; Hikino *et al.* 1986; Oshima *et al.* 1987); medicinal properties (Hu 1976; Carlson 1986; Dubrick 1983) and metabolic

processes like rate of photosynthesis, respiration and transpiration under varied solar radiation and/or temperature conditions as well as growth and yield (Hu 1976; Lee *et al.* 1980; Konsler 1986; Proctor and Tsujita 1986; Stoltz 1982; Strick and Proctor 1985).

Yoo *et al.* (2001) reported that several Himalayan species of *Panax* were taxonomically problematic due to sympatry of morphologically distinct taxa and the existence of intermediates. With the advent of molecular techniques efforts were made to resolve various taxonomic disputes by using nuclear ribosomal DNA, ITS sequences, AFLP and cpDNA restriction site variations (Wen and Zimmer 1996; Zhou *et al.* 2005; Choi and Wen 2000).

Reproductive biology

The breeding system of few members of family Araliaceae have been described as polygamo-dioecious mainly dioecious, but some plants bear a few flowers of the opposite sex or sometimes a few perfect flowers (e.g. *Stilbocarpa*); dioecious or polygamo-dioecious (e.g. *Meryta*), dioecious or polygamous (unisexual and bisexual flowers on the same plant e.g. *Schefflera*), dioecious or monoecious

(e.g. *Neopanax*, now *Pseudopanax*), and dioecious (e.g. *Pseudopanax*) (Allan 1961).

Sexual dimorphism found in *Aralia nudicaulis* (Barrett and Helenurm 1981; Bawa *et al.* 1982). The male ramets bear more flowers per inflorescence, show greater frequency of flowering and occur in higher densities than do the female ramets (Bawa *et al.* 1982; Flanagan and Moser 1985). The low flowering capacity of female ramets is due to the greater resource expenditure associated with fruit production in females (Barrett and Helenurm 1981).

Aralia hispida (Araliaceae) plants are andromonoecious (Thomson *et al.* 1989) and exhibits synchronized protandry within and among different umbels which restrict opportunities for self- and geitonogamous pollination. This could provide unisexual mutants with an outbreeding advantage and favor the evolution of dioecism (Barrett 1984; Thomson and Barrett 1981). Individual ramets pass through alternating staminate and pistillate phases during their blooming period. On average a ramet function as a male for approximately three weeks and as a female for one week. Bumble bee pollinators prefer large male phase umbels over small female phase umbels in *A. hispida* (Thomson *et al.* 1982; Thomson 1988).



A. hispida and *A. nudicaulis* are pollinated by bumble bees (*Bombus vagans*, *B. ternaries*), andrenids, halictids and syrphids (Barrett and Thomson, 1982; Bawa *et al.* 1982; Thomson, 1988).

Hedera helix (Araliaceae) flowers late in the year between September and November and provide pollen and nectar for several insect groups such as *Aculeata* (bees and wasps), *Diptera* (true flies), and *Lepidoptera* (moths and butterflies). *H. helix* is obligate entomophilous. Therefore, it has a requirement for insect pollination to produce viable fruits. *Vespula* wasps are the most effective pollinators for *H. helix* (Jacobs *et al.* 2010).

The flowers of *Raukaua anomalus* (Araliaceae) are tiny, hermaphroditic, protandrous, and occurs in clusters on short peduncles at the leaf axils. Flowering occurs sequentially and each cluster may comprise buds, flowers and fruits at the same time. *R. anomalus* is self-compatible, but within-flower dichogamy largely prevents autonomous selfing (Merrett 2005).

Flowers of *P. ginseng* were capable of producing seeds via autogamy, outcrossing or agamospermy without pollination (Koren *et al.* 1998). *P. ginseng* is a facultative apomictic plant with a diplospory

type of agamospermy. This type of apomixis occurs without a reduction in chromosome number due to disturbances in meiosis and formation of diploid megaspores (Zhuravlev *et al.* 2008). Agamospermy is common among the Araliaceae family e.g. *Acanthopanax sessiliflorum*, *A. mandshurica*, *Eleutherococcus senticosus* (Hohlov 1978; Petrov 1976).

The new Caledonian endemic Araliaceous member *Polyscias pancheri* (Baill.) was functionally dioecious, with males bearing staminate flowers and females bearing morphologically hermaphroditic flowers that have sterile pollen. Dioecism in *P. pancheri* probably evolved from andromonoecism via gynodioecism. The strong floral protandry of *P. pancheri* females appears to be a retained ancestral trait, but its adaptive significance is obscure. This was the first report of functional dioecism of this sort in Araliaceae (Schlessman *et al.* 1990b).

P. quinquefolium has bisexual flowers (Carpenter and Cottam 1982; Lewis and Zenger 1982; Schlessman 1985). *P. quinquefolius* has a mixed mating system: flowers are self-compatible (Lewis and Zenger 1983; Schlessman 1985, 1987; Mooney and McGraw 2007). The male (anthers) and female (stigma) parts of flowers are reported to mature at

different times to allow cross-pollination to occur (Small and Catling 1999). Self-pollinated flowers produce the same proportion of seeds as cross-fertilized flowers (Carpenter and Cottam 1982; Schlessman 1985). Several field studies have shown that, in the wild, more flowers are produced than fruits, and seed production is much lower than its potential. Plants of *P. quinquefolium* rarely do vegetative propagation via rhizome fragmentation (Lewis 1988).

Schlessman (1985) suggested that the high pollen-to-ovule ratio of *P. quinquefolium* promotes adequate pollination even though pollinator visits are infrequent. The female reproductive success of ginseng is probably governed by the amount of photosynthate allocated to reproduction, rather than by abundance or efficiency of pollinators. Flowers containing one, two and three ovaries produce one, two, and three-seeded fruit, respectively, but three-seeded fruits are rarely observed in wild populations (Carpenter and Cottam 1982; Schlessman 1985; Schluter and Punja 2002).

Carpenter and Cottam (1982) and Lewis and Zenger (1983) reported protandry in *P. quinquefolium*. However, Schlessman (1985) found that protandry does not exist in *P. quinquefolium*. The reduced number of fruits per flower and the reduced number of fruits per plant

are consistent with the operation of an allele effect (Hackney and McGraw 2001).

Dwarf ginseng (*P. trifolius*) is a diphasic (“sex-changing”) species in which one phase has staminate flowers and the other has hermaphroditic flowers. This is the only known gender chooser in the genus *Panax* (Schlessman 1984, Hara 1970). Both gender phases have viable pollen capable of effective fertilization. Males produce more flowers and more viable pollen per anther than hermaphrodites (Philbrick 1983; Schlessman 1987, 1990a). The phenotypic gender of hermaphrodites is extremely female-biased; it is likely that hermaphrodites function essentially as females (Schlessman 1990a). The Halictid bee *Dialictus zephyrus* Smith, *Evylaeus* sp. and the Syrphid hover flies, *Toxomerus geminates*, *Melanostoma mellinum* L., *Mesograpta boscai* Macq., have been reported as pollen vectors in natural populations of *P. quinquefolius* in the states of Missouri, New York and Wisconsin (Duke 1980a, b; Catling and Spicer 1995; Lewis and Zenger 1983; Schlessman 1985). Catling and Spicer (1995) gave quantified data of pollen transfer by pollinators. However, these pollinators probably do not transfer pollen between distant individuals (Carpenter and Cottam 1982).

Dwarf ginseng (*P. trifolium*) is pollinated by syrphid flies, andrenid and halictid bees, honeybees and bumble bees (Philbrick 1983). Although it is genetically self-compatible, strong intra-and interfloral protandry effectively prevent self-pollination (Schlessman 1990a).

Pollen Morphology

The pollen morphology of several araliacean species has been investigated (Petrovskaya-Baranova 1959; Erdtman 1952; Tseng 1971, 1973, 1974; Tseng and Shoup 1978; Tseng *et al.* 1983; Shoup and Tseng 1977; Shang and Callen 1988; Park and Lee 1989; Yongquan and Jia-heng 1989; Henwood 1991). Erdtman (1952) described the pollen of 30 species belonging to 20 genera of Araliaceae as mostly three colporate, usually reticulate, with sexine thickened at the poles such patterns seem have been accepted as the dominant pollen types in Araliaceae.

Pollen of *Reynoldsia* and *Peckeliopanax* differs from *Tetraplasandra* in the pertectate sexine and the absence of inner nexinous breaks, respectively. A pollen correlation study shows that *Munroidendron* falls readily within the genus *Tetraplasandra* but is

distinguishable from the latter by its bifurcate colpi (Tseng 1971). The palynological characters together with other data suggest that *Tupidanthus* should occupy a higher systematic position than *Plerandra*. Interspecific variations also occur among the *Plerandra* species studied (Tseng 1973).

Pollen of *Boerlagiodendron* showed a remarkable degree of differentiation of sexine into pertectate and intectate pattern and reduced apertures, which are unique within the family Araliaceae (Tseng 1974). Eight pollen types were distinguished on the basis of sexine sculpturing and structure, apertural shape, and shape and size of the grains in 48 species of *Schefflera* by Tseng and Shoup (1978). Three sexine sculpture patterns were recognized in 10 species of *Acanthopanax*: perforate–rugulose, reticulate and reticulate with a sublayer of tectum. The rhomboidal pollen shape and basic sexine pattern of *Acanthopanax* resemble closely the *hoi* type of the related genus *Schefflera* (Tseng *et al.* 1983). Two pollen types found in 33 Malesian and Australian taxa of *Polyscias*: one consisting of small grains with microreticulate tecta and lalongate ora and a second with large grains, either reticulate tecta or intectate, and lalongate, circular, or lolongate ora (Henwood 1991).

Koren *et al.* (1998) found pollen grains of *P. ginseng* varying in size, shape and the number of apertures. There is only one report concerning the pollen ultrastructure of *Panax* and *Aralia* published by Wen and Nowicke (1999). According to these authors, a close affinity of *Panax* and *Aralia* is suggested. The palynological analysis of ten species of *Panax* does not confirm the monophyly of the group *Panax* species including *P. ginseng*, *P. notoginseng* and *P. quinquefolius*, although close relationship between them was stated on the basis of morphology and the triterpenoid qualitative analysis. Similarly, close sister-pair relationship between *P. ginseng* and *P. quinquefolius* suggested by Li (1972) was not supported. However, these electron microscopic data were restricted by pollen morphology and exine ultrastructure, although specific characteristics of the vegetative cytoplasm have not been analysed (Wen and Nowicke 1999). Besides, since pollen morphological diversity was reported by light microscopy in *P. ginseng* (Koren *et al.* 1998).

Pollen heteromorphism studied by scanning electron microscope in the anthers of *P. ginseng* (Reunova *et al.* 2007). Reunov *et al.* (2008) has reported that both the bud anthers as well as open flower anthers of *P. ginseng*, *A. elata* and *Oplopanax elatus* which

were characterized by a diversity of pollen size during the reproductive period. However, larger pollen was typical of the bud anthers, whereas smaller pollen sizes were more characteristic of the open flower anthers. The vegetative cytoplasm shrinkage, intine enlargement, exine aperture deepening and accretion as well as modification of the exine were responsible for pollen-size reducing metamorphosis in *P. ginseng*, *A. elata* and *O. elatus*.

The pollen grains of *Nothopanax arboreum* are boat-shaped, with three radiating grooves. In-vitro pollen grains did not germinate in 10 per cent sugar solution. The flowers of *N. arboreum* produced small quantities of nectar on the disc. They are visited by blow-flies, and occasionally by bees (Elizabeth and Pigott 1914).

Microsporogenesis

Microsporogenesis was studied in *P. fruticosum*, *H. australiana*, *P. ginseng*, *E. senticosus*, *Tieghemopanax sambucifolius* and *Brassia actinophylla* (Gopinath 1944; Mohana Rao 1972; Lin-De *et al.* 1998; Zhuravlev *et al.* 2008). There are four to six stamens in *P. fruticosum*, five in *H. australiana* and *T. sambucifolius*, and six to eighteen in *B. actinophylla*. Flowers with numerous stamens occur occasionally

within Araliaceae, for example in some species of *Plerandra* (Eyde and Tseng 1971); *Gastonia* (Philipson 1970b) and 200 in *Tupidanthus* corresponding to the petal lobes (Sokoloff *et al.* 2007). The anthers are tetrasporangiate. The archesporium consists of two to three initials and these divide to form outer primary parietal and inner primary sporogenous layers. The parietal layer divides further and forms three to four layers of cells. The outermost of these layers forms the endothecial layer. The middle layers vary in number. In *P. fruticosa* and *H. australiana* only a single wall layer is present whereas in *B. actinophylla* there are two layers. The sporogenous cells divide further and give rise to a number of microspore mother cells. The tapetal layer is closely adpressed to the microspore mother cells. There is a great variation in the number of nuclei in the tapetal cells of the Araliaceae. In *H. nepalensis* the cells are multinucleate (Singh 1954). Binucleate tapetal cells observed in *B. actinophylla*, *E. senticosus* *P. fruticosa* whereas in *H. australiana* the cells are uninucleate (Gopinath 1944; Lin-De *et al.* 1998). In *T. sambucifolius* the tapetal nuclei are polyploidy and are formed as a result of several nuclear divisions and fusions. The tapetum was of glandular type in *E. senticosus*. When microspores were at the uninucleate stage, the tapetum began to

degenerate *in situ*. When microspores developed into 3-celled pollen grains, the tapetum completely degenerated.

Meiosis of the microspore mother cells in *P. ginseng* and *E. senticosus* was accompanied by simultaneous cytokinesis and resulting in tetrahedral tetrad (Gopinath 1944; Lin-De *et al.* 1998; Zhuravlev *et al.* 2008). The nucleus of the pollen mother cell enlarges in size and various phases of meiotic and mitotic divisions occurred. Following nuclear divisions cleavage furrows with septa formation separate the four microspores. These are enclosed for some time in the common wall of the pollen mother cell and finally become separated as individual pollen grains. The microspores when mature distinctly show two coats, an exine and an intine which is thin. In *H. australiana*, *E. senticosus* and *P. fruticosa* two germ pores were observed. The pollen grains are trinucleate at the shedding stage.

Megagametophyte Development

The members of the family Araliaceae are characterized by the presence of a solitary pendulous, anatropous ovule in each locule (Cammerloher 1910), the ovarian disc (Davis 1966; Engler 1964), and occurrence of abortive ovules (Erdtman 1952). The embryo sac

development is mono-or bisporic types (Gopinath 1944; Elizabeth and Pigott 1914; Pigott 1927) and nuclear endosperm (Schnarf 1931; Singh 1954).

Tetraplasandra is a small Hawaiian genus of seven species with remarkable diversity in ovary position, ranging from inferior to completely superior. *Tetraplasandra gymnocarpa* is the only member of the Araliaceae with a fully superior ovary (Costello and Motley 2004).

Zhuravlev *et al.* (2008) reported that in *P. ginseng* the ovary is two-locular and each loculus contains two ovules. In *P. fruticosa*, *H. australiana*, *P. pinnata* and *Heptapleuram venulosum*, the ovary is inferior with a short style and 4-5 locular. It is 10 locular in *B. actinophylla*. The ovules are pendulous, anatropous, unitegminal and tenui-or crassinucellar and are found one in each locule. In *H. australiana* in spite of the fact that four to five ovules are present only one or two develop further (Gopinath 1944). An ovary has five loculi in *E. senticosus*. Each ovary loculus has two ovules: the upper ovule and the lower ovule. The upper one is orthotropous and degenerates after the formation of archesporial cell, while the lower one is

anatropous, unitegmic and crassinucellar, and able to continue develop further (Lin-De *et al.* 1998).

A well developed hypostase has been reported in *N. arboretum* (Elizabeth and Pigott 1914), *P. fruticosa* and *P. pinnata* (Gopinath 1944). However, in *H. nepalensis* (Singh 1954) and *T. sambucifolius* such a structure is lacking. A well developed funicular obturator is present in *N. arboretum* (Elizabeth and Pigott 1914), *H. australiana* and *P. pinnata* (Gopinath 1944). Singh (1954) observed some cells rich in protoplasmic contents in the funicular region near the micropyle and considered them to be the suppressed obturator initials in *H. nepalensis*. In *T. sambucifolius* also a prominent funicular obturator is formed.

The archesporium is invariably single cell but occurrence of double archesporial cells has been recorded in all plants studied except *P. fruticosa*. The differentiation of the archesporium in *P. ginseng* occurs early (Zhuravlev *et al.* 2008). The suppression of the parietal cell formation has been recorded in some cases (Ducamp 1902) where the archesporium directly functions as the megaspore mother cell. The hypodermal archesporium cuts off a parietal and a megaspore mother cell. The order of degeneration of megaspores is not regular but shows

variations. The megasporocyte undergoes meiosis and gives rise to a linear tetrad of megaspores in *P. japonicus* (Zhengyuan and Ruiling 1993). The chalazal megaspore usually enlarges and functions as the initial for the development of the embryo-sac. But the non-functional megaspores in *H. australiana* and *P. pinnata* also develop upto the two-nucleate condition (Gopinath 1944).

Most ovaries of *P. ginseng* exhibited tetrasporic female gametophyte development. Upper loculus of ovary has a reduced embryo sac, but normal development of the upper ovule is rarely observed. Normal embryo sac develops in the lower loculus. Often ginseng plants have formed fruits with one loculus. The polygonum-type of embryo sac is developed in the ovules of *P. ginseng*. The lower anatropous, tenuinucellate ovules bears a large central megaspore mother cell. Meiotic division of megaspore mother cell leads to four linear (or sometimes three) megaspores. After that, three (or two) micropylar megaspore degenerates whereas the chalazal one enlarges and becomes a functional megaspore with vacuolated cytoplasm. Mature female gametophyte usually consists of 7 cells: 3 antipodals at the chalazal end, a single large with fused central cell and the egg

apparatus consisting of two synergids, and an egg cell at the micropylar end (Zhuravlev *et al.* 2008).

In *P. ginseng*, several deviations take place during megagametophyte development. Absence of the mother megaspore cell or its duplication is often observed. Disturbances in succession of megaspores degeneration are revealed. Sometimes there are no visible remnants of degenerated megaspores during differentiation of embryo sac. Nuclei are not separated at the beginning stages of embryo sac formation. Very often the degeneration of all megaspores in ovule is occurred and embryo sac does not form. Megagametophytes development in different ovules of one ovary is asynchronous (Zhuravlev *et al.* 2008).

Most of the members of the Araliaceae investigated so far (Ducamp 1901a, 1901b, 1902; Singh 1954; Zhengyuan and Ruiling 1993) showed polygonum type of embryo sac. However, in *P. pinnata* and *H. australiana* Gopinath noticed bisporic development whereas in *B. actinophylla* there was a tendency toward the *Oenothera* type of development. Reversed polarity in the embryo sac of *H. venulosum* has been noticed by Gopinath (1943). The egg apparatus was situated at the chalazal end, the synergids showing prominent basal vacuoles. The

three nuclei at the micropylar end remained as such without becoming cellular. In *H. venulosum* the synergids are pear-shaped in the initial stages and develop beaklike structures later. In *H. australiana* the synergids are pointed at both ends. The antipodals remain nuclear in *P. fruticosa*, *H. australiana* and *P. pinnata*. In *H. venulosum* and *B. actinophylla* they form cells simulating the synergids in shape (Mohana Rao 1972).

Double fertilization is asynchronous in *P. ginseng*. After fertilization, the synergids degenerate and their debris is observed as two darkly stained bodies near the micropyle. The egg cell fertilization precedes the central cell fertilization by premitotic syngamy. The fusion of the first sperm cell with nucleus of the central cell of the embryo sac gives rise to the primary endosperm nucleus, followed by fertilization of the egg cell by the second sperm cell. The time of pollen tube outgrowth, fertilization and the resting period of the zygote are slightly different to the same taxa for Chinese ginseng (Yong-quan and Jia-heng 1989). Similarly, in *P. japonicus* the zygote undergoes a long period of dormancy (Zhengyuan and Ruiling 1993). Most of the normal developing ovules are fertilized at the post pollination stage for about three days. Rates of zygotic and parthenogenetic embryo

development are different. After cross-pollination, embryos undergo a short period of dormancy. The unfertilized egg cell and polar nucleus were not degenerated in the ten days after flower opening (Yong-quan and Jia-heng 1989).

The entry of pollen tube is porogamous type. The endosperm is first nuclear and later wall formation sets in *H. venulosum* and *B. actinophylla* (Gopinath 1944). Structural changes of the endosperm of *Panax ginseng* from fertilization to germination were investigated by Yu and Kim (1992). The endosperm of the *P. ginseng* seed is cellular type. Since endosperm cells adjacent embryo continuously breakdown and disappear with the elongation of embryo, the real of endosperm is gradually decreased. As the anatropous ovules of immature seed with green seed coat develops more and more, ovary cells adjacent ovary cavity become abundant by the periclinal division, their size is decreased, hypotrophy of cell wall discern, and they are gradually differentiated in seed coat. Endosperm cells adjacent embryo begins to breakdown in the endosperm tissue of indehiscent seed before the beginning of the after-ripening.

Johansen (1950) records onagrad type of the embryogeny in *A. racemosa*, *A. cachemiria*, *A. cordata*, *Fatsia japonica* and *H. helix*.

However, in *P. fruticosa* and *H. australiana* the embryogeny confirms to the Chenopodial type (Mohana Rao 1972).

Seed Germination

Pretreatment is not necessary for seed germination in *P. kikuyuensis* (Araliaceae). The seed germinates within 35-45 days (Rojo 1999). The seeds have an epigeal type of germination in *P. florosa*. Seeds without seed coat start to germinate two weeks after sowing in moistened paper towels. Seeds with the seed coat intact germinate 35 days after sowing. The first pair of cotyledonous leaves are obovate, smooth and with a very distinct midrib and four veins originating from the base. The first two veins near the midrib are more pronounced than the other two. The first leaf is entirely smooth and ovately elliptic (Dayan *et al.* 2008). The seeds of *A. nudicaulis* have dormant embryos (Heit 1967). Seed dormancy in *A. nudicaulis* can be overcome by three months of stratification at low temperature (Blum 1974; Dirr and Heuser 1987).

The sprouting of ginseng seeds, under natural conditions, takes from 18 to 22 months, since in the newly ripened seeds the embryo is not yet formed. The development of the embryo takes several additional months (after ripening of the seeds) (Grushvitzky 1956).

The seeds of *P. quinquefolius* exhibit morpho-physiological dormancy (Baskin *et al.* 1995) that prevents seed germination up to 22 months (Anderson *et al.* 1993, 2002; Lewis and Zenger 1982). To germinate, seeds require an after-ripening process and cold-stratification period (i.e. warm-cold sequence of seasonal temperature changes) to allow the embryo to fully develop and then to break seed dormancy (Charron and Gagnon 1991; Lewis and Zenger 1982; Schlessman 1985). The most vulnerable stages of the life cycle of *P. quinquefolius* appear to be the seed germination and seedling establishment (Carpenter and Cottam 1982; Charron and Gagnon 1991; Lewis and Zenger 1982). An individual takes several years to reach seed-producing stage. Seed production in *P. quinquefolium* is positively correlated with age and size of the plant (Carpenter and Cottam 1982; Lewis and Zenger 1983; Schlessman 1985). Seed production varies among populations and among years, and seed production is much lower than its potential (Lubbers 2006). *P. quinquefolium* does not form a long-term seed bank. It has reported to form a short-term seed bank of five years or less (Anderson *et al.* 1984; Anderson *et al.* 2002; Charron and Gagnon 1991; Lewis 1988; Van der Voort 2005). According to Lewis and Zenger (1982) studies on *P.*

quinquefolium in Missouri, a seed has only a 0.05% chance of reaching maturity. Seeds of *P. quinquefolium* are not animal dispersed and end up on the ground near the parent plant (Lewis 1988). McGraw (2000) and Farrington (2006) reported that the depth at which seeds of *P. quinquefolium* are planted affects the germination of seed. The lowest seed germination was recorded at 0 cm (on the soil surface) and the largest amount of germination was a depth of 2.5 cm (1 inch), with rates decreasing thereafter (McGraw 2003). Moreover, seeds from red fruit germinate at nearly three times the rate of seeds from green fruits (McGraw *et al.* 2005). Depth of sowing was not found to affect the survival of seedlings after one year.

Many factors affect seed germination of *P. quinquefolium* including stratification period (Li 1995), seedling time and depth (Liu 1988), temperature (Bae 1978), and spacing (Park 1987). Seeds of *P. quinquefolium* were harvested in late August or early September and immediately stratified in a sand box (Proctor and Louttit 1995). Embryos in newly harvested seeds of *P. quinquefolium* are not fully developed and have an average length of 0.5 mm (Hovius 1996). Stolz and Synder (1985) indicated that seed germination in *P. quinquefolium* is a two-stage process. During the first stratification period under warm

temperatures of 15 to 20°C (Jo *et al.* 1988), cotyledons, hypocotyls, radicles, and epicotyls become visible and the embryos continue to develop and reach a length of 3 to 3.5 mm (Yu and Kim 1992). Seeds with fully developed embryos need a second period at a cold temperature of 1 to 2°C to overcome endogenous dormancy (Proctor and Bailey 1987). Both the warm and cold stratification may take up to 18 to 22 months (Xiao *et al.* 1987). During this two-stage stratification, seeds are very vulnerable; without proper or suitable environmental conditions seeds will either rot, fail, or be slow to germinate. Uncontrolled fluctuating temperature and moisture levels, and the presence of pathogenic organisms in the seed box cause seeds to sprout prematurely, rot, dry out, or be delayed in germination by up to 2 years after seedling, with severely reduced germination rates (Li 1995).

Calcium limestone (CaCO_3), which increases soil pH and Ca^{++} ions concentrations, has been found to significantly increase the emergence and survival of American ginseng from seeds (Nadeau *et al.* 2003; Slak 2004).

Seeds of *P. ginseng* display a triple dormancy: immature embryo, hard seed (endocarp), and physiological dormancy. Therefore, the seed need two stages of stratification, i.e. warm stratification

followed by cold stratification for germination (Li *et al.* 2000; Li 2001). After removal from the fruits, seeds were mixed with sand and stratified for over three months with periodic watering at 15-25°C. During this warm stratification, embryos grow from the tiny immature stage at harvest to large structures with a 45% embryo to endosperm ratio, after which the endocarp opens (dehiscence). Though embryos continue growing after dehiscence to a morphologically mature stage, at which there is ~85% embryo to endosperm ratio, they cannot germinate due to physiological dormancy, which can be removed by cold stratification at 4~5°C for about three months (Kwon *et al.* 2001).

Stratification of the seeds also did not give satisfactory results. Seeds stratified immediately after ripening sprouted after eight months; those stratified after four months of storing in dry condition sprouted in 19 months (Daskalov and Ilieva 1958). Soviet agriculturalist and botanists, after several years of work, developed a new and far more effective method concerned with the hastening of seed germination with gibberellic acid. In the seeds treated with gibberellic acid the length of the first stage of after-ripening is reduced from four to two months; consequently, the whole period of preparation of the seeds for sowing is reduced from eight to six months. Along with this, the

germinating power increases from 50-70% to 90-100%. The best results were obtained by the treatment of the seeds with 0.05 or 0.1% solution of gibberellic acid over a period of 24 hours (Grushvitzky and Limarj 1956; Baranov 1966).

Soil pH and Ca are considered important factors affecting American ginseng growth (Nadeau *et al.* 2003; Beyfuss 1998; Hankins 2000). Calcium is the third most abundant element in plants and is integral in providing cell rigidity, cell elongation and division, membrane permeability, and critical enzyme activation. Beyfuss (1998) suggested that 2443 kg/ha gypsum (calcium sulfate dihydrate) should be applied to soils, to increase soil Ca. Lime was added to increase soil Ca, as well as to increase soil pH.

Only a few reports are available on storage and moisture content of seeds of *P. ginseng* (Kim *et al.* 2008). After one year of cold storage, dehiscence percentage of seeds of *P. ginseng* decreased to 38.7% and undehisced seeds did not germinate (Lee *et al.* 1995). Lee *et al.* (2004) reported that the germination percentage of seeds of *P. ginseng* stored at 5°C with 30% relative humidity decreased after six years and dropped to 2.5% after nine years.

The lowest safe moisture content, below which loss of viability occurs, of the dehisced seeds of *P. ginseng* was around 7.2%, which is lower than that of recalcitrant or intermediate seeds. Undehisced seeds are more tolerant than dehisced seeds, since a significant decrease in germination percentage was not observed at 7.1% moisture content (Kim *et al.* 2008). Since informations on the seed viability and seed germination of *P. wangianus* are lacking, an attempt was, made to find out the percentage of seed viability by germination test which may be helpful in seed quality programme.

CHAPTER-3

Materials and Methods

Study Area and Climate

The study was carried out in Meghalaya state of Northeast India, which is a land-locked territory lying between 25°47' and 26°10' North latitudes and 89°45' and 92°47' East longitudes. The state is encompassed with three major hills namely, Khasi Hills, Jaintia Hills and Garo Hills. The altitude of the state varies between 100-1900 m AMSL. It has a 496 km long international boundary with Bangladesh in the south and west, whereas the state of Assam surrounds it from the north and east. The Meghalaya plateau is highly dissected and has irregular terrain in the western and northern sides, and has a continuous escarpment with steep slopes marking the southern side. The state has seven districts with thirty-eight blocks with a geographical area of 22,429 sq km.

Panax wangianus S. C. Sun were collected from Law Lyngdoh, Smit sacred grove in Nongkrem (east khasi hills) 25°31'N, 91°52'E at

an altitude 1833 m AMSL, Law Lyngdoh (Mawphlang) 25° 26'N, 91° 44' E at an altitude 1796 m AMSL and Shillong peak 25° 32' N, 91° 49' E at an altitude 1965 m AMSL in Shillong, Meghalaya (Plate – 3.1).

Climatologically, this study area belongs to the sub-tropical wet climatic region (Champion and Seth 1968). On the basis of variation of temperature, rainfall and wind, the year in the region may be divided into four distinct seasons: (1) winter (December-February), (2) pre-monsoon or summer (March-May), (3) monsoon (June-September) and (4) retreating monsoon (October and November) (Porwal *et al.* 2000; Singh and Venugopal 2010). This region receives abundant southwest monsoon from June to October. Highest rainfall is recorded in the month of July and August. The mean temperature ranges from 6 to 17°C in winter and from 15 to 24°C during summer. The data on the climatic factors were collected from Central Seismological and Meteorological observatory, Shillong station, Government of India. The average monthly mean temperature, mean maximum temperature, mean minimum temperature, rainfall and relative humidity for the years 2007, 2008 and 2009 were choose for this study (Fig. 4.2). Light intensity and relative humidity were measured using a Photometer,

LiCor Model LI-189 and thermohygrometer (TFA, Germany) respectively. Soil pH was determined immediately within 24h of collection following standard methods outlined by Anderson and Ingram (1993). The soil type is oxisols which can retain moisture content (Brady and Well 2002).

Phenological events (bud break, flowering, fruiting, shedding of leaves, fresh leaf emergence and fruit dispersal) were observed in 2007–2010 in a naturally growing population of *P. wangianus* in Nongkrem sacred grove (25° N, 91° E), Shillong, Meghalaya (Fig. 4.1).

The different phenophases and reproductive cycle were monitored for three consecutive years (2007, 2008 and 2009), with an interval of two to three weeks duration during the growing season April to September of *P. wangianus* in three populations. In each visit both the vegetative and reproductive status was recorded. The vegetative and reproductive characters were taken from the juvenile stage to fruit formation. After full expansion of the leaves, length and width of leaflets were measured and their leaflet area was measured by a Planimeter (Anderson *et al.* 1993). Age of the *P. wangianus* was recorded by counting the number of bud scale scars on the rhizome that form as a result of the annual abscission of the aerial stem. The aerial

stem is produced only once in a year per rhizome. The relationship between the age of the plant and morphological characters (height of the plant, number of prongs and leaflets and average leaflet area) and reproductive characters (length of peduncle, initiation of flower buds, mature flowers, anthesis of flowers, numbers of axillary inflorescence, young and mature fruit formation and seed dispersal) were statistically calculated by using Pearson's correlation coefficient. The mean value and standard deviation were calculated from 2016 individuals of the population of *P. wangianus* in Nongkrem sacred grove. Multiple regression analysis was calculated to determine the influence of age on morphological and reproductive characters by using Statistica version 5.0 software (Zar 1974).

Developmental aspects of flowers

The bisexual flowers of *P. wangianus* S. C. Sun at various stages 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. The samples fixed in Cornoy's fluids were transferred to 70% ethanol for preservation. 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 glutaraldehyde fixed samples were dehydrated in propanol and embedded in glycol methacrylate (Technovit 7100) (Fedder and O'Brien 1968). The paraffin and Technovit (7100) blocks 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).

Specimens for studies of floral anatomy were fixed in absolute ethanol and acetic acid (3:1, v: v), cleared in 7M NaOH, and stained with decolorized aniline blue to assess the degree of ovule development. Photomicrographs were taken by using Nikon E600 and Leitz fluorescence microscopes.

Ultrastructural details of external surfaces of the androecium and gynoecium were studied by using Scanning electron microscope (SEM). The following methods were employed for SEM studies:

1. Flower parts such as androecium, gynoecium and were dissected out with razor blades, fixed in 2-3% glutarelddehyde 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% OsO₄ for 2 hours.
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; single change in dry acetone for every 15 min.).
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 Joel (JSM-6360) Scanning electron microscope (SEM).

Transmission Electron Microscopy (TEM)

The following procedure was employed for the TEM study:

1. Flowers were collected; androecium as well as gynoecial parts 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% OsO₄ for 2 hrs.
2. After post-fixation, 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.

Pollen Count and Pollen Viability

Male inflorescences bearing flower buds about to anthesis were brought to the laboratory from the collection site in the month of June.

The cut ends of the inflorescence peduncles were immersed in water. For estimating the average pollen grain numbers produced in a flower, mature but undehisced anthers from 25 flowers were squashed in two or three drops of 25% glycerol (v/v) + 1 drop of 1% acetocarmine.

Pollen viability of fresh pollen grains was tested by using Fluorochromatic Reaction (FCR) Test (Heslop-Harrison and Heslop-Harrison 1970; Shivanna and Rangaswamy 1993).

1. Stock solution of Fluorescein diacetate solution (FDA) was prepared in acetone (2mg/ml). It can be stored in the refrigerator for months.
2. 10% sucrose solution was prepared to prevent bursting of pollen grains. 300mg/l of calcium nitrate was added into the sucrose solution to improve the response of pollen systems.
3. To 2-5ml of sucrose solution in a small glass vial drops of stock solution of FDA were added until the resulting mixture shows persistent turbidity. The mixture has to be used within 30 min. for the preparation of pollen viability otherwise most of the FDA would precipitate.
4. A drop of sucrose-FDA mixture was taken on a slide.

5. Sufficient amount of fresh pollen grains were suspended in the preparation.
6. The preparation was incubated in a humidity chamber (>90%RH) for 5-10 min.
7. At the end of the incubation period, a coverglass was lowered and observed the preparation under the fluorescence microscope with HPWB (High Performance Wide Band) filter.

Pollen grains that fluoresce brightly (which gave a bright, yellowish green) were scored as viable. And those didn't fluoresce were non viable. For calculating pollen viability, total number of viable and non-viable pollen grains was counted from 20 microscopic fields. Five replicates were maintained.

Pollen germination and Pollen tube growth

To study the pollen germination in vitro, pollen grains collected from the fresh flowers were incubated in modified Brewbaker and Kwack's medium (Dashek 2000). The concentration of sucrose (15%), boric acid (20 mg), calcium nitrate (50 mg), magnesium sulphate (30 mg), potassium nitrate (20 mg) and agar (0.6%) in modified Brewbaker and Kwack' medium induced pollen germination and tube elongation

at 23°C in both light and dark conditions. The pH of the medium was 5.6. In vivo pollen germination and pollen tube growth were checked by decolorized aniline blue fluorescent microscopic method (Shivanna and Rangaswamy 1993; Dashek 2000). Stigma receptivity was measured by the method suggested by Dafni (1992). Effect of light on germination and tube elongation were investigating by incubating the pollens in dark and white light. The light intensity was 530-750 lux. Pollen were incubated for 17 hours in the germinating medium and then fixed by putting a drop of FAA (Formalin: glacial acetic acid: 50% ethanol 1:1:10) and safranin on the incubated pollen. Per treatment 10 slides were maintained. Data on pollen germination were recorded by scoring 10 microscopic fields chosen randomly per slide. All the 10 slides were scored for data recording. Thus, altogether 100 observations were made for an inflorescence. Pollen germination was determined by taking mean of all the 100 readings. Pollen tube elongation was measured in 100 pollen tubes per slide, from ten different microscopic fields. The mean length of the 1000 tubes so measured gave the tube growth. The data were analyzed statistically by one – way ANOVA.

Pollination

Insect visitors on the umbels were observed throughout the day at 30 min. intervals in three flowering seasons (2007-2009) from April-May. Pollinators and casual visitors were distinguished on the basis of the pollen load on their body parts under a microscope (Kearns and Inouye 1993). Pollinators were fixed in 3% glutaraldehyde-PO₄ buffer (pH 7.2) for identification. Pollinators were identified up to family or order levels only with the help of Zoological Survey of India, Eastern Circle, Shillong, Meghalaya, India. Breeding experiments such as autogamy, geitonogamy, xenogamy and agamospermy were tested using controlled pollination studies. In order to observe the rate of natural fruit-set, 100 inflorescence on different plants were tagged and were followed until fruit development.

To test for autogamy, flowers were emasculated using a fine pair of forceps and then bagged. The bags were opened on the day of anthesis and the flowers manually pollinated by gently brushing the flowers which were again re-bagged. To effect cross-pollination on emasculated and bagged flowers (n = 100), pollen grains were used from different plants and for geitonogamous self-pollination (n = 100) pollen grains were applied from other applied from other flowers of the



same plant. To test for agamospermy, flowers were emasculated and insects were excluded with fine mesh bags.

In all manual pollinations, the bags were removed after 48 h to monitor flower retention. The index of self-incompatibility (ISI) was expressed as the ratio of the number of fruits formed through manual self-pollination to those formed through cross-pollination (Zapata and Arroyo 1978) at the end of 18-20 day. Foraging behavior of insects and birds was recorded.

Seed Moisture Determination

Fresh seeds were weighed and dried in an oven at 105°C for 24 hours and then weighed again and percent moisture content was calculated on wet basis (Roberts and Roberts 1972).

Seed Germination

The seeds of *P. wangianus* were collected from matured ripened fruits in September, 2010 from Law Lyngdoh, Smit sacred grove in Nongkrem and the natural subtropical forest at Upper Shillong. The fleshy and fibrous fruit pulp was removed by hand. The seeds were then completely washed several times with tap water to remove any

adhering fibres followed by initial soaking of seeds in water for 24 hours.

The presoaked seeds were surface sterilized using 1.05% (w/v) sodium hypochlorite containing few drops of Tween 20 for 20 minutes. The presoaked seeds were scarified with the help of a sharp surgical blade. The scarified seeds were then washed thoroughly in distilled water to remove any debris over the seed coat. Three sets were prepared separately viz., without any treatment, GA₃ treatment and addition of calcium limestone. 50 seeds soaked overnight in a 0.1% solution of gibberellic acid over a period of 24 hours and then washed with tap water. At the end these seeds were sown in rectangular trays filled with soil to study seed germination under laboratory conditions at the North-Eastern Hill University Campus, Shillong (latitude 25° 34' N and longitude 92° 54' E, 1450 AMSL). Remaining 50 seeds were sown in lime mixed soil (0.2 mg/gm). Seeds were kept moist by watering once in a day and they were maintained at room temperature (20-25°C). Seed germination rates were recorded upto December from the date of sowing beyond which the seeds ceased to germinate.

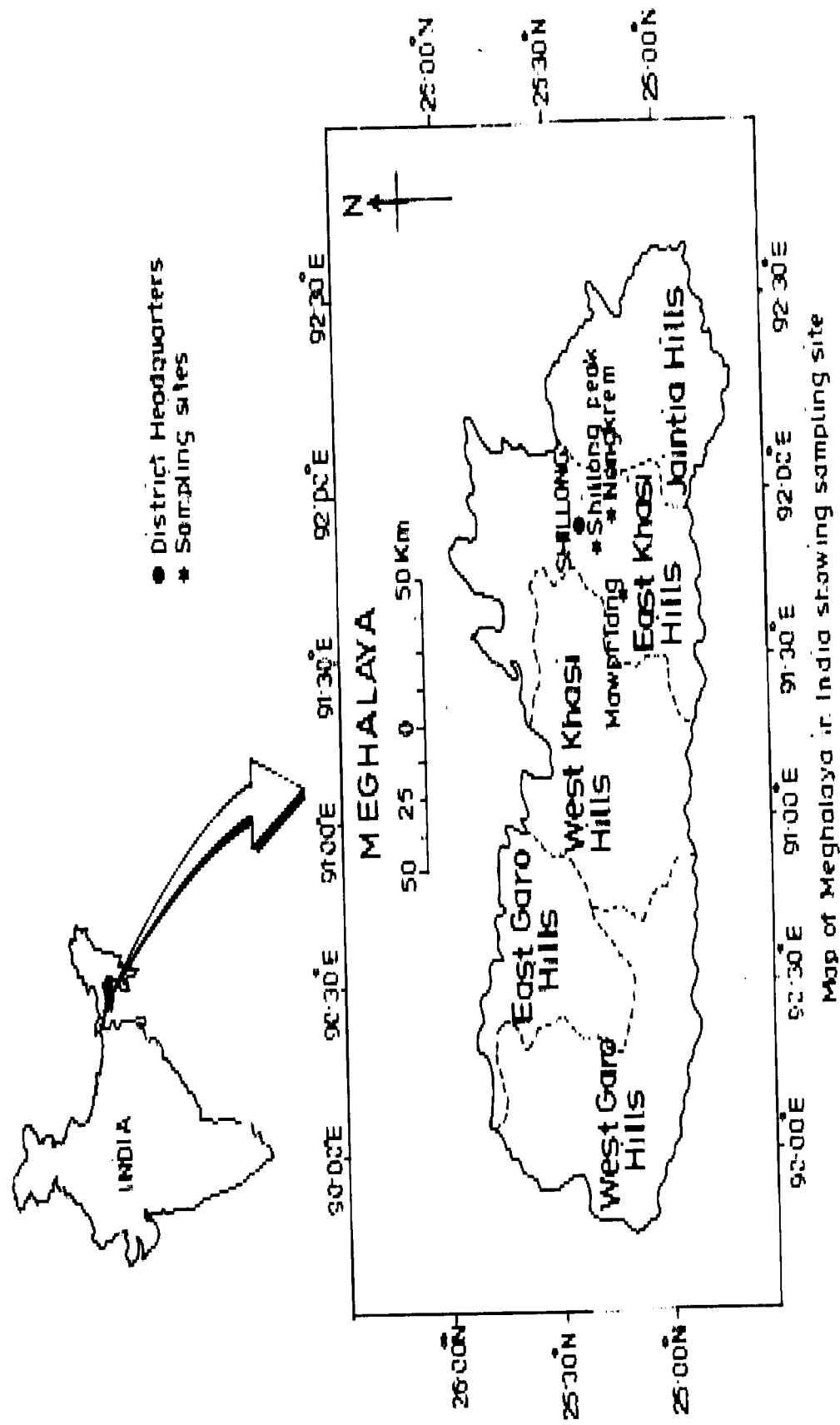
Visible emergence of radicle was taken as the criterion for germination (Kaliamoorthy and Rao 1994). The data on the percentage

of seed germination in response to varying concentrations of GA₃ treatment was recorded.

Plate – 3.1

Map of Meghalaya state showing the collection sites of samples.

Plate – 3.1



CHAPTER-4

Relationship between age, size, fecundity and climatic factors in *Panax wangianus*

4.1 Introduction

The genus *Panax* L. (Araliaceae) contains 18 species worldwide (Wen and Zimmer 1996; Wen 2001; Lee and Wen 2004; Reunov *et al.* 2008), out of which two species grow in eastern North America (Seemann 1868; Burkill 1902; Graham 1966; Proctor and Bailey 1987) and the other species in eastern central Asia (Burkill 1902; Hara 1970; Wen and Zimmer 1996). In Asia, Southeastern China and Eastern Himalaya is the centre of diversity for *Panax* (Burkill 1902; Hara 1970; Hu 1976; Wen and Zimmer 1996). The Chinese have been using ginseng for over 2000 years as a tonic, a stimulant and a fatigue-resistance medicine (Wen and Nowicke 1999). The wild roots of *Panax* are believed to be more potent than cultivated roots in Asia and thus the rhizomes of most species are harvested from natural habitats (McGraw *et al.* 2010).

The two American species, *Panax trifolius* and *P. quinquefolius*, were defined on the basis of their morphology, molecular and pollen ultrastructure. Numerous studies of *P. quinquefolium* and *P. ginseng* have examined various ecological relationships (Lewis and Zenger 1982; Lewis 1984; Anderson *et al.* 1984; Anderson 1996; Hackney 1999; Shahi 2007; Van der Voort 2005; Stathers and Bailey 1986), reproductive biology (Carpenter and Cottam 1982; Lewis and Zenger 1982; Schlessman 1985, 1987), chemical composition (Shim *et al.* 1983; Tomoda *et al.* 1985; Hansen and Boll 1986; Hikino *et al.* 1986; Oshima *et al.* 1987), medicinal properties (Hu 1976; Carlson 1986; Dubrick 1983) and growth, yield and rates of photosynthesis, respiration and transpiration under varied solar radiation and/or temperature conditions (Hu 1976; Lee *et al.* 1980; Konsler 1986; Proctor and Tsujita 1986; Stoltz 1982; Strick and Proctor 1985).

Wen (2001) and Shu (2007) demonstrated the high level of morphological and reproductive plasticity in eleven species of *Panax* with respect to the shape and size of rhizome, number of leaves, leaflet shape, pubescence of leaves and number of flowers in an umbel inflorescence. Guo *et al.* (2010) reported the morphological variations

in stem, leaf, root, flower and fruit in the cultivated populations of *P. notoginseng*. The retention of ancestral juvenile characters by mature plants was reported in few members of family Araliaceae such as *Schefflera racemifera* and *S. longipetiolata* by Fiasch and Frodin (2006) and Venugopal and Ahuja (2011). The young leaves are linear in *S. veitchii* however; *S. racemifera* and *S. longipetiolata* produce linear leaves during maturity.

Yoo *et al.* (2001) reported that several Himalayan species of *Panax* have been taxonomically problematic due to sympatry of morphologically distinct taxa and the existence of intermediates. With the advent of molecular techniques efforts have been made to resolve various taxonomic disputes by using nuclear ribosomal DNA, ITS sequences, AFLP and cpDNA restriction site variations (Wen and Zimmer 1996; Zhou *et al.* 2005; Choi and Wen 2000).

Relationship between age, size, reproduction and demographic studies of *P. quinquefolius* has extensively been studied in North America (Mooney and McGraw 2009; Charron and Gagnon 1991). However, *Panax wangianus* growing in sacred groves of Meghalaya in wild conditions has not been thoroughly investigated on its growth, behaviour and reproductive biology. Therefore, the present

investigation reveals that the morphological and reproductive changes in relation to the age of the plant and the effect of climatic factors on flowering, fruiting and seed production. This study will be helpful to understand the reproductive biology for its conservation and sustainable utilization. The objective of this study was to determine how plant size affects flowering phenology and to evaluate the effect of climatic factors on flowering, fruiting and seed production.

4.2 Morphological and reproductive variations in relation to the age of the plant

P. wangianus grows in a colony of a few plants under shady forest floor with rich leaf litter in Nongkrem sacred grove (Plate – 4.1). *P. wangianus* had a whorl of digitately compound leaf at the summit of aerial shoot; the leaf (prong) consists of three to eight palmately compound leaflets with a petiole, of which the terminal three to five leaflets are larger than the lateral two to three leaflets (Plate – 4.2 a–f). Leaves are exstipulate or occasionally with persistent stipules; leaflets are bristly along veins and veinlets on both surfaces, narrowly lanceolate to broadly linear. Rhizome is creeping and elongated with thick internodes (Plate – 4.3 c). The flowers were arranged in an

umbel inflorescence on a long peduncle arising from the centre of the leaf attachment at the top of the aerial stem. The flowers were bisexual and pentamerous with an inferior ovary. The fruits were berry.

The height of aerial shoot ranges from 30 cm to 112 cm depending upon the age of the plant. Similarly the aerial shoot diameter as well as rhizome diameter also varies from 0.3 cm to 1.5 cm and 0.5 cm to 3.5 cm respectively in different age classes (Table 4.1). Morphological variations were observed in natural conditions based on prong number (Plate – 4.2 a–f). Leaflet area increased concurrently with age. Plants of age class 1-10 years had one prong with three to five leaflets (Plate – 4.2 a) and 10-15 years had two prongs with six to ten leaflets respectively (Plate – 4.2 b). The average leaflet area of one-pronged and two-pronged plants was 2 cm² and 4 cm² respectively. Plants of age class 16-24 years had three prongs with 9-15 leaflets (Plate – 4.2 c) and 25-34 years age class contained four prongs with 12-21 leaflets (Plate – 4.2 d). The average leaflet area of three-pronged and four-pronged plants was 8 cm² and 16 cm² respectively. While plants of age class 35-50 years and above 50 years had five prongs with 22-36 leaflets and six prongs with 30-42 leaflets respectively (Plate – 4.2 e, f). The average leaflet area of five-pronged

and six-pronged plants was 32.5 cm² and 37.5 cm² respectively. Petiole length also increases with age of the plant. It ranges from 3cm to 32cm depending upon age and number of prongs of the plant (Table 4.1). Occasionally only one to two percent one-pronged and two-pronged plants were of more than 40 years old. It indicated that neoteny do exist in *P. wangianus* (Plate – 4.3 a, b).

In Nongkrem sacred grove, plants of age class 1-10 years and 10-15 years were either juvenile or vegetative stages only i.e. they were non-fecundity. During 2007-2009, the plants did not flower until they were grown and attained the minimum age of 16 years. Plants older than 16 years with more than three prongs were producing flowers from April to early May (Plate – 4.2 d). The length of peduncle of terminal inflorescence also varies from 15 cm to 40 cm in different age classes. The number of flowers in an umbel was 13-56 and 25-60 flowers in the age class 16-24 and 25-34 years respectively (Table 4.2). Plants older than 35 years had two to three axillary inflorescences in addition to the terminal inflorescence. The number of flowers in an umbel varied from 25-73 flowers. The numbers of axillary inflorescence were five to eight (Plate – 4.2 f) and the number of flowers ranged from 45-75 in the 50 years old plant. The length of

peduncle of axillary inflorescence was 7 cm in age class 35-50 years and 9 cm in age class 50 years and above (Table 4.2).

The number of style and stigmatic lobes varies in each inflorescence (Table 4.2). Generally each ovary consists of one to five carpels. In the age class 35-50 years and above 50 years, the number of carpels varied from one to five which would be readily observed from the exterior by counting the number of lobes in fruits. Each carpel had single pendulous, anatropous and unitegmic ovule. The stigma was dry with unicellular papillate outgrowths. In the age class of 16-24 years, the flowers had one to two stigmatic lobes; the flowers of age class 25-34 years had one to three numbers of stigmatic lobes. While plants of age class of 35-50 years and above, the number of stigmatic lobes in each inflorescence ranged from one to five. The terminal inflorescence matured first followed by axillary inflorescence.

Young fruits were green in color which appeared in July and matured during September. As fruits mature, it became bright red on the bottom and black on the top. The color of pedicel also changed from green to crimson red. Fruits contained one to five seeds. The number of fruits produced by the age class 16-24 years and age class 25-34 years was one to three and four to nine respectively. Age class

35-50 years and above produced 6-17 and 7-30 number of fruits respectively. In *P. wangianus* the percentage of one-seeded fruit is 26%, two-seeded fruit 31%, three seeded fruit 22%, four-seeded fruit 12% and five seeded fruits are 9%. But the ability to produce flowers that mature into fruit increased with the age of the plant. Phenological and reproductive characters revealed that most of the individuals of age class 35-50 and 50 years above plants contributed most of the flowering, fruiting and seed production.

4.3 Effect of different climatic factors on phenology of *Panax wangianus* in Nongkrem sacred grove

The Nongkrem sacred grove is spread over an area of about 6 ha. The increased in temperature about 4°C favoured the sprouting of aerial shoot from the rhizome during March (spring season) after dormancy. The vegetative growth continues up to the end of May (pre-monsoon or summer season). Flower buds appear from late April to May (Fig. 4.1). Anthesis occurs in the month of June (i.e. beginning of the monsoon season) during which period the monthly mean temperature 20.77°C, monthly mean precipitation 299 mm and monthly mean relative humidity 82% (Fig. 4.2). Small plants (three-prong and four-prong) have a shorter

flowering season while large individuals (five-prong and six-prong), extend their blooming. The young fruits are observed in July-August (i.e. peak monsoon season) during this period monthly mean temperature 20.47°C, monthly mean precipitation 388 mm and monthly mean relative humidity 89% was maximum. However, the mature fruits are produce in the month of September to October (Fig. 4.1) during these months monthly mean temperature 19.26°C, monthly mean precipitation 177.85 mm and monthly mean relative humidity 78.75% is recorded (Fig. 4.2). The dispersal of seeds take place in the month of October-November (retreating monsoon period to the beginning of winter), during this period there is decrease in monthly mean temperature 16.55°C, monthly mean precipitation 79.45 mm as well as in monthly mean relative humidity 89% (Figs. 4.1 and 4.2). Light level ranges from 5.18 – 100.48 $\mu\text{moles m}^{-2} \text{sec}^{-1}$ with an average of 33.79 $\mu\text{moles m}^{-2} \text{sec}^{-1}$ during the life span of *P. wangianus* from March-October. The color of corolla of *P. wangianus* also varied depending upon the intensity of sunlight. Those plants grow in bright sun-light, the flowers would appear pinkish; on the other hand, plants which are growing in shade, the color of flowers is greenish-white. The soil of the Nongkrem sacred grove is loamy, reddish brown in color, lateritic in origin and acidic (pH 4.5 - 4.62). The soil type is oxisols

where moisture content is 30-40% even during the dry season. Humus increases its water holding capacity up to 50-60%.

4.4. Statistical correlation between the age, morphological and reproductive traits of the *P.wangianus*

In *P. wangianus*, during the study period from 2007-2009, a strong positive correlation was observed between the age versus height of the plant ($r^2 = +0.93$), aerial shoot diameter ($r^2 = +0.92$), rhizome diameter ($r^2 = +0.94$), total number of leaflets ($r^2 = +0.97$), breadth of leaflet ($r^2 = +0.98$), average leaflet area ($r^2 = +0.96$) and petiole length ($r^2 = +0.94$) (Table 4.3).

Similarly, the reproductive parameters also showed positive correlation with the age of the plant. The value of correlation coefficient of length of peduncle ($r^2 = +0.95$), length of peduncle of axillary inflorescence ($r^2 = + 0.78$), number of carpels ($r^2 = + 0.81$), number of stigmatic lobes ($r^2 = +0.92$), number of flowers ($r^2 = +0.88$), number of axillary inflorescence ($r^2 = +0.69$) and number of fruits ($r^2 = + 0.93$) were also very high (Table 4.4).

4.5 Discussion

The vegetative as well as reproductive, phenological relationships in *P. wangianus* showed that nearly 90% of the features correlated with age. Wen (2001) and Shu (2007) demonstrated that the high level of morphological and reproductive plasticity in eleven species of *Panax* with respect to the shape and size of rhizome, number of leaves, leaflet shape, pubescence of leaves and number of flowers in an umbel inflorescence (Tables 4.5 and 4.6). Guo *et al.* (2010) also reported that the morphological variations in stem, leaf, root, flower and fruit in the cultivated populations of *P. notoginseng*. However, in population of *P. wangianus* growing in Nongkrem sacred grove, there is a clear-cut correlation between the different age classes with respect to the height of the plant, aerial shoot diameter, rhizome diameter, total number of leaflets, breadth of leaflet, average leaflet area, petiole length, length of peduncle, length of peduncle of axillary inflorescence, number of carpels, number of stigmatic lobes, number of flowers, number of axillary inflorescences and number of fruits (Tables 4.1 and 4.2).

But with respect to the age classes in *P. wangianus* the height of the aerial shoot did not vary much in age class 1-10 years and 10-15

years with one and two-pronged plants respectively. However, in age class 16-24 years, 25-34 years, 35-50 years and 50 years and above, there was a steady increase in the height of aerial shoot, aerial shoot diameter, rhizome diameter, petiole length and breadth of the leaflet (Table 4.1). The height of aerial shoot of *P. wangianus* showed similar growth trend when compared to *P. japonicus* and *P. vietnamensis*. While in the remaining six species *P. ginseng*, *P. zingiberensis*, *P. notoginseng*, *P. pseudoginseng*, *P. quinquefolius* and *P. trifolius*, the height of aerial shoot ranged from 5-60 cm (Wen 2001; Shu 2007).

The number of prongs in *P. wangianus* was 1-6 while Wen (2001) and Shu (2007) reported 1-5 in *P. quinquefolius* and in *P. ginseng*, *P. japonicus*, *P. zingiberensis*, *P. notoginseng*, *P. pseudoginseng*, *P. vietnamensis*, *P. stipuleanatus*, *P. bipinnatifidus*, *P. quinquefolius* and *P. trifolius* it ranged from 3-7 (Table 4.5).

Anderson *et al.* (1993) mentioned that in *P. quinquefolium* one-prong plant of two years of age possessed three to five leaflets; two-prong plants (with 10 leaflets) ranged from three to six years of age, three-prong plants (with 15 leaflets) ranged from seven to nine years of age, and four-prong plants (with 20 leaflets) ranged from 10 to 11 years of age. However, in *P. wangianus* plants of age class 1-10 years

had one prong with three to five leaflets and 10-15 years had two prongs with six to ten leaflets respectively. Plants of age class 16-24 years had three prongs with 9-15 leaflets and 25-34 years age class contained four prongs with 12-20 leaflets. While plants of age class 35-50 years had five prongs with 22-36 leaflets. Lewis and Zenger (1983) observed that four and five-prong plants represented the oldest individual of a population of *P. quinquefolium*. But in *P. wangianus*, six-prong plants with 30-42 leaflets fell under the category of 50 years and above age class. Similar observations were reported in *P. quinquefolius*, using the combination of leaf area and leaf number which are the good indicators of age (Furedi 2004; McGraw and Furedi 2005). In *P. quinquefolius* the maximum number of leaflets was 20 (Anderson *et al.* 1993). While in *P. ginseng*, *P. japonicus*, *P. zingiberensis*, *P. notoginseng* and *P. pseudoginseng*, it ranged from 9-42 (Shu 2007; Wen 2001).

In Nongkrem sacred grove, only one to two percent was one-pronged and two-pronged plants of *P. wangianus* exhibited neoteny and their age was above 40 years (Plate – 4.3 a, b). The retention of ancestral juvenile characters by mature plants was reported in few

members of family Araliaceae e. g. *S. racemifera* and *S. longipetiolata* by Fiasch and Frodin (2006).

Lewis and Zenger (1982); Carpenter and Cottam (1982); Lewis (1984); Anderson *et al.* (1993) and Gagnon (1999) reported that the height of aerial shoot, number of leaves and leaflet and leaflet area increased with which were the good predictors of age in *P. quinquefolium* as estimated by the number of bud scars on the rhizome. Similar observations have been encountered in *P. wongianus* (Table 4.1). The maximum age of *P. quinquefolium* estimated by Anderson *et al.* (1993) was 25-30 years and in *P. ginseng* it was 140-150 years (Grushvitskii 1961; Khrolenko *et al.* 2007); while in *P. wongianus* it was above 50 years. Rhizome damage could make the plant appear younger than its actual age, but growth probably would continue with adventitious roots assuming the storage and absorption functions (Anderson *et al.* 1993).

Similarly the reproductive features were also positively correlated with length of peduncle, length of peduncle of axillary inflorescence, number of carpels, number of stigmatic lobes, number of flowers, number of axillary inflorescences, number of fruits and flowering season, age versus fecundity of the plant and ripening of

fruits etc. In *P. wangianus*, the length of peduncle of terminal inflorescence ranged from 15-40 cm while in *P. ginseng*, *P. japonicus*, *P. zingiberensis*, *P. notoginseng* and *P. pseudoginseng* it ranged from 7-30 cm (Shu 2007; Wen 2001).

Farrington (2006) reported two pronged plants also showed fecundity in *P. quinquefolium*. However, in *P. wangianus* the fecundity has been observed from three-pronged plants and onwards.

Schlessman (1985) reported three and four-pronged plants tended to flower earlier to one and two-pronged plants. In *P. quinquefolius*, 20-50% of two-year-old plants have inflorescences (Proctor *et al.* 2003). Fiebig *et al.* (2001) reported that there were 32 to 79 flowers in an inflorescence in a three-year-old plant of *P. quinquefolius*. Shu (2007) and Wen (2001) reported that the numbers of flowers per plant ranged from 20-100 in *P. ginseng*, *P. japonicus*, *P. notoginseng* and *P. pseudoginseng*. Out of literature surveyed of 11 species of *Panax*, the only species *P. trifolius* is a dioecious plant in which male and female umbel consists of 15-30 flowers and 3-14 flowers respectively (Wen 2001). But in the case of *P. wangianus*, all the flowers in an umbel were hermaphrodite. Only in the three-pronged (six + leaflets) plants, the inflorescence consisted of 13-56 number of

flowers. The earliest flowering plants were 16 years of age; they had few flowers and only rarely formed fruit, but after 24 years of age, all plants developed flowers and most had fruits. Five and six- pronged plants produced more flowers and fruits, whereas one and two-pronged plants remained vegetative or in juvenile stage (Table 4.2).

Anderson *et al.* (1993) reported only one axillary inflorescence in *P. quinquefolium* and the length of peduncle of axillary inflorescence was one cm and rarely does it produce fruits. Shu (2007) and Wen (2001) reported 1-5 numbers of axillary inflorescences in *P. japonicus*, *P. pseudoginseng*, *P. vietnamensis* and *P. bipinnatifidus*. While in *P. wangianus* the numbers of axillary inflorescence is two to eight and the length of peduncle of axillary inflorescence has been measured from seven cm to nine cm depending upon the age of the plant and all the axillary inflorescence also possessed fruits. The young fruits of *P. wangianus* collected in July remained greenish and turned into red bottom and black on the top after six to eight weeks. But in *P. quinquefolium* all the ripened fruits are reddish in color (Proctor *et al.* 2003).

In addition to the present investigation, as well as that of others (Anderson *et al.* 1993; Carpenter and Cottam 1982; Lewis and Zenger

1982), has shown that fruit production is correlated with plant size. The initiation of flower and fruit production varies in different population of *P. quinquefolium*. Anderson *et al.* (1993) reported that the fruit production started in four year old plants of *P. quinquefolium* in Illinois and produced 30-40 numbers of fruits. While Carpenter and Cottam (1982) reported fruit production began after eight years in *P. quinquefolium* in Wisconsin. Farrington (2006) mentioned that all the plants irrespective to age produce berries containing one to three seeds. In *P. wangianus* fruit production started after 16 years and it increased with age of the plant. Plants of 50 years and above produced 7-30 numbers of fruits. Schlessman (1985) reported that flowers containing one, two and three ovaries produced one, two and three-seeded fruit, respectively, and the ratio of two-to one-ovuled flowers increases with plant size (age). In *P. wangianus* the percentage of one-seeded fruit is 26%, two-seeded fruit 31%, three seeded fruit 22%, four-seeded fruit 12% and five seeded fruits are 9%. Stoltz and Garland (1980) also reported about one-seeded (16.3%), two-seeded (77.0%), three-seeded (6.5%) and four-seeded (0.2%) fruits in *P. quinquefolium*.

Shu (2007) reported all the species of *Panax* in China are two carpellate and bistigmatic except *P. japonicus* which have two to five

carpels and two to five styles growing in China. Wen (2001) reported one to three carpellate conditions except in *P. trifolius*. Wen *et al.* (2001) also mentioned pentacarpellate condition was an evolutionarily liable and developmental abnormality in Araliaceae. Philipson (1970a) and Eyde and Tseng (1971) also reported two to five carpellate conditions is common in Araliaceae. The pluricarpellate condition (upto 20 carpels) is a primitive condition in Araliaceae. The American ginseng with one styled flowers represent an intermediate step in the evolution of functional maleness. But Asiatic ginseng is functional hermaphrodite because all the individuals of *P. wongianus* in the population growing in Nongkrem sacred grove showed one to five carpellate conditions.

Several climatic variables such as temperature (Ashton *et al.* 1988; Pfeifer *et al.* 2006), precipitation (Borchert *et al.* 2004; Dominguez and Dirzo 1995; Inouye *et al.* 2003; Opler *et al.* 1976; Pfeifer *et al.* 2006; Stevenson *et al.* 2008; Stiles 1977; Tyler 2001) and other variables (e.g. soil nutrient concentration, Dahlgren *et al.* 2007) can trigger flowering. The biological advantages of extended blooming include: (a) a reduced risk of reproductive failure; (b) an increased chance of mating with more individuals; and (c) a better control of the

relative investment in flowers and fruits (Bawa 1983). The color of corolla of *P. wangianus* also varied depending upon the intensity of sunlight. Those plants grow in bright sun-light, the flowers would appear pinkish; on the other hand, plants which are growing in shade, and the color of flowers is greenish-white. Guo *et al.* 2010 and Liu *et al.* 1992 proved that *P. notoginseng* is shade-loving plant with C₃ cycle, which is sensitive to sunshine and dislikes direct irradiation by sun. The favourable climatic conditions and protection over a long period of time have played a major role in growth and reproduction of *P. wangianus* in Nongkrem sacred grove. While approaching the winter, the leaves and aerial stem senescence because of low rainfall and moderate temperature. Venugopal and Liangkuwang (2007) and Singh and Venugopal (2010) have reported that relative humidity played a secondary role for the tree species in the sub-tropical wet climate. However, this does not apply for the understory shaded herbaceous plant growth. Therefore, increase in temperature, rainfall and relative humidity shows its effect on the sprouting of aerial shoot from the rhizome and further growth and reproduction in *P. wangianus*.

Larger plants (in terms of age class 35-50 years and above 50 years) are reproductively more successful because of its vegetative growth and flowering phenology that influences the more production of fruits and seed set in Nongkrem sacred grove. Morphological variations were observed in natural conditions based on prong numbers and the number of carpellate conditions. Age class was significant for to predict the size of the plant and its reproductive capacity. Therefore, from conservation and management point of view, the age class 35-50 years and above 50 years is the most important for population sustainability. Plants of age class 35-50 years may facilitate the floral rewards to the pollinators. Therefore, in *P. wangianus*, there is a clear-cut correlation between the age of the plant and pentacarpellate condition, formation of axillary inflorescences with more productivity. The color of flowers of *P. wangianus* also varied depending upon the sunlight intensity. The soil moisture content 30-40% of oxisols is enough to trigger the vegetative growth after dormancy during the month of March. The onset of flowering is primarily related to temperature and precipitation. Climatic factors such as temperature, precipitation and relative humidity show synergistic effect on both the vegetative and reproductive phases in *P. wangianus*. Nongkrem sacred

grove is undisturbed climatic climax forest in which environment conditions are stable in terms of precipitation, temperature and relative humidity. Therefore, there is a relationship existing between the development of vegetative and reproductive features with environmental conditions.

Table 4.1 Morphological features of *Panax wanganus* growing in Nongkrem sacred grove

Age Class	No. of prongs	No. of individuals (n) and % of population	Height of aerial shoot (cm)	Aerial shoot diameter (cm)	Range of rhizome diameter (cm)	Total no. of leaflets	Breadth of leaflet (cm)	Average leaflet area (cm ²)	Range of petiole length (cm)
1-10 years	1	187= 9.27%	20.00 ± 6.20	0.3±0.02	0.5-0.7	3.0-5.0	0.4-0.8	2.00 ± 0.12	3.0-4.0
10-15 years	2	243=12.05%	20.00 ± 6.20	0.4±0.03	0.7-1.5	6.0-10.00	0.4-1.0	4.00 ±0.02	4.0-7.0
16-24 years	3	482=23.90%	30.00 ± 6.20	0.5±0.02	0.8-1.5	9.0-15.0	1.0-2.0	8.00 ± 0.58	6.0-10.0
25-34 years	4	602=29.86%	80.00 ± 6.20	0.7±0.06	1.0-2.0	12.00-21.00	2.0-3.0	16.00±1.73	10.0-15.0
35-50 years	5	418=20.73%	95.00 ± 9.09	1.0±0.01	1.0-3.0	22-36	3.0-4.0	32.5 ± 3.76	26-30
50 years and above	6	84=4.16%	110.00 ±6.20	1.5±0.12	2.0-3.5	30-42	3.5-4.5	37.5 ± 3.25	28-32

± indicates standard deviation.

Table 4.2 Reproductive features of *Panax wangianus*

Age Class	No. of prongs	Length of peduncle of terminal inflorescence (cm)	Length of peduncle of axillary inflorescence (cm)	No. of carpels/plant	Range of number of stigmatic lobes/plant	Range of number of flowers/plant	Range of no.of axillary inflorescence/plant	Range of no. of fruits/plant
1-10 years	1	—	—	—	—	—	—	—
10-15 years	2	—	—	—	—	—	—	—
16-24 years	3	15	—	1-3	1.0 -2.0	13-56	—	1.0-3.0
25-34 years	4	27	—	1-3	1.0-3.0	25-60	—	4.0-9.0
35-50 years	5	38	7	1.0-5.0	1.0-5.0	25-73	2.0-3.0	6.0-17.0
50 years and above	6	40	9	1.0-5.0	1.0-5.0	45-75	5.0-8.0	7.0-30

— indicates no flowering.

Table 4.3 r^2 value of different morphological features versus age of the plant

VEGETATIVE PARAMETERS							
Dependent variable	Height of the plant	Aerial shoot diameter	Rhizome diameter	Total No. of leaflets	Breadth of leaflet	Average leaflet area	Petiole length
Age of the plant	r^2	r^2	r^2	r^2	r^2	r^2	r^2
	0.93	0.92	0.94	0.97	0.98	0.96	0.94

**Table 4.4 r^2 value of different reproductive features versus age of
the plant**

REPRODUCTIVE PARAMETERS							
Dependent variable	Length of peduncle of terminal inflorescence	Length of peduncle of axillary inflorescence	No. of carpels	No. of stigmatic lobes	No. of flowers	No. of axillary inflorescence	No. of fruits
Age of the plant	r^2	r^2	r^2	r^2	r^2	r^2	r^2
	0.95	0.78	0.81	0.92	0.88	0.69	0.93

Table 4.5 Morphological variations in different species of *Panax* reported by Anderson *et al.*, (1993), Grushvitskii (1961), Khrolenko *et al.* (2007), Shu (2007) and Wen (2001a)

Characters	<i>Panax wangianus</i>	<i>Panax ginseng</i>	<i>Panax japonicus</i>	<i>Panax zingiberensis</i>	<i>Panax notoginseng</i>	<i>Panax pseudoginseng</i>	<i>Panax vietnamensis</i>	<i>Panax stipuleanatus</i>	<i>Panax bispinnatifidus</i>	<i>Panax quinquefolius</i>	<i>Panax trifolius</i>
Age (years)	1.0-50	140-150	–	–	–	–	–	–	–	25-30	–
No. of prongs	1.0-6.0	3.0-6.0	3.0-5.0	3.0-7.0	3.0-6.0	4	3.0-5.0	3.0-4.0	3.0-5.0	1.0-5.0	3
Height of aerial shoot (cm)	20-110	30-60	50-100	20-60	20-60	50	40-100	–	–	20-50	5.0-20
Aerial shoot diameter (cm)	0.3-0.12	–	–	–	–	–	–	–	–	–	–
Range of rhizome diameter (cm)	0.5-3.5	–	–	–	–	–	–	–	–	–	–
Total number of leaflets	3.0-42	9.0-30	15-25	9.0-32	9.0-42	12.0-16.0	–	–	–	3.0-20	–
Breadth of leaflet (cm)	0.4-4.5	–	–	–	–	–	–	–	–	–	–
Average leaflet area (cm ²)	2.0-37.5	–	–	–	–	–	–	–	–	–	–
Range of petiole length (cm)	3.0-32	–	–	–	–	–	–	–	–	–	–

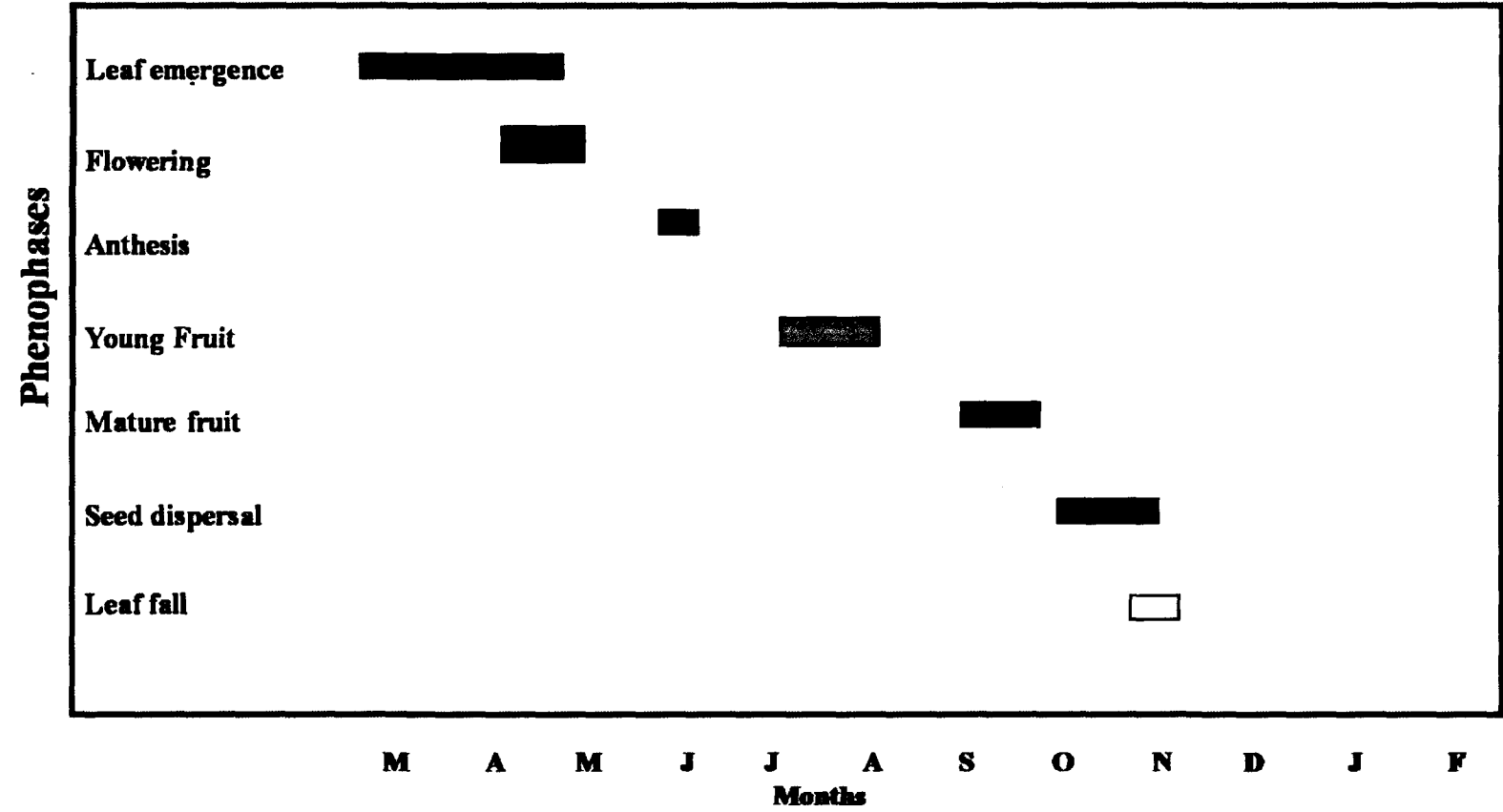
–indicates data not available.

Table 4.6 Reproductive variations in different species of *Panax* reported by Anderson *et al.* (1993), Fiebig *et al.* (2001), Shu (2007) and Wen (2001a)

Characters	<i>Panax wangianus</i>	<i>Panax ginseng</i>	<i>Panax japonicus</i>	<i>Panax zingiberensis</i>	<i>Panax notoginseng</i>	<i>Panax pseudoginseng</i>	<i>Panax vietnamensis</i>	<i>Panax stipuleanatus</i>	<i>Panax bipinnatifidus</i>	<i>Panax quinquefolius</i>	<i>Panax trifolius</i>
Length of peduncle of terminal inflorescence (cm)	15-40	15.0-30.0	12.0-21.0	24-26	7.0-25	12	—	—	—	—	—
Length of peduncle of axillary inflorescence(cm)	7.0-9.0	—	—	—	—	—	—	—	—	1	—
Number of carpels/plant	1.0-5.0	2	2.0-5.0	2	2	2	—	—	—	2.0-3.0	—
Range of number of stigmatic lobes/plant	1.0-5.0	—	—	—	—	—	—	—	—	—	—
Range of number of flowers/plant	13-75	30-50	50-80	—	80-100	20-50	—	—	—	32-79	Male umbels = 15-30; female umbels = 3-14
Range of number of axillary inflorescences/plant	2.0-8.0	0	2.0-4.0	—	—	1.0-3.0	2.0-4.0	—	2.0-5.0	1	—
Range of number of fruits/plant	1.0-30.0	—	—	—	—	—	—	—	—	30-40	—

—indicates data not available.

Fig. 4.1 Phenology of *Panax wangianus* S.C. Sun



**Fig. 4.2 Showing different climatic factors in the study area
of *Panax wangianus* during 2007-2009**

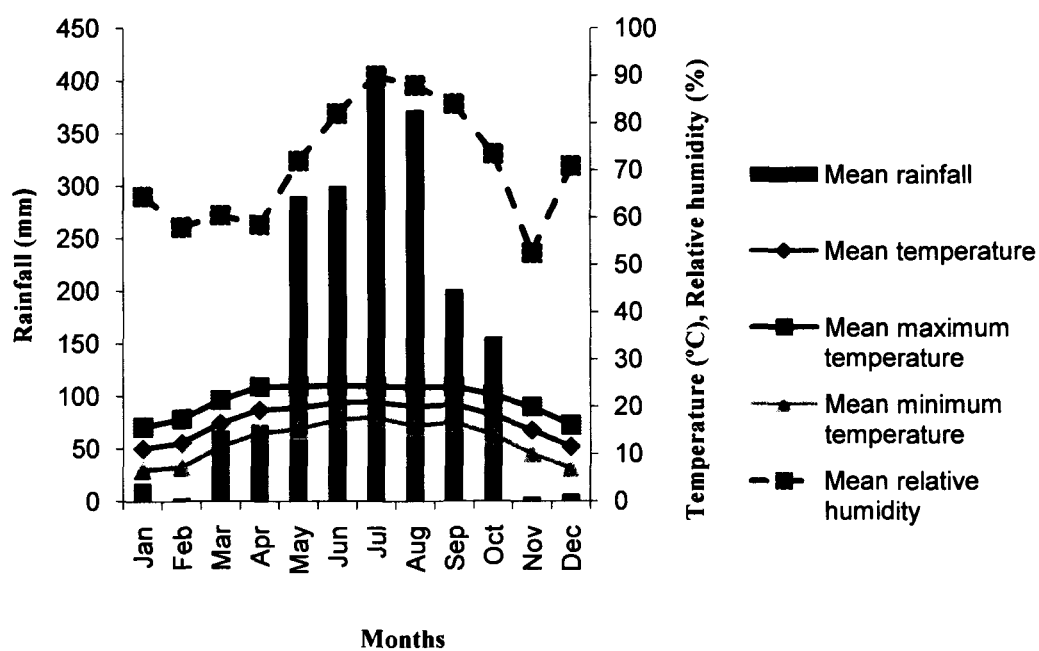


Plate – 4.1

- a . A colony of a few plants of *P. wangianus* growing under shady forest floor with rich leaf litter in Nongkrem sacred grove. Bar = 15 cm.**

Plate – 4.1

***Panax wangianus* S. C. Sun**



Habit of plants

Plate – 4.2

- a-d** Showing different age classes of plant with one to four digitately compound leaves (prongs = PR) and variable number of palmately compound leaflets ranges from 5 to 21. The terminal leaflets are larger than the lateral leaflets. Note the well developed terminal inflorescence in the four pronged plant. (Bar: a = 2 cm; b = 3.2 cm; c = 4.8 cm; d = 5.7 cm).
- e-f** Showing the well developed and matured plants of age class 35-50 years and above 50 years with five prongs (33 leaflets) and six pronged (38 leaflets) respectively. Note all the seven axillary inflorescences and one terminal inflorescence possessed 30 numbers of fruits. (Bar: e = 11 cm; f = 11.40 cm).

Plate – 4.2

Morphological variations in natural conditions on
the basis of the number of prong

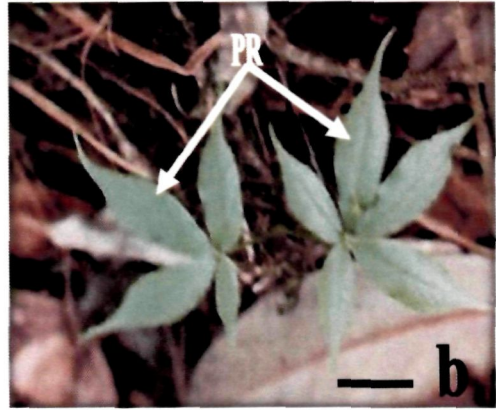
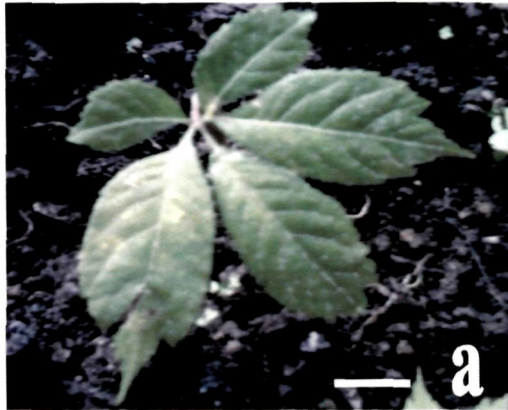
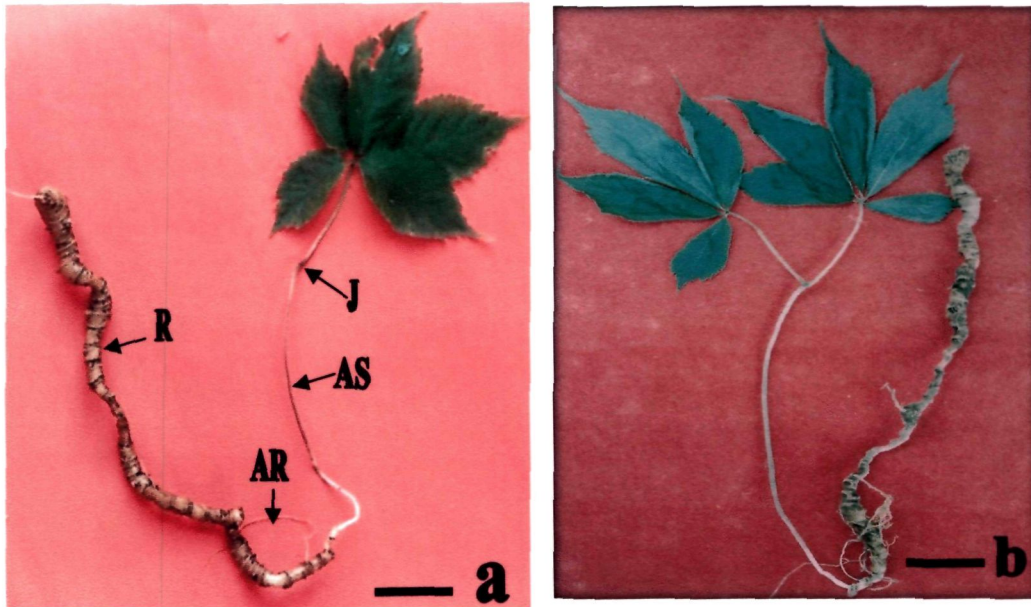


Plate – 4.3

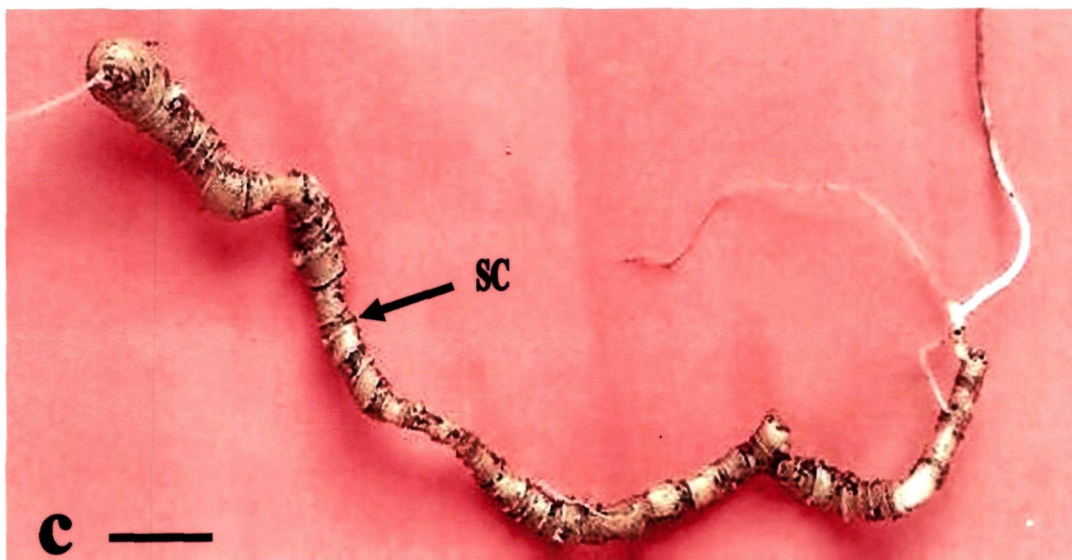
- a-b** An adult, 48 years old plant of *P. wangianus* exhibiting neoteny character by producing one prong and two prongs. (AS = Aerial shoot; J = joint between petiole and aerial shoot; R = Rhizome and AR = adventitious root). Bar; a = 4.6 cm; b = 5.0 cm.
- c** An enlarged view of creeping and elongated rhizome showing the bud scale scars on the rhizome after each season. The age of the plant was recorded by counting the number of bud scale scars (SC) on the rhizome. Bar = 8.5 cm.

Plate – 4.3

Panax wangianus S. C. Sun



Neoteny



An enlarged view of rhizome

CHAPTER-5

Breeding System in *Panax wangianus*

5.1 Introduction

Pollination is the transference of pollen from the anthers (male structure) of a flower to the stigma (the receptive part of the female structures) of the same or another flower, mediated by abiotic or biotic means. The outputs of pollination are expressed in number and quality of seeds, which are essential incomes for population growth. Furthermore, through pollen dispersal, gene flow along or among populations is also involved in this process, playing its role in the genetic composition of the populations (Rozzi *et al.* 1997). The pollination process was first addressed in the 18th century by Christian Konrad Sprengel.

Two types of pollination occur in plants: Abiotic pollination and biotic pollination. Abiotic pollination refers to situations where pollination is mediated without the involvement of other organisms. Only 10% of flowering plants are pollinated without animal assistance.

The most common form of abiotic pollination, anemophily, is pollinated by wind. This form of pollination is predominant in grasses, most conifers, and many deciduous trees. Hydrophily is pollinated by water and occurs in aquatic plants which release their pollen directly into the surrounding water. Of the 20% of abiotically pollinated species, 98% is by wind and 2% by water. About 80% of flowering plants show biotic pollination. Biotic pollination (animal-mediated pollination) where both plants and animals communities can mutually benefit from each other. Animals actively or passively transfer pollen grains, containing the male gametes, from the stamen to the stigmas, where the pollen grains can germinate and fertilise the female gamete (egg cell of the embryo sac) in the ovule. Animals are attracted by colour of petals and pheromones and find their food and shelter in the flowers, and many animal species are dependent on flowers.

Pollinators can be as different as insects, birds, bats and other small mammals (Proctor 1978; Faegri and van der Pijl 1979), and even lizards (Traveset and Saez 1997; NyHagen *et al.* 2001). Insects are the dominant pollinators on earth (Faegri and van der Pijl 1979); at least 70 percent of all angiosperms are insect-pollinated (Kearns and

Inouye 1993). Therefore, animal-mediated pollination has profound importance to maintain different types of ecosystems.

Not all flower visiting insects have the same potential to affect pollination; differences in behavior and morphology affect how well they pick up pollen from anthers and transfer it to stigmas of flowers. To be effective, pollen must be picked up in sufficient quantities and transferred to the appropriate location on another plant of the same species, and it must be transferred while still viable. Many pollinators have special structures on the body for collecting pollen. Body hairs are an important means of trapping and transferring pollen, bees, which have numerous hairs that are often highly branched.

Great diversity exists in the flowers that have adapted to insect visitation, and likewise among the animals that visit the flowers are potential pollinators which also have several adaptations (Loew 1895; Knuth 1898; Proctor 1978; Faegri and van der Pijl 1979). Therefore, the diversity of both these two levels may be important for pollination. Changes in species richness and functional diversity (the number of groups of similar species) of plants and insects, and changes in plant population size and structure can affect pollination (Rathcke and Jules 1993; Buchmann and Nabhan 1996; Kremen and Ricketts 2000).

Continuous loss of pollinator species due to the habitat destruction, pesticide, parasitism/diseases etc. may lead to a worldwide "pollination crisis" (Buchmann and Nabhan 1996). For many plant species a decline in pollination quantity and quality will result in a lower seed set (Agren 1996; Kearns and Inouye 1993; Kwak *et al.* 1998; Steffan-Dewenter and Tschardtke 2000; Tomimatsu and Ohara 2002) and is thus detrimental to the survival of plant populations.

Abiotic factors have profound influence on native pollinators. Weather can play a substantial role in the ecology of pollinators. All flower-visiting insects are temperature-constrained, and rely on good weather to allow them to forage. Bad weather (i.e. cold or rain) prevents many flower visitors from visiting flower species and stops pollen transfer (Kearns and Inouye 1993). Cloudy weather decreases the activity of many pollinators. Many insects use polarized light (only visible in blue skies) as a means of navigation and need clear patches of sky to do this (Gullan and Cranston 2000). Along with weather conditions, the climate of a region is also important.

Pollination can be accomplished by cross-pollination or by self-pollination: Cross-pollination, also called allogamy occurs when pollen is delivered to a flower from a different plant. Plant adapted to outcross

or cross-pollinate often have taller stamens than carpels or use other mechanisms to better ensure the spread of pollen to other plants' flowers. Self-pollination occurs when pollen from one flower pollinates the same flower or other flowers of the same individual. It is thought to have evolved under conditions when pollinators were not reliable vectors for pollen transport. Self-pollination may include autogamy, where pollen moves to the female part of the same flower; or geitonogamy, when pollen is transferred to another flower on the same plant.

Pollen viability is an important parameter since pollen must be viable and capable of germination at the time of pollination in order to have successful seed set. Heslop-Harrison *et al* (1984) defined viability as "the competence of individuals of a given pollen population to deliver male gametes to the embryo sac".

Pollen germination studies are essential for the estimation of quality of the pollen required for controlled pollination. Artificial germination of the pollen grains is a surest test of pollen fertility, which is important for the understanding of any breeding programme. Since aniline blue fluorochrome (ABF) has affinity for callose (a carbohydrate in pollen tubes), the aniline blue fluorescence (ABF)

method has been an important technique for studying the progressive phases of pollen tube growth, enabling visualization of callose in pollen tubes (Dumas and Knox 1983).

5.2 Floral biology

The umbel inflorescence of *Panax wangianus* consists of 13–75 flowers (Plate - 5.1 a, b) which appear from late April to May. Flowers are small, bisexual, odor-less, protandrous with five (or rarely four) calyx teeth, five petals, five stamens and an inferior ovary with one to five styles. The length of stigma is 2 mm (Table 5.2). The color of corolla of *P. wangianus* also varies depending upon the intensity of sunlight. Those plants grow in bright sun-light, the flowers would appear pinkish; on the other hand, plants which are growing in shade, and the color of flowers is greenish-white (Plate – 5.1 a–d).

Anthesis occurs in the month of June, between 07.00 A. M. to 04.00 P.M. Small plants (three-pronged and four-pronged) have a shorter flowering season with 3-5 open flowers in a day while large individuals (five-pronged and six-pronged), extend their blooming and the number of open flowers in a day varied from 20–30 (Plate – 5.1 a, b). Within inflorescences, flowers matured centripetally over a period

of one to three weeks. As buds opened, the petals reflexed, filaments of the stamens ceased elongation and the anthers dehisced. At about the same time, the styles reached maximum length and their apparently receptive stigmatic tips recurved at 180°. After the styles matured, the stamens and later the petals abscised. The entire sequence is completed in two or three days.

Each ovary consists of one to five carpels which would be readily observed from the exterior by counting the number of furrows and ridges on the ovary (Plate – 5.8 b). Each carpel has single pendulous, anatropous and unitegmatic ovule. The stigma is dry with unicellular papillate outgrowths (Heslop-Harrison and Shivanna 1977) (Plate– 5.7 b). The number of stigmatic lobes ranges from one to five (Plate – 5.2 a–e). The flowers have nectariferous epigynous disc which cover the ovary top and usually confluent with style bases (Plate– 5.7 a).

5.3 Pollen viability and Pollen germination

In *P. wangianus* long viscin threads (range 150-600 µm) hold numbers of pollen grains together in relatively large clumps (Plate – 5.3 a). The distribution of the threads was unequal, i.e., some grains had many and others had few threads. Pollen grains were tricolpate

with reticulate sculpturing. The exine of pollen grain is with short columellae and thick foot layers (Plate – 5.3 b). Longitudinal section of pollen grain shows long polar columellae and the endexine is thickened above and below the endoaperture, forming costa endocolpi (Plate – 5.3 c). The total number of pollen grains in a flower ranges from 2000–2500. The mean number of pollen grains per flower was 1895, and the mean number of ovules was 3. Thus, the pollen to ovule ratio was 1895:3 (Table 5.2). Pollen viability by fluorochromatic reaction test confirmed that 43% of pollen grains were viable while the remaining 57% pollen grains were sterile on the day of anthesis (Plate – 5.3 d). Sterile pollen grains were shriveled, lacked cytoplasmic contents and did not fluoresced brightly with FDA and contained starch as reserve material. *In vitro* pollen germination study also revealed that 43% pollen grains were germinated and produces $113.2 \mu\text{m} \pm 3.27$ tube length in modified Brewbaker and Kwack's medium. Callose plugs are found in the pollen tubes of *P. wangianus* (Plate – 5.4 d). A close examination of culture in modified Brewbaker and Kwack's medium indicated that the germination of pollen grains commenced 12 hours after dusting but the maximum pollen germination was obtained about 17 hours of incubation (Plate – 5.4 a–c). Varying concentrations of the

media had no effect on the pollen germination and length of the germination tubes. There is swelling at the tip of the pollen tube has been observed. The length of stigma is 2 mm. Though 43% of viable pollen grains are available, sufficient viable pollens are not landed on the stigma (Plate – 5.7 c, d) through visitors because of difference in height of anther and receptive surface of stigma as well as recurvature of stigma.

5.4 Observation of floral visitors

The sticky nature of pollen grains of *P. wangianus* is due to the presence of pollenkitt (Plate – 5.3 a) which makes them adhere strongly to the anther; the possibility of wind pollination can be ruled out; therefore, the most likely agents of pollen grain transfer are insects. The flowers are pollinated by bees and flies belonging to Halictidae and Syrphidae respectively (Plates – 5.5 a, b and 5.6 a). They visited the flowers between 07.30 A. M. to 04.00 P. M. Visitors of the Syrphidae have well developed hind legs for landing on the inflorescence of *P. wangianus* (Plate - 5.5 c) and they spend the longest time on individual flowers per visit, with averages ranging from 5.0 to 58.0 seconds in fine weather. Similarly, halictid bees remained on the flowers for comparatively long periods, ranging from

35.0 to 50.0 seconds per single visit. However, during the rainy season flies and bees were completely inactive. All pollinators are pollen carriers and their inter plant movement facilitates cross-pollination. Observations of different body parts indicate that bee have a higher amount of pollen grains on the scopal hairs of the legs, mouth parts and on the ventral abdomen (Plate – 5.6 b, c).

5.5 Breeding Mechanism

The species has a mixed breeding system of autogamy and xenogamy, suggesting self-compatibility. The percentage of fruit set through autogamy, geitonogamy and xenogamy was 49%, 36% and 40% respectively (Fig. 5.1). Plants that were bagged and emasculated to test for agamospermy produce no fruits (Table 5.1). Results of the autogamy and self-pollination treatments provide strong evidence of self-compatibility in *P. wangianus* and both with and without mechanical assistance from pollen vectors.

The fruits are berry. Young fruits were green in color (Plate – 5.8 a) which appeared in July-August and matured during September to October. As fruit mature, it became bright red on the bottom and black on the top. The color of pedicels also changed from green to crimson

red (Plate – 5.8 c). The number of fruits produced by *P. wangianus* was 1–30. Populations of *P. wangianus* typically have low fruit-to flower ratio, so few fruits mature per plant. These fruits can have 1–4 seeds or less frequently 5 seeds. The percentage of one-seeded fruit is 26%; two-seeded fruit 31%, three-seeded fruit 22%, four-seeded fruit 12% and five-seeded fruits are 9%.

5.6 Fruit / Seed dispersal

Fruits of *P. wangianus* abscise by October or November staying near the parent plant (i.e. gravity seed dispersal) (Plate – 5.9 b). Seedlings were found to be clustered within a meter or less of the parent plant and fruits dispersed less than 50 cm or < 1 m from the parent plant. The dispersal of seed is by animals (birds) (Plate – 5.9 a). Seed dispersal is passive.

5.7 Discussion

P. wangianus has umbel inflorescence with 13-75 hermaphroditic flowers which are small and closely spaced, the architecture and recurved styles are facilitated for cross-pollination by pollinators mainly in five-pronged and six-pronged plants. Navarro and Guitián (2002) also reported that morphological and functional traits affect the

reproductive success of the plant. In *P. wangianus*, larger plants (in terms of age class 35-50 years and above 50 years) are reproductively more successful due to its vegetative growth, number of flowers in an umbel, formation of axillary inflorescences and floral rewards to the pollinators which influence the more production of fruits and seed set in Nongkrem sacred grove. The colour of corolla of *P. wangianus* also varied depending upon the intensity of sunlight. Those plants grow in bright sun-light; the flowers would appear pinkish which has not been reported in other species of *Panax*. These pink colour flowers invite more pollinators than the greenish-white flowers produced by the shade growing plants.

P. wangianus would be classified as obligate xenogamy (the highest outcrossing level) because the pollen-ovule ratio 1895: 2 has been recorded (Table 5.2). According to Cruden (1977), pollen-ovule ratios of xenogamous species range from 1,062: 1 to 19,525: 1. Schlessman (1985) suggested that the high pollen-ovule ratio in *P. quinquefolium* promotes adequate pollination even though pollinator visits are infrequent.

Pollen grains of *P. wangianus* are tricellular. The exine of pollen grain is with short columellae and thick foot layers (Wen and Nowicke

1999). The stigma is dry with unicellular papillate outgrowths (Heslop-Harrison and Shivanna 1977). According to Went and Willemse (1984), tricellular pollen prefers a dry stigma. In *P. wangianus* the pollens remain viable only up to 3 days after dehiscence. This suggests that the pollen grains are in a highly active metabolic state and as a result, the biochemical changes probably occur over time. Fresh pollen grains collected during the anthesis gave optimal pollen germination and tube elongation in *P. wangianus*. Pacini *et al.* (1997) reported in most of the angiosperm species the pollen viability decreases with time.

Pollen of *P. wangianus* is prolate spheroidal, subprolate with costa ectocolpi and lalongate endoaperture the polar spheroidal, subprolate with costa ectocolpi and lalongate endoaperture the polar margins thickened and forming costa endocolpi (Wen and Nowicke 1999).

No pollen germination and tube growth were occurred in the stored pollens in *P. wangianus*. Fresh pollen grains collected during the anthesis gave optimal pollen germination and tube elongation in *P. wangianus*. The pollen germinability is influenced by flower age, drying time and pollen source (Randolf and Robert 1986). However,

in *Asclepias exaltata*, flower age has little effect on pollen germinability because of the presence of pollinia (Shannon and Wyatt 1986).

Trinucleate pollen grains survive for shorter duration than binucleate pollen grains and takes long time for germination in vitro (Mulcahy and Mulcahy 1983; Stanley and Linskens 1974). Similarly in *P. wangianus* the germination of trinucleate pollen grains commenced 12 hours after dusting but the maximum pollen germination was obtained about 17 hours of incubation

Pollen germination can be substantially altered by the temperature. Pollen of most plants show optimum germination and tube growth between 20-30°C (Johri and Vasil 1961). In the case of *P.wangianus*, pollen grains germinated only at 23°C. The sucrose concentrations required for optimal germination varies from species to species e.g. *Solanum aviculare*, *S.laciniatum*, *S.sisymbriifolium* require 10% sucrose; *Corylus avellata* require 10 to 25% sucrose (Kim *et al.* 1985). In *P. wangianus*, the concentrations of sucrose (15%), boric acid (20 mg), calcium nitrate (50 mg), magnesium sulphate (30 mg) and potassium nitrate (20 mg) and agar (0.6%) induced pollen germination and tube elongation whereas the other concentrations of

above mentioned chemicals and percentage of agar did not cause any effect at all.

Pollen grains germinate in a wide range of pH but the best results are obtained at pH 5.5-6.5 (Johri and Vasil 1961). Similarly, in *P. wangianus* optimal germination and tube elongation were seen at pH 5.6. For adjusting the pH uses KOH and H₂SO₄ because sodium (e.g., NaOH) often inhibits the growth of pollen tubes considerably. After preparing the medium if the pH is 5.6, it is preferable that it is not adjusted (Higashiyama 2000). Lights of different color influenced pollen germination and tube elongation in *Schima wallichii* (Chauhan and Katiyar 1996). But in *P. wangianus* pollen germination and tube elongation were more or less similar in both light and dark conditions.

In *P. wangianus* bursting of pollen and occasionally the pollen tubes were observed to eject their contents. In addition to this was various pollen tube deformities viz. bloating or ‘bulla’ formation resulting in the swelling of the tip of the pollen tube. Similar findings have been reported for pollen tubes in several angiosperm species (Dickinson 1978; Yang *et al.* 1999). Pollen tube grown in a coiled manner was also observed in *P. wangianus* frequently which was due to unstraight tube wall. Callose, which is an important polysaccharide

component of the pollen tube wall (Kyu-Ock and Bradford 1998; Pierson *et al.* 1994; Franklin-Tong *et al.* 1996; Ferguson *et al.* 1998), is present in the pollen tubes of *P. wanganus*.

The presence of viscin threads in pollen grains of *P. wanganus* is the first report for family Araliaceae. Wen and Nowicke (1999) examined pollen grains of *P. wanganus* by light microscopy, scanning and transmission electron microscopy but they have not noticed the presence of viscin threads. Pollen grains with adhering viscin threads only occurred in two families e.g. Onagraceae and Ericaceae, (Skvarla *et al.* 1978; Cruden and Jensen 1979; Hermann and Palser 2000; Harder and Johnson 2008). Viscin threads have also been reported in the pollen grains of Late Cretaceous and Paleocene of South America (Pocknall and Jarzen 2009). In *P. wanganus* the pollen: ovule ratio 1895: 3 is high. While species with aggregated pollen or viscin threads tend to have much low pollen: ovule ratios (166:1) indicating that individual grains of species with aggregated pollen have a comparatively high chance of contributing to the next generation of sporophytes (Cruden 2000; Erbar and Langlotz 2005; Harder and Johnson 2008).

The pollenkitt is deposited on the pollen grains of *P. wangianus* (Plate – 5.3 a). Pollenkitt is to be found nearly in all angiosperm plants. Jebb (1998) also reported that pollen grains of *Trevesia valida* (Araliaceae) form regular ellipsoidal aggregation of 60 pollen grains which are held together by pollenkitt. But, in *P. wangianus* both types of pollen adhesive clumped the pollen grains in a mass like “massulae”. They play an important role in pollination. They help to adhere to the body of the insect and transfer the large number of pollen grains at a time like pollinia do in Asclepiadaceae and Orchidaceae (Cruden and Jensen 1979). A similar observation has been reported in members of family Onagraceae (Hesse 1978a). In entomophilous species the quantity of pollenkitt is more than the anemophilous species (Hesse 1977, 1978a, b, 1979a, b, 1980).

P. wangianus exhibits reverse herkogamy which enhances the chances of cross-pollination by the insects belonging to the order Diptera. Small gap between the stigma and anther provides passage of insects (mainly bees and flies belonging to family Halictidae and Syrphidae respectively) for pollination in *P. wangianus* as similar in *P. quinquefolius* and *P. trifolius* (Carpenter and Cottam 1982; Catling and

Spicer 1995; Duke 1980a, b; Lewis and Zenger 1983; Schlessman 1985).

The variation in the time of anther dehiscence and stigma receptivity indicate protandry forms of dichogamy in *P. wangianus*. This sort of pattern of synchronized protandry is well known in other members of the Araliaceae, such as *Aralia hispida* (Thomson and Barrett 1981; Barrett 1984). Schlessman (1985) found protandry does not exist in populations of *P. quinquefolium*. Lewis and Zenger (1983) and Carpenter and Cottam (1982) reported protandrous condition in *P. quinquefolium*. In *P. wangianus*, however, the protandrous flowers still allow self-pollination. Even with xenogamy, autogamy and geitonogamy occur, only about one-quarter (25%) of flowers of *P. wangianus* in Nongkrem sacred grove set fruit. The production of fruits and seeds through autogamy is more than geitonogamy and xenogamy in three-pronged and four-pronged plants while five-pronged and six-pronged plants produce more fruits and seeds through geitonogamy and xenogamy. *P. wangianus* is incapable of agamospermous reproduction as indicated by the zero fruit production of plants (Table 5.1). However, agamospermy is common among the Araliaceae e.g. *Acanthopanax sessiliflorum*, *Aralia mandshurica*, *Eleutherococcus*

senticosus and *P. ginseng* (Zhuravlev *et al.* 2008; Hohlov 1978; Petrov 1976). Self-pollinated flowers produce the same proportion of fruits as outcrossed (cross-fertilized) flowers in *P. quinquefolium* (Carpenter and Cottam 1982; Schlessman 1985). But in *P. wanganus* the percentage of fruit set through autogamy, geitonogamy and xenogamy was 49%, 36% and 40% respectively.

In *P. wanganus* fruits are developed by the outermost flowers of the inflorescence in three-pronged and four-pronged plants while in five-pronged and six-pronged plants 50% flowers produce fruits. The position of developing fruits within the inflorescence affects fruit development (Bawa and Webb 1984). In *P. wanganus* mature fruits are bright red on the bottom and black on the top as similar in *P. japonicus*, *P. vietnamensis*, *P. stipuleanatus* and *P. assamicus* whereas, in other species of *Panax*, mature fruits are red in color except yellow color fruits in *P. trifolius* (Wen 2001; Basnet and Dey 2008; Shu 2007; Chen *et al.* 2010).

Table-5.1 Results of breeding systems in *Panax wangianus*

Treatment	No. of plants	No. of flowers	No. of fruits	No. of seeds/fruit	Average No. of seeds/plant
Autogamy	25	100	49	2-5	98-245
Geitonogamy	25	100	36	1-3	36-108
Xenogamy	25	100	40	1-4	40-160
Agamospermy	25	100	0	0	0

Table-5.2 Floral Characters of *Panax wangianus*

Floral Characters	Observations
Inflorescence	Umbel
Flowering period	April-May
Flower type	Hermaphrodite
Flower color	Greenish-white or pinkish
Odor	Odor-less
Range of No. of flowers / inflorescence in three-pronged plant	13-56
Range of number of flowers / inflorescence in four-pronged plant	25-60
Range of No. of flowers / inflorescence in five-pronged plant	25-73
Range of No. of flowers / inflorescence in six-pronged plant	45-75
No. of calyx / flower	5
No. of corolla / flower	5
No. of anthers / flower	5
Average No. of pollen grains / anther	503
Average No. of pollen grains / flower	1895
Percentage of pollen viability/ flower	43%
No. of carpels / flower	1-5
No. of ovules/carpel	1
Pollen-ovule ratio	1895:3
Pollen shape	Prolate spheroidal
Ovary type	Inferior
Anthesis	07.00 A.M.-04.00 P.M.
Type of dichogamy	Protandry
Stigma type	Dry with unicellular papillate structures
Length of stigma(mm)	2 mm
Stigma receptivity	3-6 days

Fig. 5.1 Showing mean number of fruit formation, seed set per fruit and total number of seeds per plant through autogamy, geitonogamy and xenogamy respectively in *Panax wangianus*.

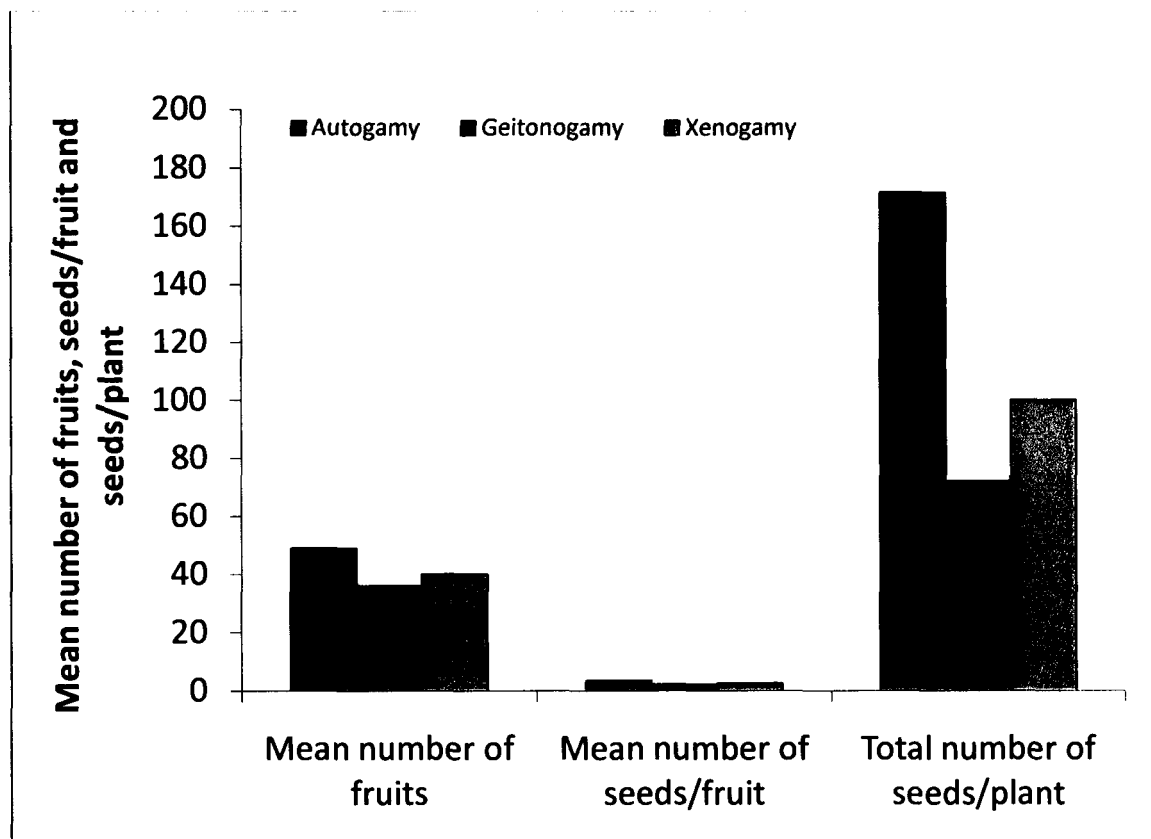


Plate – 5.1

- a- b** An enlarged view of umbel inflorescences of four- pronged and six-pronged plants respectively. Each inflorescence is composed of 13–75 numbers of greenish white flowers. The four-pronged plants produce 3-5 open flowers in a day while in six-pronged plants the number of open flowers in a day varied from 20–30. Note the occurrence of anthesis in centripetal manner. Bar; a = 2 cm; b = 2.5 cm.
- c** An inflorescence in which the terminal umbel is detached and showing six axillary inflorescences with pink color flowers growing in the bright sunlight. Bar = 3.5 cm.
- d** An enlarged view of axillary umbel with pink color flowers. Bar = 1.5 cm.

Plate – 5.1

Variation in color of inflorescence of *P. wangianus*

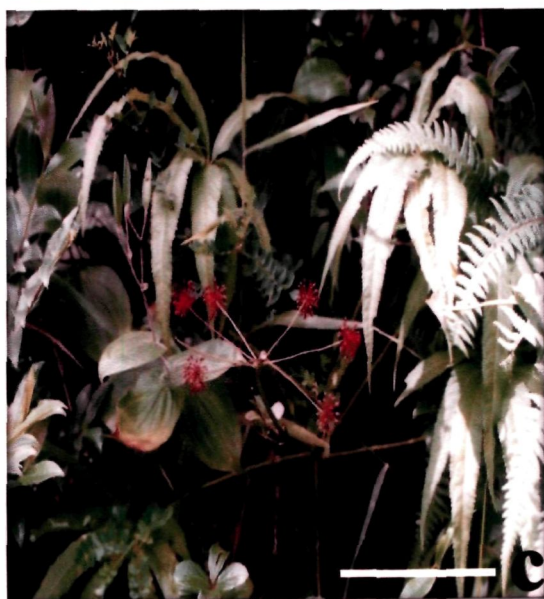


Plate – 5.2

a-e Scanning electron microscope (SEM) photograph of flowers of *P. wangianus* showing one to five styles. The number of style and stigmatic lobes varies in each inflorescence. The length of stigma is 2 mm. Arrow indicates the deposition of pollen grains on the floral disk in unistigmatic and tetrastigmatic flowers. (FI = anther filament).

Plate – 5.2

Uni-pentastigmatic condition in the flower of

Panax wangianus

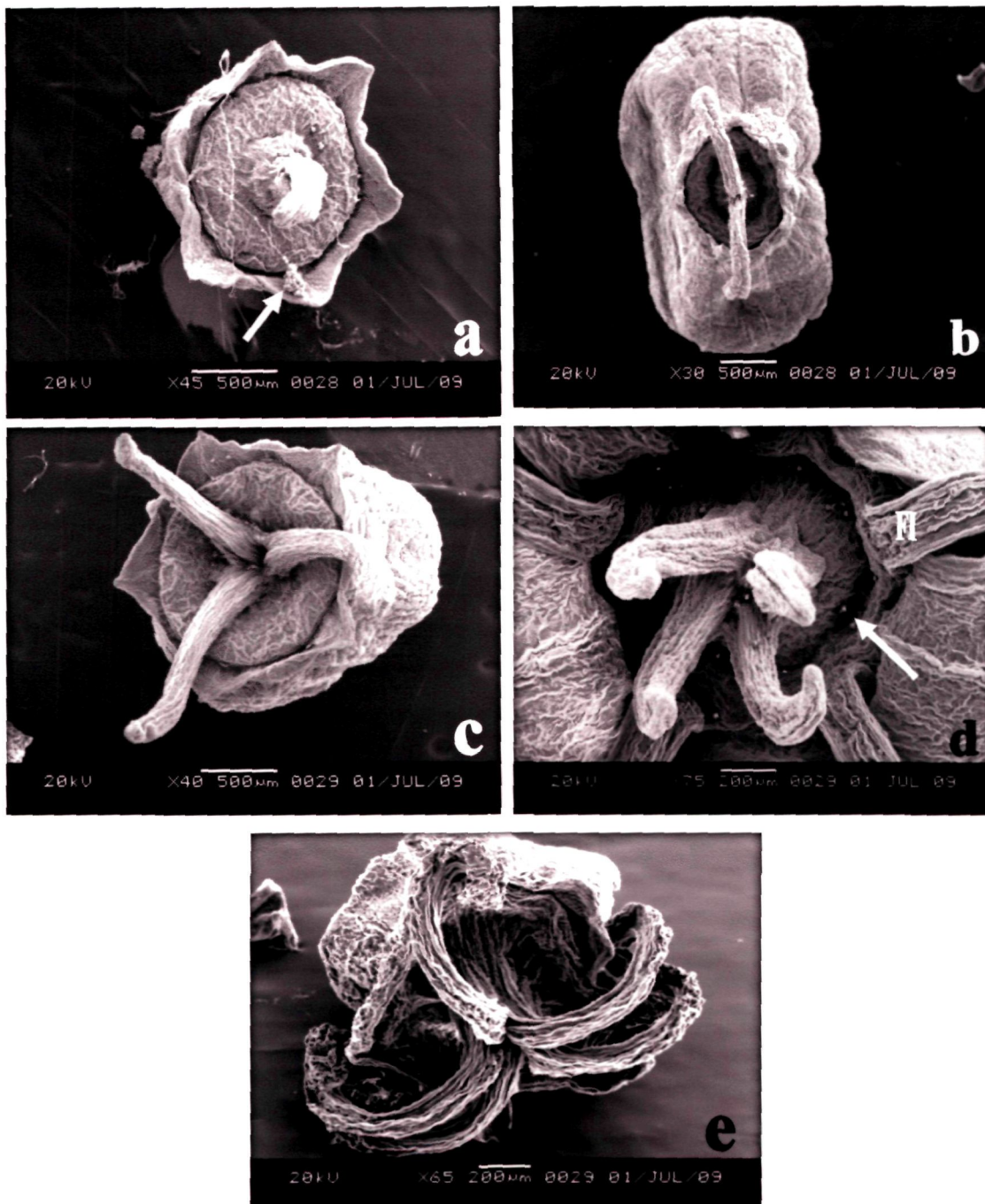
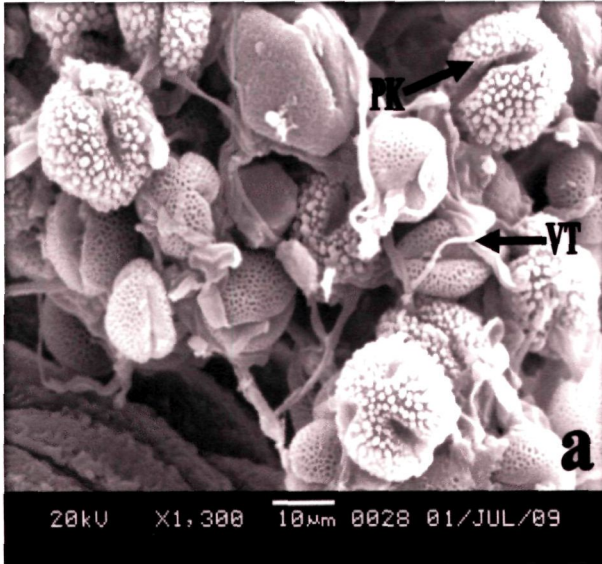


Plate – 5.3

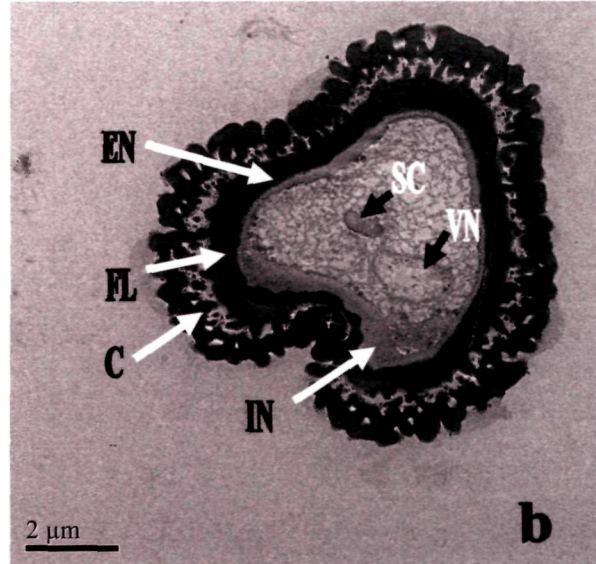
- a** SEM photograph showing the mass of pollen grains which are held together by viscin threads (VT). Note the deposition of Pollenkitt (PK) on the young pollen grains.
- b** TEM photograph of tricellular pollen grain showing short columellae (C) and thick foot layers (FL). Note the sperm cells (SC) and vegetative nucleus (VN). (EN = endexine; IN = intine).
- c** Longitudinal section of a bicellular pollen grain. The polar columellae are elongated and the endexine is thickened above and below the endoaperture, forming costa endocolpi (arrow). The generative cell is very much elongated and “U” shaped with distinct nucleus. Note the part of cut portion of vegetative nucleus
- d** Pollen viability (43%) by FCR test, the fertile pollen grains emit greenish yellow fluorescence under fluorescent microscope (UV light of 430 nm). Bar = 150 μ m.

Plate – 5.3

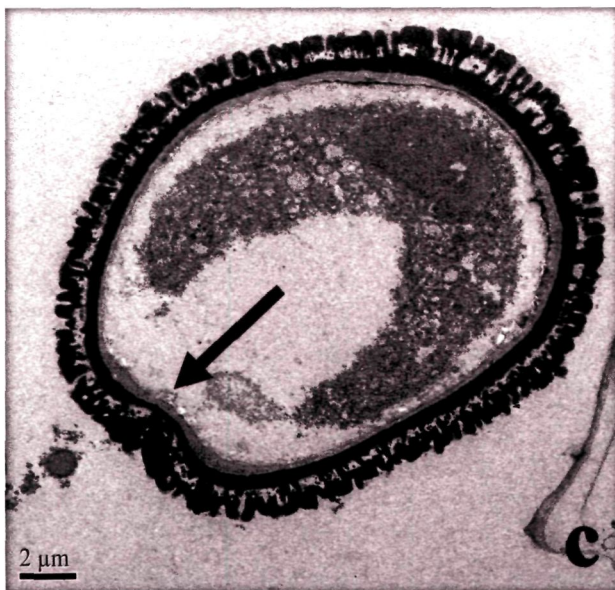
Panax wangianus S. C. Sun



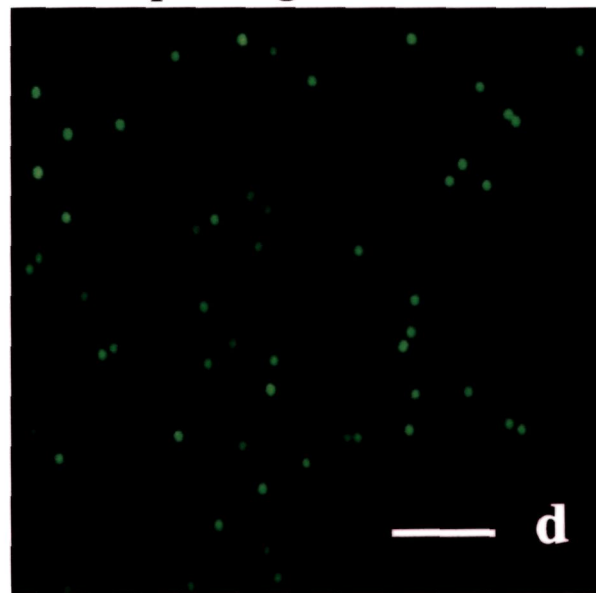
**SEM photograph of
pollen grains**



**TEM photograph of a
pollen grain**



**Longitudinal section of
whole pollen grain**

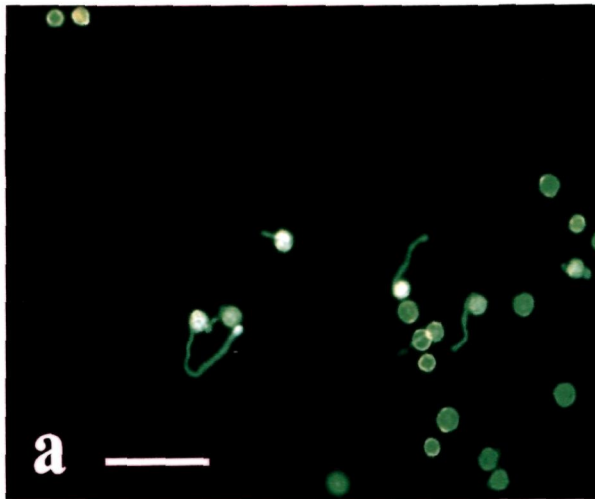


Pollen Viability

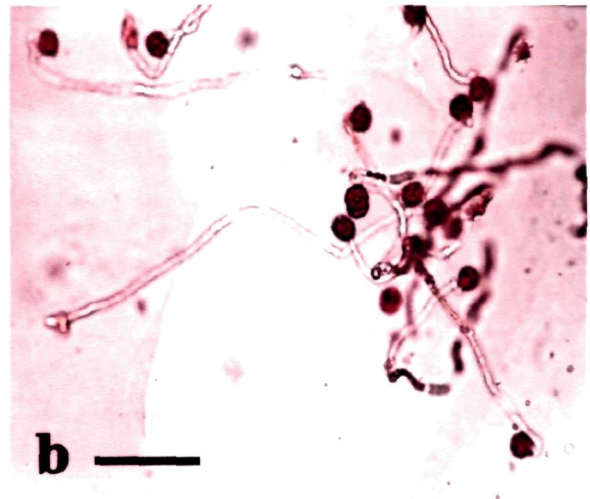
Plate – 5.4

- a** Pollen germination in modified Brewbaker and Kwack's medium after 12 hours stained by decolorized aniline blue (after exposure to UV light of 430 nm under fluorescent microscope). Bar = 150 μm .
- b** Pollen germination after 15 hours. Bar = 170 μm .
- c** Pollen germination after 17 hours (average length of pollen tubes 115.7 μm). Bar = 100 μm .
- d** Auto fluorescence of callose plugs in the pollen tube (under UV light). Bar = 50 μm .

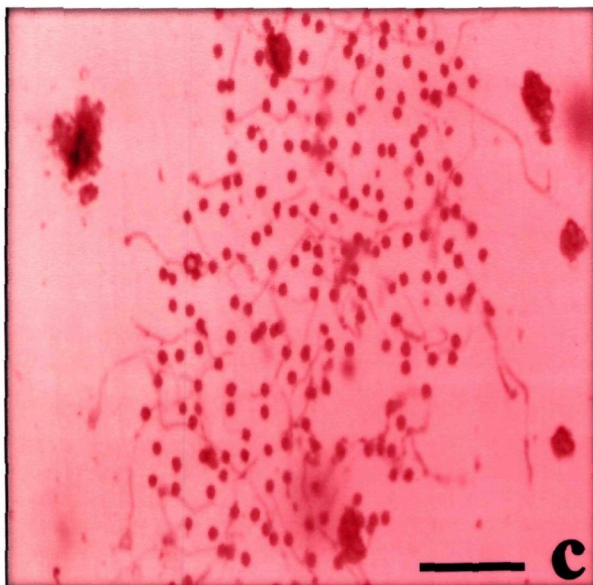
Plate – 5.4
***Panax wangianus* S. C. Sun**



**Pollen germination
after 12 hours**



**Pollen germination
after 15 hours**



**Pollen germination after
17 hours**



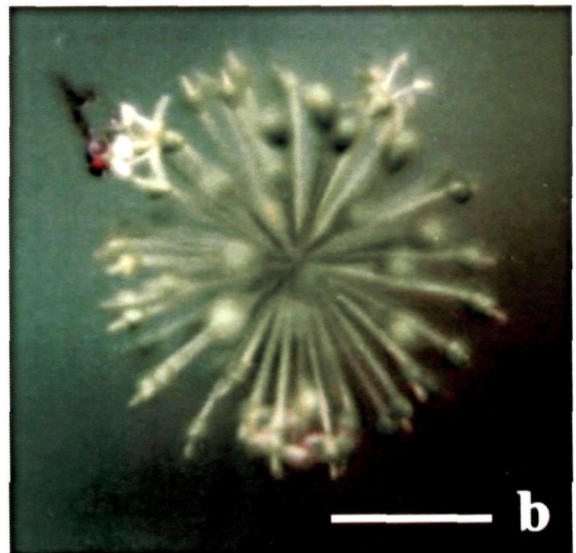
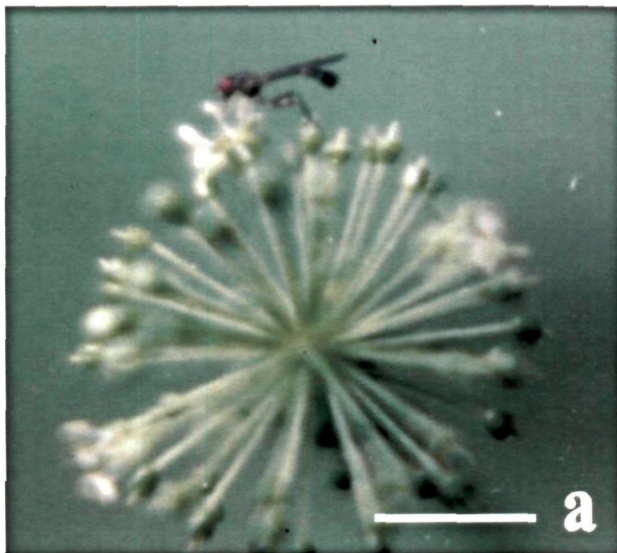
**Callose plugs in
the pollen tube**

Plate – 5.5

- a, b** A fly belonging to family Syrphidae (Order-Diptera) for pollination on the umbel of *P. wangianus* . Bar = 3.6 cm.
- c** A magnified view of fly belonging to the family Syrphidae showing well developed hind legs for landing on the inflorescence of *P. wangianus*. Bar = 6 cm.

Plate – 5.5

Pollinators



Pollination by fly belonging to the family Syrphidae



An enlarged view of fly belonging to family Syrphidae

Plate – 5.6

- a** **A magnified view of bee belonging to the family Halictidae.**

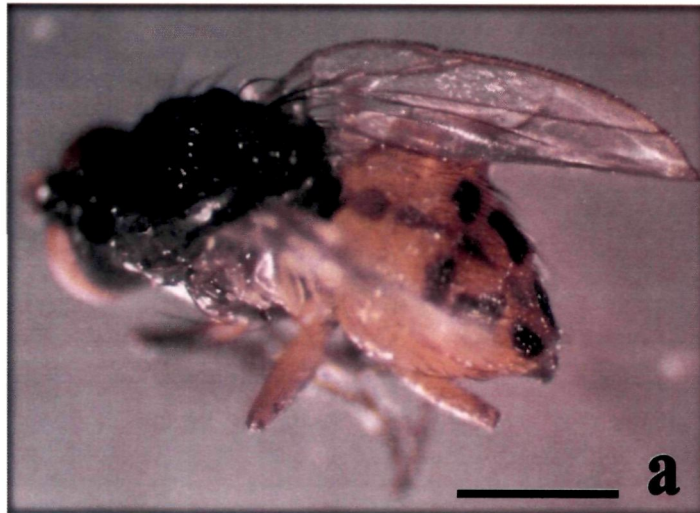
Bar = 4 cm.

- b** **SEM photograph of bee belongs to the family Halictidae (Order-Diptera). Arrows indicating deposition of pollen grains near its mouth organ, abdomen and on legs.**

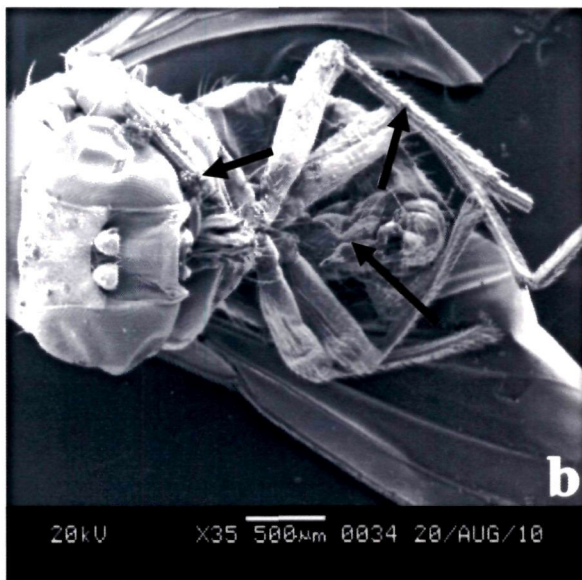
- c** **SEM photograph of scopal hairs of the legs of bee carrying the pollen grains.**

Plate – 5.6

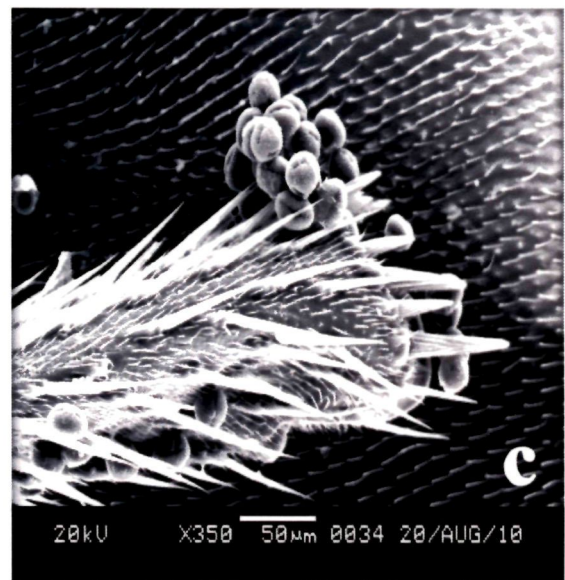
Pollinators



An enlarged view of bee belonging to family Halictidae



SEM photograph of bee



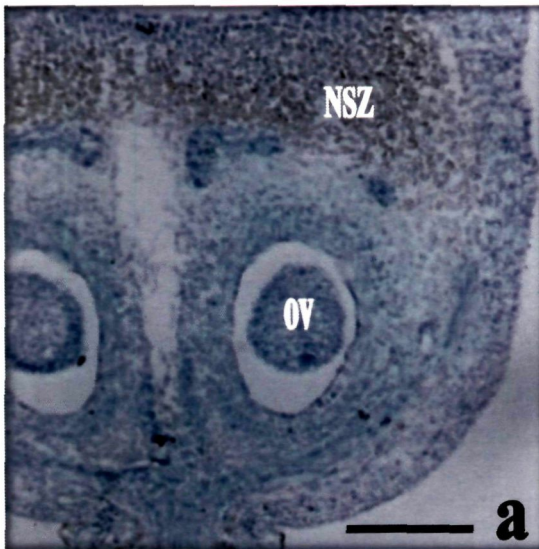
Pollen grains on the
body of bee

Plate – 5.7

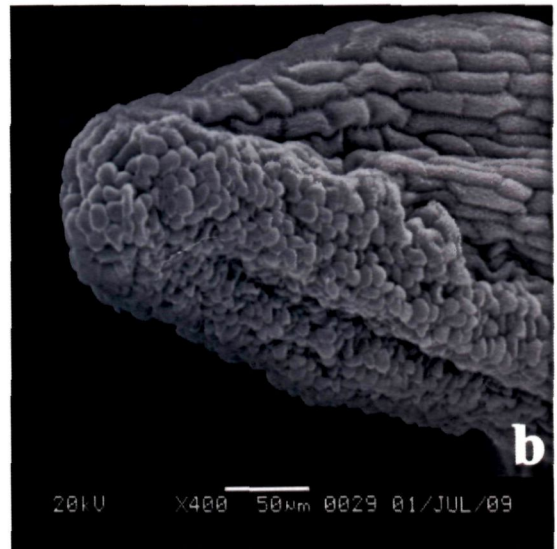
- a** L.S. of ovary showing nectar secreting zone (NSZ) in the epigynous disc. Note single ovule (OV) in each locule.
Bar = 4 cm.
- b** SEM photograph of stigma dry with unicellular papillate structures.
- c** Pollen load on the stigma is very low, that too, on the receptive surface of the stigma contains only few pollen grains. Bar = 2.5 cm.
- d** SEM photograph of receptive surface of stigma. Arrows indicates pollen grains.

Plate – 5.7

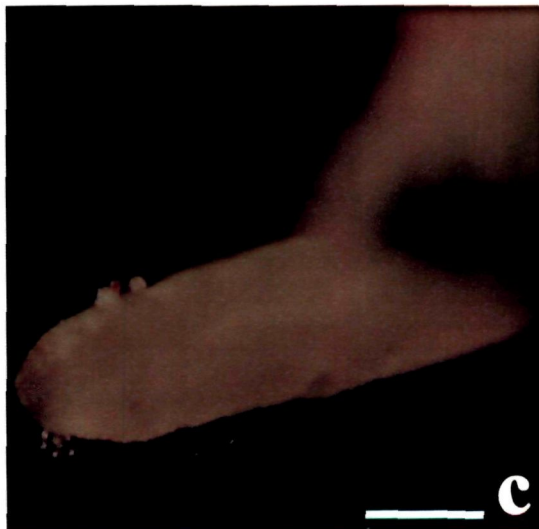
***Panax wangianus* S. C. Sun**



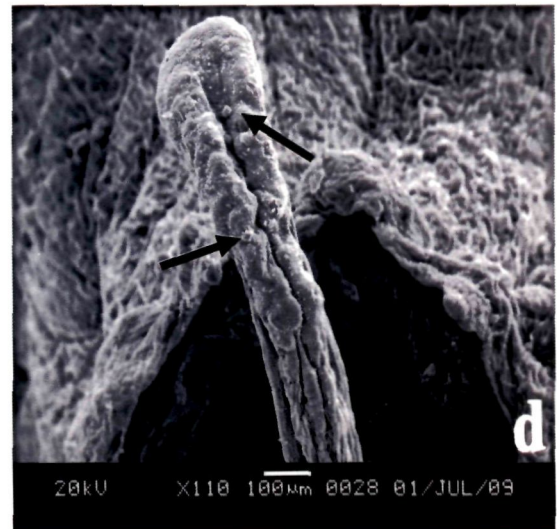
**L.S. of ovary showing
Nectar secreting zone**



SEM photograph of stigma



**Pollen grains on the
receptive surface of stigma**



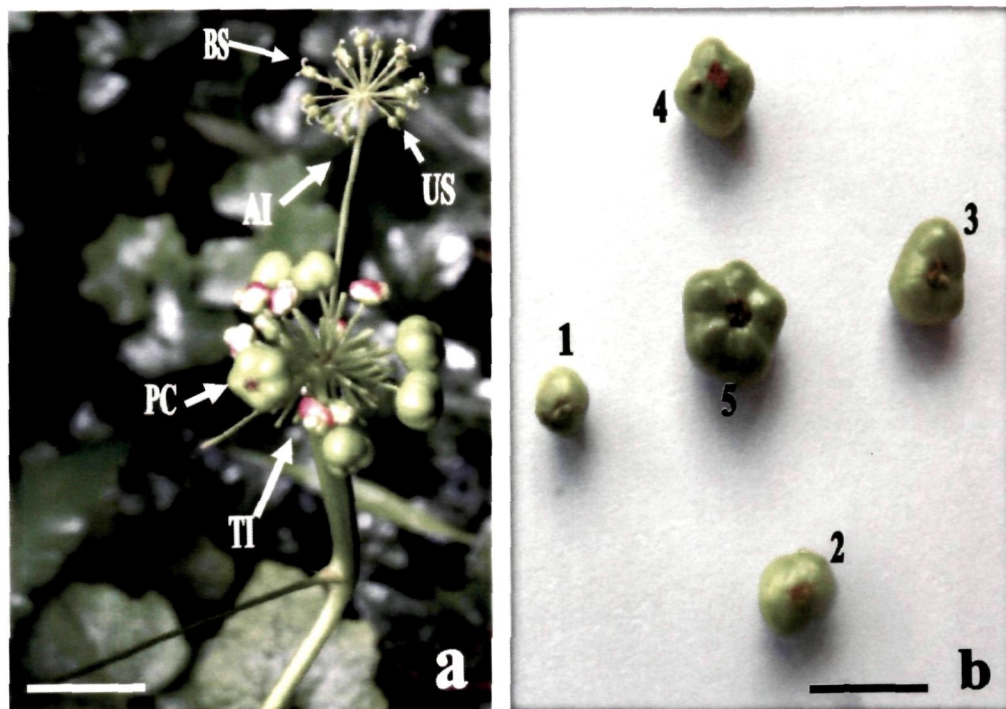
**SEM photograph of
receptive surface of
stigma**

Plate – 5.8

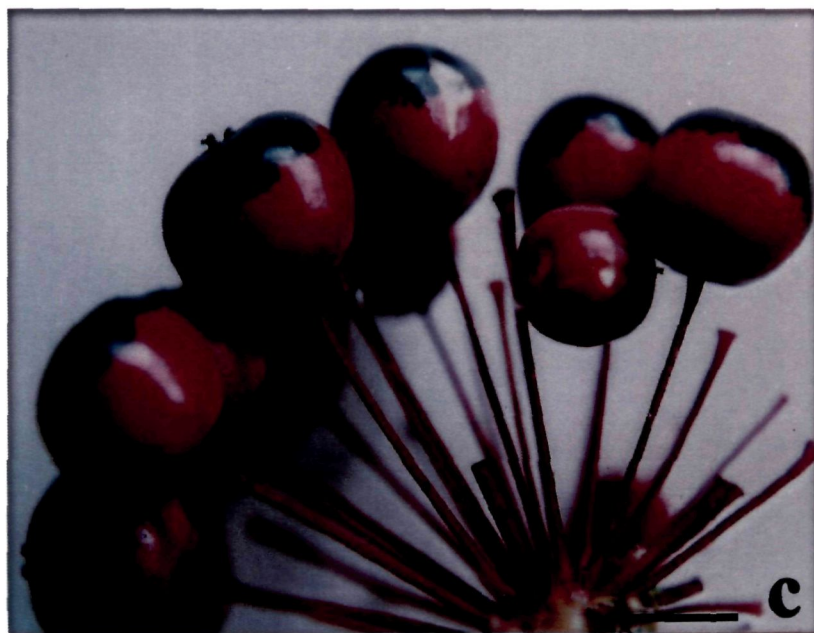
- a** An enlarge view of inflorescence showing the terminal inflorescence (TI) and axillary inflorescences (AI) and unistigmatic (US) to pentastigmatic as well as uni-pentacarpellate (PC) condition. Terminal inflorescence bearing young green fruits. Note the terminal inflorescence mature earlier than the axillary one and the pink color fruits are abortive fruits. (BS = bistigmatic). Bar =10 cm.
- b** Young greenish fruits with uni-pentacarpellate condition. Note the variation in the number of stigmatic lobes. (1 = unicarpellate; 2 = bicarpellate; 3 = tricarpetlate; 4 = tetracarpellate and 5 = pentacarpellate condition). Bar = 1cm.
- c** An enlarged view of mature fruits. The berries are bright red on the bottom and black on the top. Note the color of pedicel also changed from green to crimson red. Bar = 5.5 cm.

Plate – 5.8

***Panax wangianus* S. C. Sun**



Young greenish fruits with uni -pentacarpellate condition



Mature fruits

Plate – 5.9

a **Mature fruits are eaten by birds. (P = Pulp and S= Seed).**

Bar = 9.50 cm.

b **Showing the naturally bending of aerial stem of**

***P. wangianus* (i.e. gravity seed dispersal). Bar = 11 cm.**

Plate – 5.9

Panax wangianus S. C. Sun



CHAPTER-6

Structure and development of Anther

6.1 Introduction

There are variations in the flower structure of the members of the family Araliaceae e.g. reduction of calyx, increase in number of stamen whorls, increase of overall floral meristem or only of stamen number, increase or decrease of carpel number, congenial petal fusion with postgenital corolla closure into a massive calyptras, appearance of flower dissymmetry or asymmetry during flower development or even from its very beginning (Nuraliev *et al.* 2010). Flowers of most members of Araliaceae possess five stamens (Sokoloff *et al.* 2007). There are seven to twelve stamens in *Trevesia palmata*, four to thirty in *Osmoxylon*, six to eighteen in *Brassia actinophylla*, twenty seven stamens in *Schefflera subintegra* and twenty two stamens in *S. hemiepiphytica* (Xiang and Lowry 2007; Grushvitzky and Skvortsova 1973; Frodin and Govaerts 2004). Flowers with numerous stamens

occur occasionally within Araliaceae, for example, more than 500 stamens in *Plerandra pickeringii* (Oskolski *et al.* 2010a); 250 stamens in *S. cabalionii* (Lowry II 1989); 200 stamens in *Tupidanthus* corresponding to the petal lobes (Sokoloff *et al.* 2007) and 100 stamens in *Gastonia spectabilis* (Philipson 1970b). The embryology of angiosperms has been extensively reviewed and published by Schnarf (1931), Davis (1966), Maheshwari (1950) and Johri *et al.* (1992). The embryology of some of the members of family Araliaceae viz., *Panax fruticosum*, *Hedera australiana*, *Polyscias pinnata*, *Heptapleurum venulosum*, *B. actinophylla*, *Panax ginseng*, *Tieghemopanax sambucifolius* and *Eleutherococcus senticosus* have been studied in detail (Ducamp 1902; Cammerloher 1910; Elizabeth and Pigott 1914; Pigott 1927; Singh 1954; Gopinath 1944; Lin-De *et al.* 1998; Zhuravlev *et al.* 2008). The anthers are tetrasporangiate. This chapter aims to study the structure and development of the androecium and microsporogenesis in *Panax wangianus*.

6.2 Development of the Stamen

The young flower buds during in its developmental stages contained five anther primordia enveloped by sepals (Plate – 6.2 a). The anther primordia are roundish or globular in outline but soon they

become more or less rectangular in shape because of accelerated cell division in the four corners (Plate – 6.2 b).

A plate of four to five hypodermal cells differentiates into an archesporial initial in each corners of the anther primordium. An archesporial initial is radially elongated, and can be distinguished from the other cells by its conspicuous nucleus, nucleolus and dense cytoplasm. The number of archesporial cells increases in number by additional anticlinal divisions. The entire archesporial initials divide periclinally. The cells derived from the first periclinal division are all of equal sized daughter cells results into the formation of primary parietal and primary sporogenous layers respectively. The inner row of cells is much larger than the outer ones. The former becomes the primary sporogenous cells and later the primary parietal cells.

The primary parietal cells undergo periclinal divisions to form secondary parietal layers of which the outer secondary parietal layer divides periclinally to form endothecium and middle layer. Therefore, the anther wall development is dicotyledonous type. The middle layer undergoes one more periclinal division so that the middle layer is bilayered. Finally, the anther wall constitutes epidermis, endothecium, middle layers and tapetum (Plate – 6.2 c). In young anther, the

endothecial layer is rectangular, smaller than middle layers and tapetal layers. The cells of endothecial and middle layers contain few vacuoles. By the time of anther wall dehiscence, the cells of the endothecium have become enlarged and have developed fibrous thickenings (Plate – 6.5 b). The middle layers consist of two rows of medium-sized cells which are ephemeral. The cell walls are thin. Usually, it begins to degenerate when the early stages of the meiosis in the microspore mother cells (MMCs) (Plate – 6.4 a) and its by products are utilized for the differentiation of microspores. During the disintegration of middle layers, callose like substances are observed in middle layers under fluorescent microscope which indicates the callose is secreted in middle layer and transported to MMCs through tapetum. It proves that middle layers play an important role in producing callose apart from tapetum and MMCs. Concomitant with the differentiation of anther wall layers, the primary sporogenous cells undergoes several divisions in different planes to form sporogenous tissue which is enclosed by anther wall layers.

The tapetal initials are much larger than endothecial layer and middle layers. Tapetal cells are radially elongated; bi- and tetranucleate and sometimes shows polyploidy condition with dense cytoplasm and

prominent nucleus, nucleolus and small-sized vacuoles (Plates – 6.2 d and 6.3 a). The tapetal cells that abutting at the connective side arise from the cells of the connective tissues, which are much larger in size and biseriate, indicate the dual origin of tapetum. Thus, the tapetum originated from (1) the derivatives of the parietal cells (parietal tapetum) and (2) the connective cells (connective tapetum). Both the parietal tapetum and connective tapetum are of secretory type. The parietal tapetum is binucleate whereas the connective tapetal cells show multinucleate condition. The parietal tapetum becomes prominent and provides nourishment to the microspores. The tapetum persists even after the microspores attain their maximum size (Plate – 6.5 a).

The cells of sporogenous tissue are polygonal in outline and attached close each other within the locule. Later on, more or less all sporogenous tissues undergo several divisions to form MMCs. The cells of sporogenous tissue have dense cytoplasm, nucleus, nucleolus and have few small vacuoles.

During the onset of meiosis in the MMCs, a special callose wall layer is secreted around each mmc (Plate – 6.3 b). The MMCs undergo two consequent meiotic divisions and cytokinesis is of simultaneous

type (Plate – 6.4 b). Tetrads are formed due to the simultaneous formation of centripetal furrows after meiosis II. The tetrads almost filled the entire space of the anther locules. The tetrads were arranged in tetrahedral and isobilateral manner. Each tetrad was surrounded by a thick, asymmetrical callose envelope (Plate – 6.4 c). The callose wall is fluorescent when stained with decolorized aniline blue after exposure to UV light at 430 nm under fluorescent microscope (Plate – 6.3 c, d). When callose was dissolved, the microspores soon separated from each other (Plate – 6.4 d). Wall formation around pollen grain initiated during cytokinesis. The pollen grains are tricolpate with thick exine (Plate – 6.5 c) and thin intine. In the young spore, the nucleus is centrally situated and embedded in a dense cytoplasm. The nucleus of the microspore divides to form a large vegetative and a small generative cell with two nuclei during shedding. The dehiscence of anther takes place through a longitudinal lateral slit in the anther wall (Plate – 6.1 b).

6.3 Discussion

The number of stamens in *P. wangianus* is five (Plate – 6.1 a) similarly as in most members of Araliaceae (Sokoloff *et al.* 2007). While there are seven to twelve stamens in *T. palmata*, four to thirty in

Osmoxylon and six to eighteen in *B. actinophylla* (Xiang and Lowry 2007). Flowers with numerous stamens occur occasionally within Araliaceae, for example, more than 500 stamens in *P. pickeringii* (Oskolski *et al.* 2010a); 250 stamens in *S. cabalionii* (Lowry II 1989); 200 stamens in *Tupidanthus* corresponding to the petal lobes (Sokoloff *et al.* 2007) and 100 stamens in *G. spectabilis* (Philipson 1970b). The development of the five stamens is synchronous in *P. wangianus*. The anther is tetra-locular in *P. wangianus*. Similarly in *P. fruticosa*, *H. australiana*, *P. ginseng*, *E. senticosus* and *B. actinophylla* the anther shows a four locular nature in cross-section (Gopinath 1944). Tetrasporangiate anthers have been reported in majority of angiosperm families (Bhandari 1984).

The development of anther wall is dicotyledon type in *P. wangianus*. The young anther wall comprises of an epidermis, endothecium, two middle layers and a tapetum. *P. wangianus* consist of two middle layers similar as in *B. actinophylla* whereas in *P. fruticosa* and *H. australiana* there are single middle layer (Gopinath 1944).

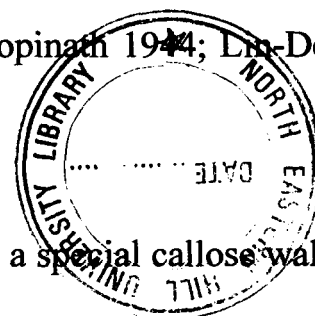
According to Periasamy and Swamy (1966) the tapetum is of dual origin in most of the angiosperms e.g. in *Anemone rivularis*

(Bhandari 1968), *Salvia mellifera* (Carlson and Stuart 1936), *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). Similarly, in *P. wangianus*, a part of tapetum is derived from the single layer primary parietal cell, whereas another part is derived from the multilayered connective tissue which proves dual origin of tapetum.

There is a great variation in the number of nuclei in the tapetal cells of the Araliaceae. In *P. wangianus* uni-tetranucleate glandular tapetal cells found. In *Hedera nepalensis* the cells are multinucleate (Singh 1954). Binucleate tapetal cells observed in *B. actinophylla*, *E. senticosus* and *P. fruticosa* whereas in *H. australiana* the cells are uninucleate (Gopinath 1944; Lin-De *et al.* 1998). In *T. sambucifolius* the tapetal nuclei are polyploidy and are formed as a result of several nuclear divisions and fusions.

In *P. wangianus* meiosis in MMCs is accompanied by simultaneous cytokinesis. Many authors deduce microsporogenesis type from tetrad configuration: tetrahedral tetrads are normally associated with simultaneous microsporogenesis, whereas isobilateral,

decussate, T-shaped or linear tetrads are associated with successive microsporogenesis. Tetrad configuration appears to be a reliable indicator of microsporogenesis type (Rudall *et al.* 1997; Furness and Rudall 1999, 2000). However, in *P. wangianus* simultaneous type cytokinesis leads to the formation of tetrahedral and isobilateral tetrads. Caddick *et al.* (1998) also reported in *Stenomeris*, simultaneous microsporogenesis does not always produce tetrahedral tetrads, because the configuration of the tetrad is strongly influenced by three factors: orientation of meiotic spindles, timing of the two meiotic divisions and timing of cell plate formation. Therefore, tetrad configuration is apparently not always a reliable indicator. While *P. ginseng*, *T. sambucifolius* and *E. senticosus* produce tetrahedral or decussate type of tetrads (Mohana Rao 1972; Gopinath 1944; Lin-De *et al.* 1998).



During the onset of meiosis in the MMCs, a special callose wall layer is secreted around each MMC which is fluorescent when stained with decolorized aniline blue after exposure to UV light at 430 nm 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 *P. wangianus* the callose wall facilitates the transfer of material from the tapetum to the MMCs. Once the callosic wall disintegrates, microspores are freed. Pollen grains are three-celled at the time they are shed. The same condition has been reported in *H. australiana*, *E. senticosus* and *P. fruticosa* (Gopinath 1944; Lin-De *et al.* 1998).

In angiosperms, mature pollen grains are divided into two groups: bicellular and tricellular type (Maheshwari 1950; Knox 1979). However, mature pollen grain possesses three cells in most taxa of the Araliaceae (Gopinath 1944; Singh 1954; Lin-De *et al.* 1998). As a member of the Araliaceae, *P. wangianus* also belongs to the tricellular type. The pollen grains were tricolporate, presenting a small generative cell with large nucleus.

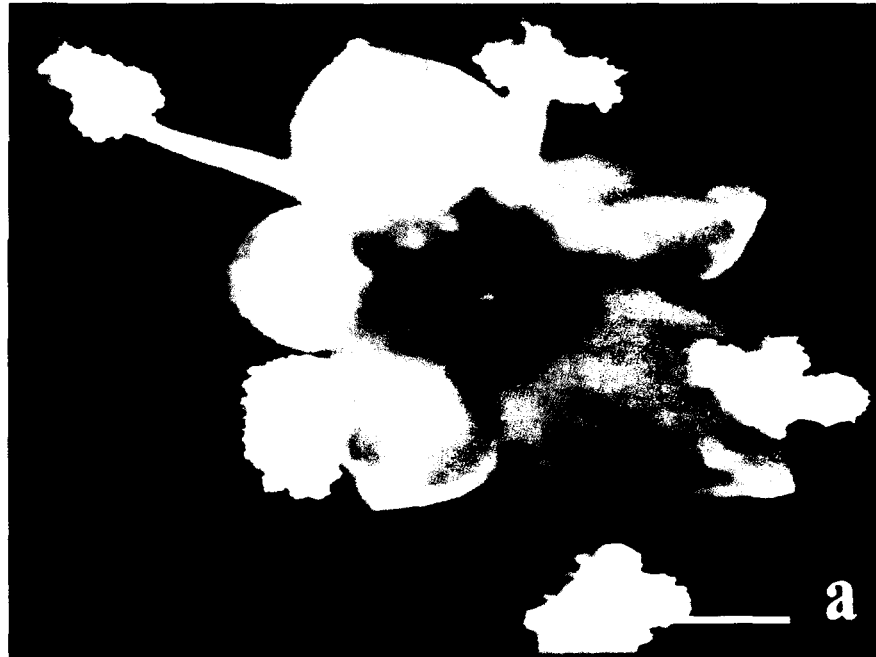
Plate – 6.1

- a** **An enlarged view of a flower of *P. wangianus* showing pentamerous condition. Note the pollen grains in the dehiscent anther lobes. Bar = 3 cm.**

- b** **SEM photograph of lateral dehiscence of an anther lobe.**

Plate – 6.1

***Panax wangianus* S. C. Sun**



An enlarged view of a flower



Lateral dehiscence of anther

Plate – 6.2

- a Longitudinal section of a young flower bud showing anther primordia (AP); and the central gynoecial primordium (GP) enveloped by union of thalamus and sepals (SE) indicate hypogyny. Bar = 30 μ m.**
- b Cross section of a young flower bud showing the tetrasporangiate anther lobes and two stigmatic lobes (SL). Bar = 45 μ m.**
- c Cross section of a young undifferentiated anther lobe showing the tiered arrangement of epidermis (EP), endothecium (EN), middle layers (ML), tapetum (TP) and the central sporogenous tissue (ST). Bar = 50 μ m.**
- d A mature anther lobe contains central zone of microspore mother cells (MMCs) which is enclosed by tapetum. Note the dense cytoplasm, prominent nuclei and radially stretched tapetal cells. (PT = parietal tapetum and CT = connective tapetum). Bar = 50 μ m.**

Plate – 6.2

Panax wangianus S. C. Sun

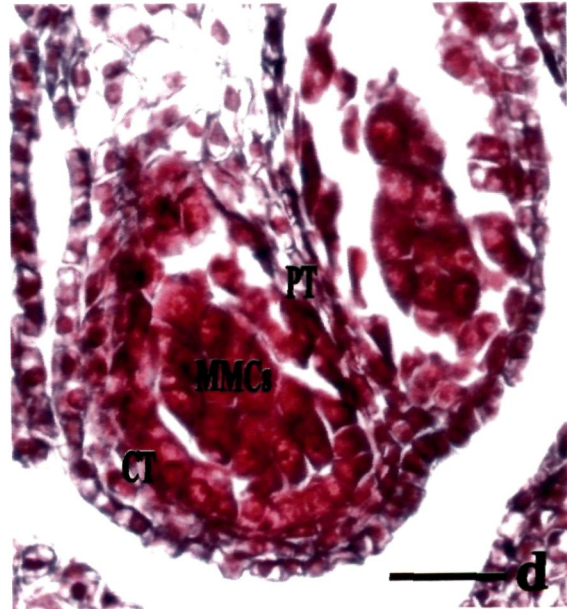
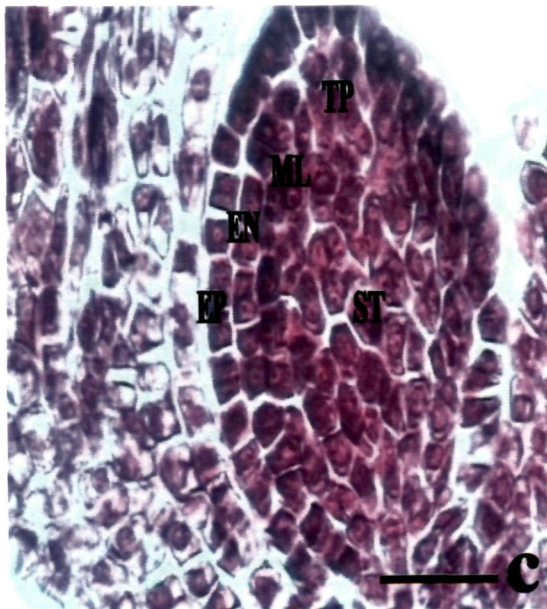
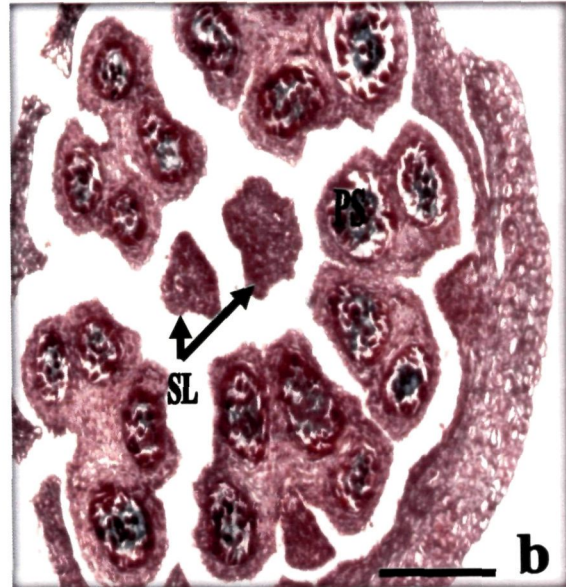


Plate – 6.3

- a** Tapetal cells are bi- and tetranucleate (TN) and sometimes contain ployploidy nuclei with dense cytoplasm and prominent nucleus and nucleolus. Bar = 45 μm .
- b** During the onset of meiosis in the microspore mother cells, a special callose wall (CW) layer is secreted around each microspore mother cell. Note the granular cytoplasm and few small vacuoles in the microspore mother cells. Bar = 52 μm .
- c** Autofluorescence emitted by Callose deposition. Arrows show granular emittance of fluorescence from the middle layers indicates the site of synthesis. Bar = 50 μm .
- d** Autofluorescence emitted by callose around the microspore mother cells (MMCs). Bar = 50 μm .

Plate – 6.3

Panax wangianus S. C. Sun

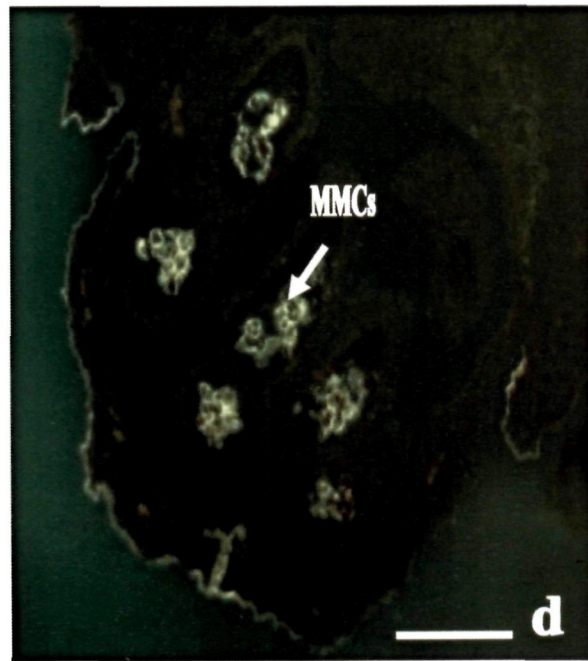
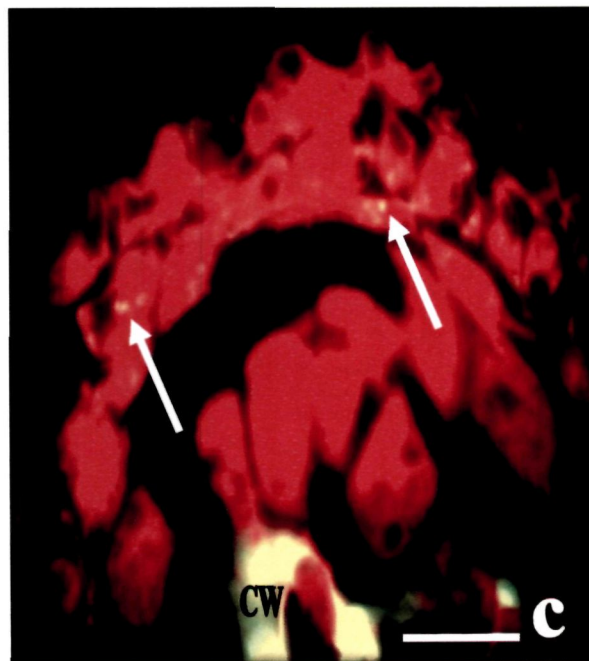
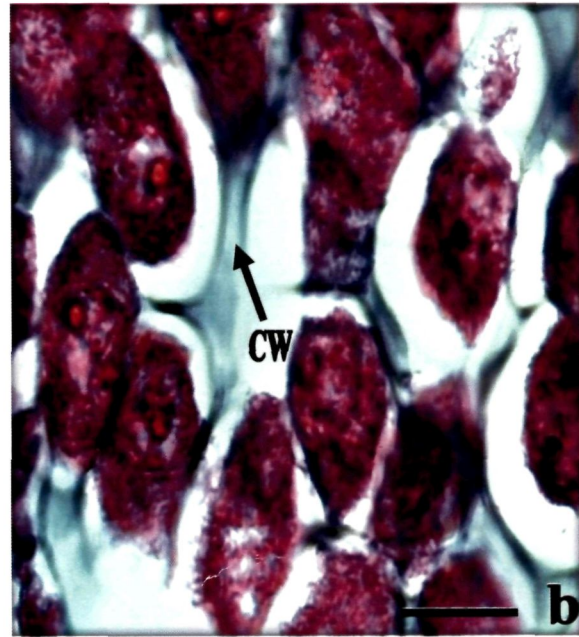
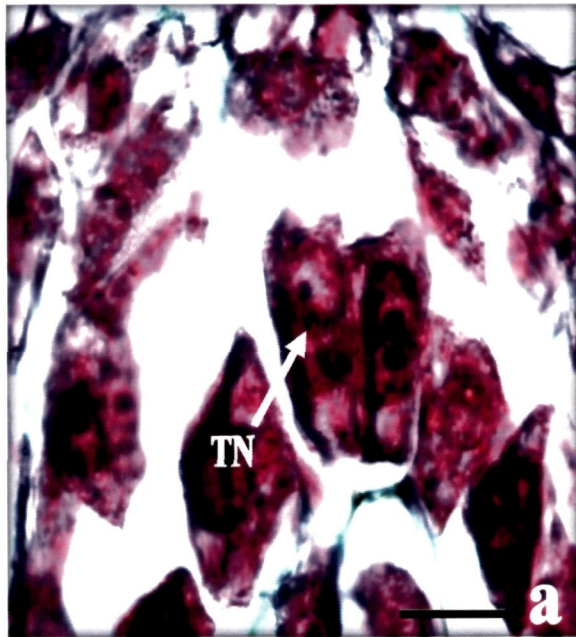


Plate – 6.4

- a** **Cross section of anther with microspore mother cells (MMCs) before meiosis and some MMCs are in the prophase stage. Note the thickenings in the endothecium (EN) and the middle layers are disintegrated and disappeared before meiosis. Bar = 60 μm .**
- b** **Meiosis is simultaneous, all the four daughter nuclei (DN) located within the meiocyte before cytokinesis. Bar = 45 μm .**
- c** **The four products of meiosis enclosed still within the callose wall (CW) before wall formation. Bar = 68 μm .**
- d** **After wall formation the separated pollen grains (PG) within the callose walls (CW). Bar = 56 μm .**

Plate – 6.4

Panax wangianus S. C. Sun

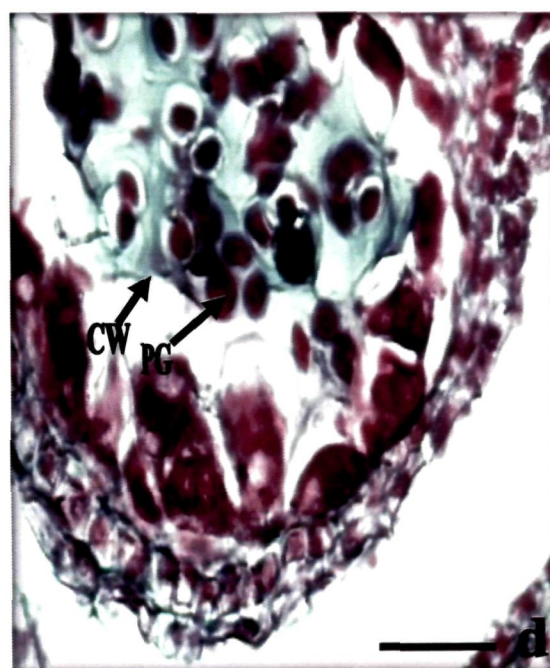
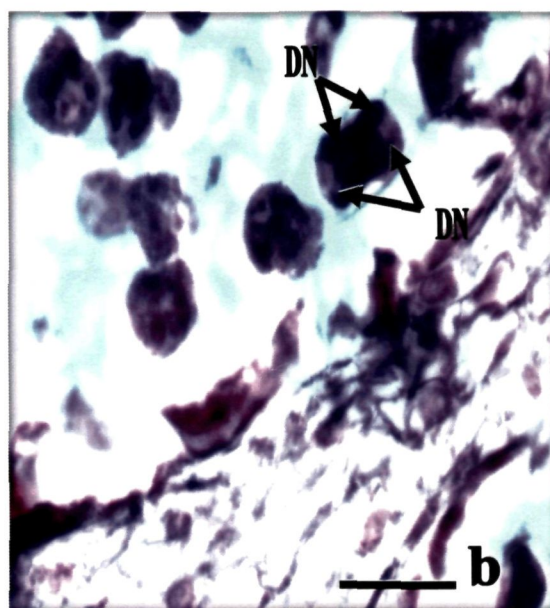
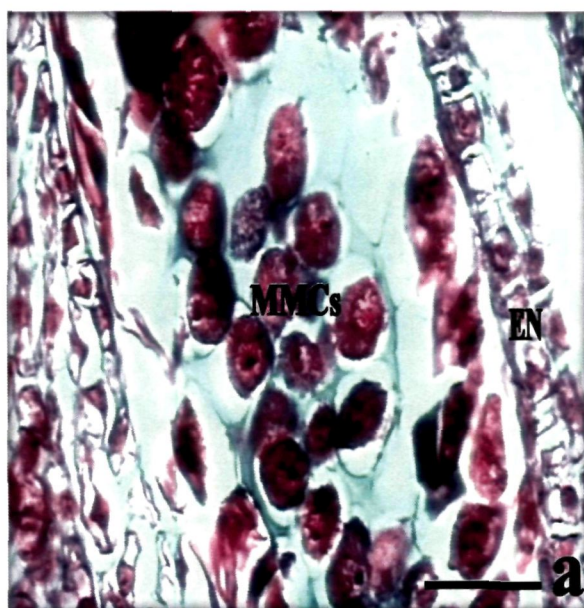
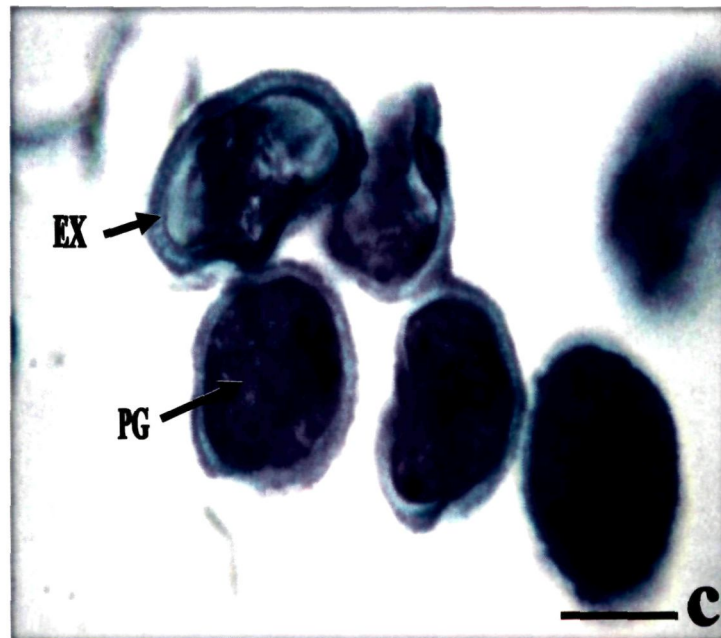
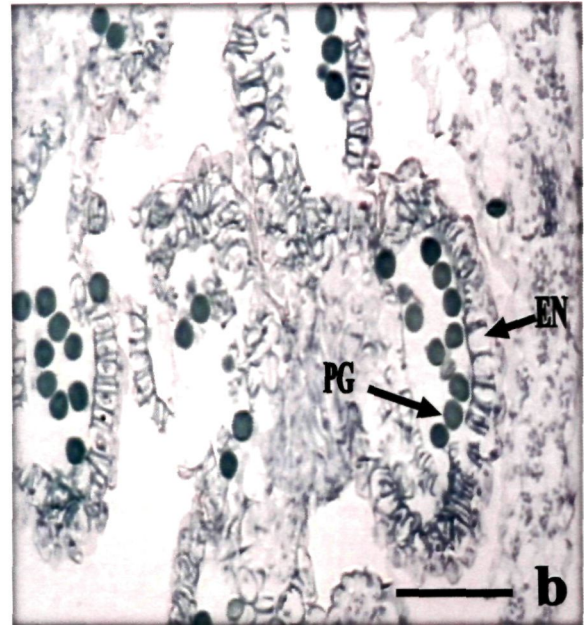
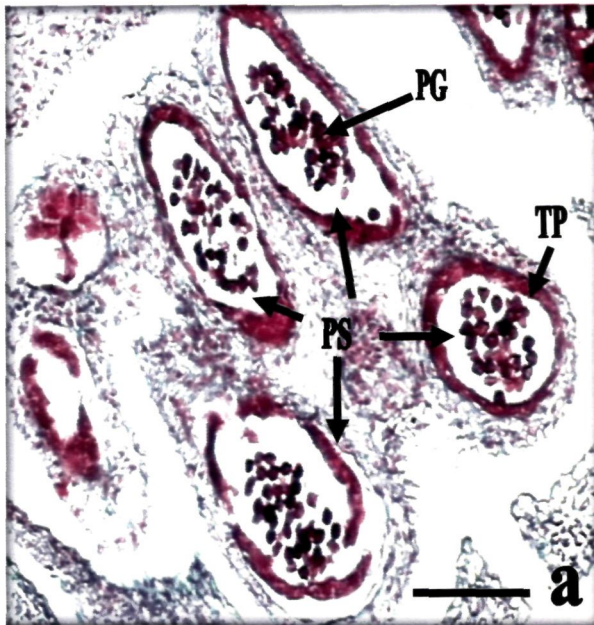


Plate – 6.5

- a** The four neighboring pollen sac (PS) show synchronized in development and contains young pollen grains (PG). At the time of pollen grains release, tapetum (TP) is still persisting.
Bar = 60 μm .
- b** Dehiscence of mature anthers which contains mature pollen grains (PG). Anther wall consist of only endothecium (EN).
Bar = 65 μm .
- c** Exine (EX) formation in mature pollen grains (PG). Bar = 55 μm .

Plate – 6.5

Panax wangianus S. C. Sun



CHAPTER-7

Development of Female Gametophyte and post-fertilization changes in embryo-sac

7.1 Introduction

The family Araliaceae represents an important group for investigating general patterns of variation in flower structure in relation to the genetic background of regulation of floral development because it shows a wide range of variation in floral meristem, ovary position and type of organ fusion such as congenital and postgenital fusion (Philipson 1970a; Eyde and Tseng 1971; Costello and Motley 2004).

Flowers of most Araliaceae members generally possess pentacarpellate condition. Flowers with numerous carpels (more than 13-25 carpels) occur in a few species of *Osmoxylon*, *Gastonia*, *Reynoldsia*, *Schefflera subintegra* and *S. hemiepiphytica* (Philipson 1970b, 1979; Eyde and Tseng 1971; Grushvitzky and Skvortsova 1973; Frodin and Govaerts 2004). *Tupidanthus calyptratus* is probably

the most extreme example of polymery among asteroids, in which 60-138 carpels are found (Sokoloff *et al.* 2007).

The occurrence of unilocular ovaries is reported in a few members of Araliaceae, such as in *Arthrophyllum*, *Cuphocarpus* and *Osmoxylon micranthum* which is derived from bi-or multilocular ovaries through a reduction in the number of carpels i.e. these gynoecia are pseudomonomerous (Baumann-Bodenheim 1955; Magin 1977, 1980; Philipson 1970a, 1979; Oskolski *et al.* 2010b).

The ovules are solitary, pendulous, anatropous in each locule (Cammerloher 1910) just below the epigynous disc (Davis 1966; Engler 1964), and sometimes abortive ovules are found in the same locule (Erdtman 1952); mono or bisporic type of embryo sac development (Gopinath 1944; Elizabeth and Pigott 1914; Pigott 1927) and nuclear endosperm (Schnarf 1931; Singh 1954) are characteristic features of family Araliaceae. This chapter deals with the structure of gynoecium, differentiation of embryo sac and subsequent to post fertilization changes in the ovule of *Panax wangianus*.

7.2 Structure of Pistil

P. wangianus is a monoecious plant. Ovary is inferior with each locule uniovulate. Nectariferous epigynous disc cover the ovary top.

The number of stigmatic lobes varies in different flowers of the same inflorescence. So, the number of stigmatic lobes always corresponds to the number of carpels in each ovary. The number of carpels varied from one to five, which would be readily observed from the exterior by counting the number of lobes in fruits. The stigma is spatulate, dry, receptive surface hollow covered with unicellular papillate outgrowths.

7.3 Structure of ovule, embryo sac formation and fertilization

In *P. wangianus* the number of locules ranges from one to five in the gynoecium. Each locule contains single, pendulous and anatropous ovule (Plate – 7.1 a). The ovules are unitegmic and tenuinucellate with distinct funicular obturator (Plate – 7.1 b). The obturator is the distinct near the micropylar region. The nucellar primordium becomes differentiated from the placental mass (Plate – 7.2 a). As development proceeds, the ovules become anatropous (Plate – 7.2 b) so that the micropyle, nucellus and chalazal region are lying parallel to the funiculus. The integument develops in the initial stages as a ring of cells at the base of the nucellus. In later stages it became two to three celled thick (Plate – 7.2 a). In the ovary wall and

integument the secretory canals are found where ginsenosides formation occurs (Plate – 7.1 c, d).

The obturator is formed through proliferation of cells from the funicle. The cells are rich in protoplasmic contents. They enlarge and arch over the micropyle. The cells of the surface layer of obturator elongated and rod shaped with distinct nucleus. Thus, at the time of fertilization the funicular obturator covers the micropylar part of the ovule so that it guides the pollen tube to grow towards the micropyle. A few sub-dermal cell layers of obturator contain calcium oxalate crystals. During post-fertilization stages the cells of the obturator become shriveled and are depleted of their contents. After the formation of the endosperm, the obturator tissue is crushed and gradually degenerate and disappear.

The archesporial initial is differentiated hypodermally in the nucellus which coincides with the initiation of the integument (Plate – 7.2 c, d). The archesporial directly function as the megaspore mother cell.

In the course of the development, the megaspore mother cell enlarges in size. The nucleus passes through the normal meiotic changes and forms the dyad. The next homoetypic division in the dyad

results in the formation of a linear row of tetrad of megaspores, the upper three invariably degenerate and the chalazal megaspore functions to form the embryo-sac. It enlarges further and the nucleus undergoes three cycles of mitotic divisions and results in 8-nucleate embryo-sac. Thus, the development of the embryo sac confirms to the Polygonum or Normal type. During differentiation of the embryo-sac, the nucellus is completely absorbed by the embryo sac haustoria and thus it comes in contact with the integument (Plate – 7.4 a). The chalazal end of the embryo-sac tapers into a narrow pouch-like structure, where the three antipodal cells are located (Plate – 7.3 c). The antipodal cells are uninucleate and ephemeral. At the micropylar end the egg apparatus and two polar nuclei are present. The polar nuclei lie in close vicinity of the egg apparatus.

The cells of egg apparatus consists of an egg cell and two synergids (Plates – 7.3 a, b and 7.4 b). Each synergid is globular to ovoid in structure. Both synergids share the same cell wall in their contact region. At the extremely micropylar pole the wall is strongly thickened, forming a lunar or sickle shaped structure known as “Filiform Apparatus” (FA) (Plate – 7.4 b). From the FA towards the base of the synergids the wall gradually thin. The chalazal half of the

synergid is surrounded by the plasma membrane only. The FA increases the surface area of the plasma membrane. A single large vacuole occupies the chalazal pole of the synergid while most of the cytoplasm, cell organelles and nucleus restricted just below the FA in the micropylar region.

The egg cell is club shaped. It is 6μ larger than the synergids. 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 and nucleus while the micropylar end is occupied by one or two small vacuoles with a thin layer of cytoplasm. The egg cell and synergids show distinct polarity in their distribution of nucleus, cytoplasm and vacuoles.

The central cell occupies the largest portion of the embryo sac, with a large central vacuole. Its cytoplasm is confined to a thin layer along the embryo sac wall and accumulates near the egg apparatus and the antipodal cells. The central cell is surrounded by a partial cell wall. The cell wall is absent at the micropylar pole while the cell wall against the nucellus or integuments is usually thick and regular.

After entry of pollen tubes into the two synergids and release of its contents i.e. sperms cells, the synergids start to disintegrate slowly (Plate – 7.5 a, b). The fusion of egg cell nucleus and one of the sperm cells results into the formation of zygote. The polar nuclei remain near together or closely adpressed for a long time and fuse to form the secondary nucleus just before fertilization. A considerable time elapses between fertilization and the first division of the zygote.

7.4 Zygote

The zygote is characterized by prominent nucleus, nucleolus and dense cytoplasm (Plate – 7.6 a). This feature indicates the zygote is the site of very active metabolism which includes synthesis of mRNA and protein. After fertilization, there is a formation of distinct cell wall around the zygote. The zygote of *P. wangianus* enlarges in size (Plate – 7.6 a) about ten times more than the egg cell after fertilization. This enlargement is due to the increase in the content of cytoplasm and nucleus of the zygote.

7.5 Formation of endosperm and embryo

The fusion product of two polar nuclei with the second male gamete or sperm cell constitutes the primary endosperm nucleus (PEN) (Plate – 7.6 b) which is triploid in nature located just below the zygote. The PEN shows precocious development than the zygote. It undergoes several mitotic divisions resulting into a nuclear condition; all nuclei are suspended in a cytoplasmic strand around the central vacuole and contain more than one nucleolus. Nucleolar fragmentation is of common occurrence. When the ovule is young, the endosperm is initially nuclear with two distinct highly folded haustoria in which several nuclei are suspended (Plate – 7.7 a, b). Subsequently, centripetal wall formation sets in and the endosperm becomes cellular (Plate – 7.6 c, d). The cells are large, polyhedral, thin-walled and vacuolated with prominent nuclei. At maturity they become packed with reserve food materials mainly starch and proteins in nature. The starch was localized with IKI (Iodine potassium iodide) and PAS reaction (Plate – 7.9 a, b) while the protein bodies were stained with mercuric bromophenol blue (Plate – 7.9 c), which gave positive reaction. As the ovule (seed) enlarges in size, the haustoria are

unfolded (Plate – 7.8 a, b) and absorb nutrients from the ovary wall. The endosperm is very large and occupied entire portion of the seed.

A characteristic feature has been observed in the endosperm in *P. wangianus* in which both the nuclear and cellular endosperm remains together in the mature seeds (Plate – 7.10 b). In *P. wangianus*, the cellular region of endosperm proper produces several haustoria to absorb nutrients (Plate – 7.10 a). The integuments (testa) are totally absorbed by the endosperm haustoria. Therefore, in *P. wangianus*, the seed coat is derived by the innermost layers of the locule by undergoing lignifications to protect the embryo and endosperm which is also a significant feature in *P. wangianus*.

The zygote begins to divide mitotically in a definite pattern and attained the globular shaped configuration (Plate – 7.11 a–d). The suspensor is seven to eight celled long at globular stage. 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 heart shaped (Plate – 7.12 a) which is the characteristics feature of the dicotyledons. There is accumulation of nucleic acids and protein in the cotyledonary primordia of heart-shaped embryo. The embryo is present

in the nuclear part of endosperm (Plate – 7.12 b, c) so that it can draw the nutrition easily. The embryo probably exerts some physical and physiological forces that prevent the nuclear endosperm to become cellularization.

7.6 Discussion

In *P. wangianus* ovary is inferior as similar in other members of the family Araliaceae except *Tetraplasandra gymnocarpa* where the ovary is completely superior (Costello and Motley 2004). It is 1-5 locular in *P. wangianus* while 4-5 locular in *P. fruticosa*, *Hedera australiana*, *Polyscias pinnata*, *Heptapleuram venulosum* and 10 locular in *Brassia actinophylla*. The ovary is 2-locular and each loculus contains 2 ovules in *P. ginseng*. The ovules are pendulous, anatropous and are found one in each loculus. In *H. australiana* in spite of the fact that four to five ovules are present only one or two develop further. In *Eleutherococcus senticosus*, an ovary has 5 loculi. Each ovary locule has 2 ovules; the upper ovule and the lower ovule. The upper one is orthotropous and degenerates after the formation of archesporial cell, while the lower one is anatropous, unitegmic and crassinucellar, and able to continue developing. It may be suggested that uni-ovulate condition is evolutionary advanced feature in *P. wangianus*. Therefore,

in *P. wangianus* the carpel is unilocular and uniovulate condition which is derived from the bi or multiovulate condition.

Shu (2007) reported all the species of *Panax* in China were two carpellate and bistigmatic except *P. japonicus* which has two to five carpels with two to five styles growing in China. Wen (2001) reported one to three carpellate conditions except in *P. trifolius*. Wen *et al.* (2001) mentioned pentacarpellate condition was an evolutionarily liable and developmental abnormality in Araliaceae. Philipson (1970a) and Eyde and Tseng (1971) also reported two to five carpellate is common in Araliaceae. The pluricarpellate condition (upto 20 carpels) is a primitive condition in Araliaceae. The American ginseng with one-styled flowers represents an intermediate step in the evolution of functional maleness. Asiatic ginseng is a functional hermaphrodite because all the individuals of *P. wangianus* in the population growing in Nongkrem sacred grove show one to five carpellate conditions.

According to Gopinath (1944) in *B. actinophylla*, *H. australiana*, *H. venulosum*, *P. fruticosa* and *P. pinnata*, a hypodermal archesporium differentiates which cuts off a parietal cell. However, in *Tieghemopanax sambucifolius* it appears that the archesporium directly functions as the megaspore mother cell. A single archesporial cell has

been observed in *P. wangianus* as similar in *P. ginseng* and *T. sambucifolius*. Double archesporial cells have also been reported in *B. actinophylla*, *H. australiana*, *H. venulosum* and *P. pinnata*. The occurrence of multiple archesporium is reported in many plants belonging to Rhizophoraceae, Nyssaceae, Onagraceae, Rosaceae, Compositae, Rubiaceae, Umbelliferae, Liliaceae and Cornaceae (Home 1914; Hakansson 1923; Bouman 1984; Jonsson 1881).

A well developed hypostase has been reported in *Nothopanax arboretum*, *P. fruticosum* and *P. pinnata* (Elizabeth and Pigott 1914; Gopinath 1944). However, in *H. nepalensis*, *T. sambucifolius* and *P. wangianus* such a structure is lacking (Singh 1954).

A funicular obturator is present in *N. arboretum*, *H. australiana*, *P. pinnata* and *T. sambucifolius* (Elizabeth and Pigott 1914; Gopinath 1944; Mohana Rao 1972). Singh (1954) observed some cells rich in protoplasmic contents in the funicular region near the micropyle and considered them to be the suppressed obturator initials in *H. nepalensis*. In *P. wangianus* also a prominent funicular obturator is formed. The obturator tissue also has been recorded in Euphorbiaceae, Labiatae and in Thymelaceae (Bushnell 1936; Murthy 1940; Kaushik 1940).

Most of the members of the Araliaceae (*T. sambucifolius*, *H. nepalensis*, *E. senticosus*, *P. ginseng* and *P. wangianus*) investigated so far showed Polygonum type of embryo sac (Ducamp 1901a, 1901b, 1902; Singh 1954; Mohana Rao 1972; Zhuravlev *et al.* 2008). According to Swamy and Krishnamurthy (1975), the development of embryo-sac formation in *P. wangianus* belongs to the supra-homeotypic (Polygonum type) category. However, Gopinath (1944) noticed bisporic development in *P. pinnata* and *H. australiana* whereas in *B. actinophylla* there was a tendency toward the *Oenothera* type of development. Reversed polarity in the embryo sac of *H. venulosum* has been noticed by Gopinath (1943).

In *P. wangianus* both synergids are globular to ovoid in structure while in *H. venulosum* the synergids are pear-shaped in the initial stages and develop beaklike structures later. In *H. australiana* the synergids are pointed at both ends. At the micropylar pole the cell wall is strongly thickened, forming the FA in *P. wangianus* and *P. japonicus* (Zhengyuan and Ruiling 1993). The morphology of the filiform apparatus is highly variable among species. In *Torenia* (Van der Pluym 1964) it is spherical; in *Petunia* (Went 1970) and *Helianthus* (Newcomb and Steeves 1971) it is wedge-shaped while in *P.*

wangianus it is lunar or sickle shaped. Filiform apparatus facilitates the transport of nutrients into the synergid cells, pollen tube reception, chemotropic attraction of the pollen tube towards the embryo sac (Gunning and Pate 1969; Newcomb and Steeves 1971; Willemse and Went, 1984; Huang and Russell, 1992).

Three antipodal cells are located opposite the egg at the chalazal end of the embryo sac. The antipodal cells are uninucleate and ephemeral in *P. wangianus* as similar in *P. fruticosa*, *H. australiana* and *P. pinnata* (Mohana Rao 1972). The antipodal cells are involved in the transfer of nutrients from the nucellus to the central cell and serve as storage for the developing endosperm and embryo. These cells also produce and secrete growth-controlling substances which regulate the development of the endosperm (Willemse and Went, 1984).

The central cell is the largest cell in embryo sac and is highly vacuolated, sharing common walls with the egg apparatus and with the antipodal cells at the beginning of development. The central cell plays a critical role for the development of endosperm after triple fusion (Willemse and Went 1984).

The zygote show distinct polarity in its distribution of nucleus and organelles at the chalazal pole. The structure of zygote in *P.*

wangianus is similar to that of *Gossypium*, *Capsella* (Jensen 1964, 1968; Schulz and Jensen 1968; Cocucci and Jensen 1969). The cytoplasm, nucleus and nucleolus are deeply stained with Azure B staining. This feature indicates the zygote is the site of very active metabolism which includes synthesis of mRNA and protein in *P. wanganus*. After fertilization, there is a formation of distinct cell wall around the zygote. Jensen (1968), Mogensen and Sultan (1979) and Mogensen (1972) also reported the formation of complete PAS-positive cell wall around the zygote after fertilization in cotton, *Nicotiana* and *Quercus*. The zygote of *P. wanganus* enlarges in size about 10 times more than the egg cell after fertilization. This enlargement is due to the increase in the content of cytoplasm and nucleus of the zygote. It can be substantiated that to put the zygote into the nutritionally favourable medium i.e. endosperm. Moreover, the endosperm development is precocious in *P. wanganus*. Similar phenomenon was reported in *Datura stramonium* (Satina and Rietsema, 1959) *Cypripedium insigne* (Poddubnaya-Arnoldi, 1967) *Jasione Montana* (Erdelska and Klasova 1978) and some members of the family Lentibulariaceae, Orbachaceae and Scrophulariaceae.

The endosperm is first nuclear and later wall formation sets in *P. wangianus* as similar in other members of family Araliaceae viz. *P. fruticosa*, *P. pinnata*, *H. australiana*, *H. venulosum*, *B. actinophylla*, *T. sambucifolius*, *E. senticosus* and *P. ginseng* (Gopinath 1944; Mohana Rao 1972; Zhuravlev *et al.* 2008). In the majority of the angiospermous families, the endosperm, eventually, becomes cellular. In *Limnanthes* and *Oxyspora paniculata*, however, free-nuclear condition persists until the endosperm is almost completely consumed by the developing embryo (Mathur 1956; Subramanyam 1951).

However, in a few families Cruciferae, Cucurbitaceae, Fabaceae and Proteaceae, wall formation is no doubt initiated but does not proceed beyond the central region. Thus, the central chalazal region remains free-nuclear. Since the cellular region grows further by cell divisions and the nuclear region by free nuclear divisions, the distinction between the two regions is maintained for some time. Cell formation in the nuclear region commences after the cellular part has attained the maximum dimensions (Vijayaraghavan and Prabhakar 1984). A characteristic feature has been observed in the endosperm in *P. wangianus* in which both the nuclear and cellular endosperm remains together in the mature seeds.

In *P. wangianus*, the cellular region of endosperm proper produces several haustoria to absorb nutrients as similar in *Buckinghamia* and *Banksia* (Rao 1967). The integuments (testa) are totally absorbed by the endosperm haustoria during its development. Therefore, in *P. wangianus*, the seed coat is derived by the innermost layers of the locule by undergoing lignifications to protect the embryo and endosperm which is also a significant feature in *P. wangianus*.

The zygote begins to divide mitotically in a definite pattern and attained the globular shaped configuration. The suspensor is seven to eight celled at globular stage in *P. wangianus*. While in *Pedicularis* it is made up of 20 cells. The suspensor plays a very dynamic role in nourishing the embryo proper at specific developmental stages. 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 heart shaped which is the characteristics feature of the dicotyledons. There is accumulation of nucleic acids and protein in the cotyledonary loci of heart-shaped embryo (Schulz and Jensen 1968). The embryo is always located in the nuclear part of endosperm so that it can draw the nutrition easily. The embryo probably exerts some

physical and physiological forces that prevent the nuclear endosperm to become cellularization in *P. wangianus*. I could not trace the ontogeny of the proembryo development. However, the embryo development is of Solanad type in *E. senticosus* (Lin-De *et al.* 1998). Johansen (1950) recorded Onagrad type of the embryogeny in *Aralia racemosa*, *A. cachemiria*, *A. cordata*, *Fatsia japonica* and *Hedera helix*; the embryogeny confirms to the Chenopodiad type in *P. fruticosa* and *H. australiana* (Mohana Rao 1972).

Plate – 7.1

- a A single pendulous ovule (OV) in each carpel (CP). (FU = funiculus). Bar = 70 μm .**
- b Cross section of ovule showing the tenuinucellus, single integument (IN) and part of the obturator (OB). Note the protandrous condition of stamen. (NU = Nucellus). Bar = 72 μm .**
- c, d Secretory canals (SC) in the ovary wall (OW) as well as in integument (IN) where the ginsenosides are synthesized. Bar = 50 μm .**

Plate – 7.1

***Panax wangianus* S. C. Sun**



A single pendulous ovule in each carpel



Ovule showing the nucellus, integument and obturator

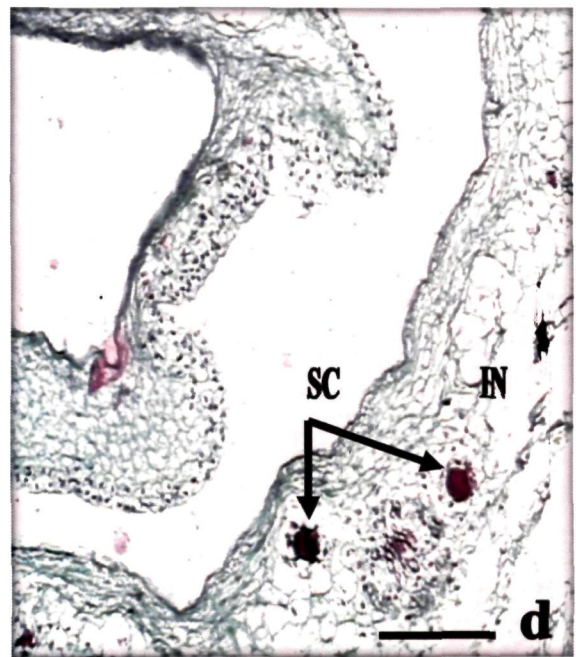


Plate – 7.2

- a** **Differentiation of integument (IN) and nucellus (NU) in the ovule primordium. Bar = 45 μ m.**

- b** **Because of overgrowth in the chalazal region the ovule becomes anatropous. (IN = Integument; NU = nucellus; FU = funiculus and OB = obturator). Bar = 50 μ m.**

- c** **T.S. of ovule with archesporial initial (AI) and integumentary initial (II). Bar = 40 μ m.**

- d** **An enlarge view of archesporial initial (AI) and integumentary initial (II). Bar = 40 μ m.**

Plate – 7.2

Panax wangianus S. C. Sun

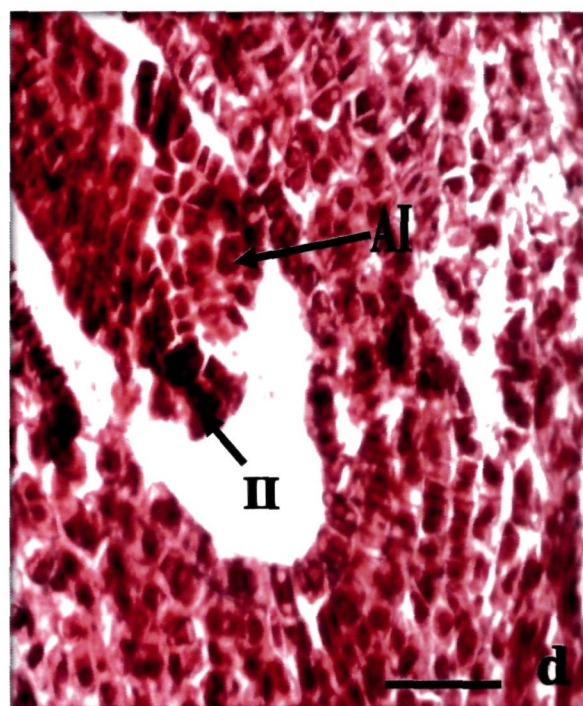
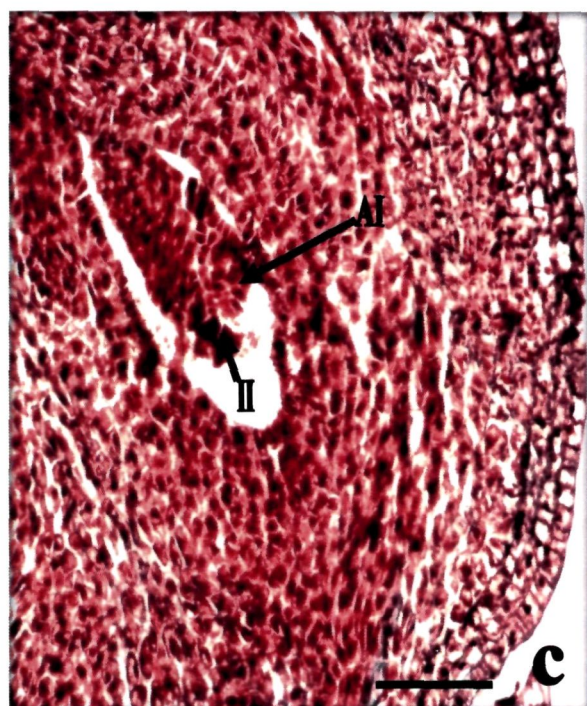
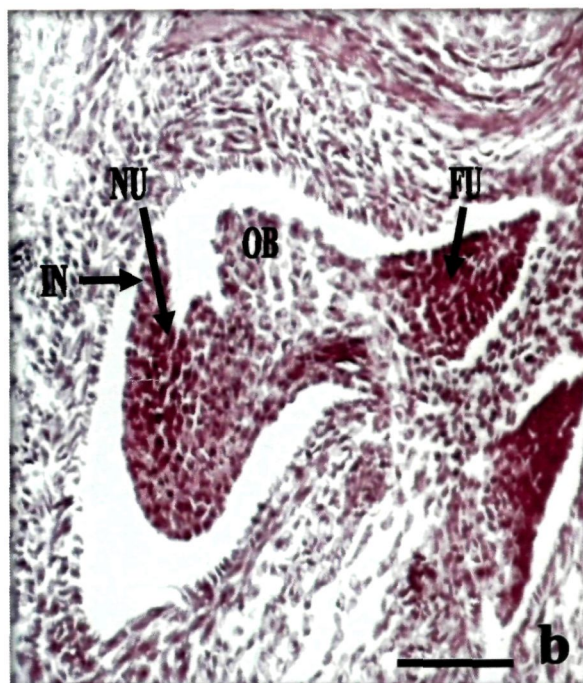


Plate – 7.3

- a Embryo sac with two synergids (SY). Note the polarity in the location of nucleus and cytoplasm in the synergids. Bar = 55 μm .**
- b Embryo sac with an egg cell (EC) at micropylar end. Note the polarity in the location of nucleus and cytoplasm in the egg cell. Arrow indicates antipodal cells at chalazal end. Bar = 55 μm .**
- c An enlarge view of chalazal region showing antipodal cells (AC). Bar = 20 μm .**

Plate – 7.3

Panax wangianus S. C. Sun

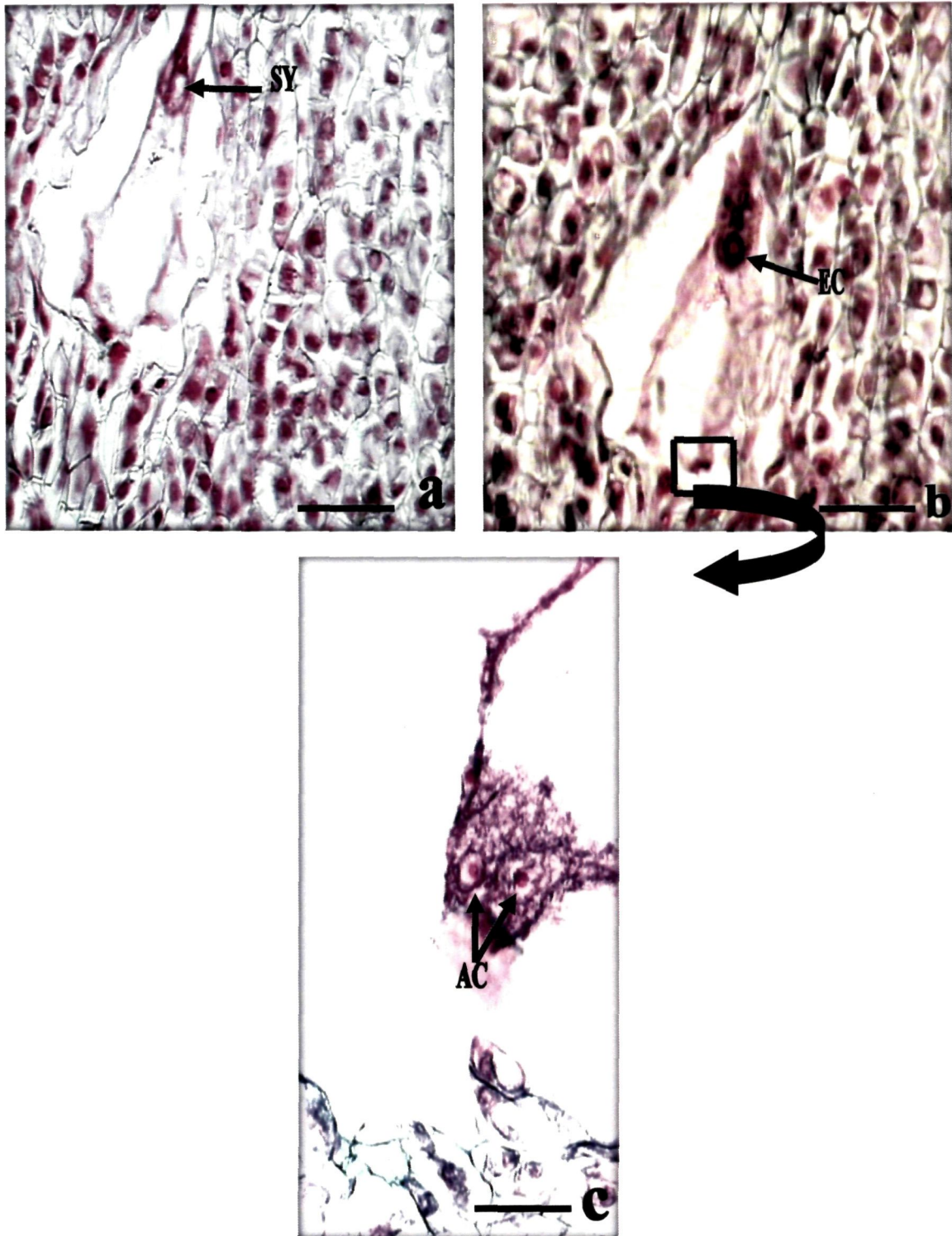


Plate – 7.4

- a** The embryo sac haustoria (EHS) absorb the nutrients from the integuments (IN). (DIN = part of the digestive integument; MP = micropyle and OB = obturator). Bar = 55 μm .
- b** An enlarge view of embryo sac haustoria. Note two synergids (SY) at the micropylar end with lunar- shaped filiform apparatus (FA). Bar = 55 μm .

Plate – 7.4

Panax wangianus S. C. Sun

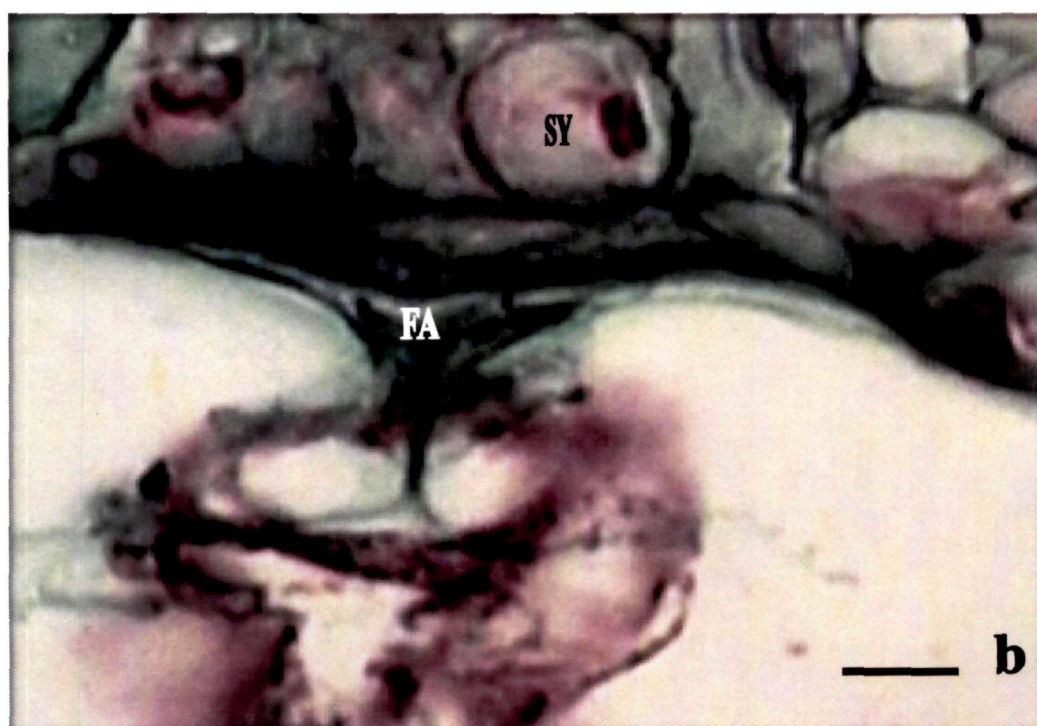
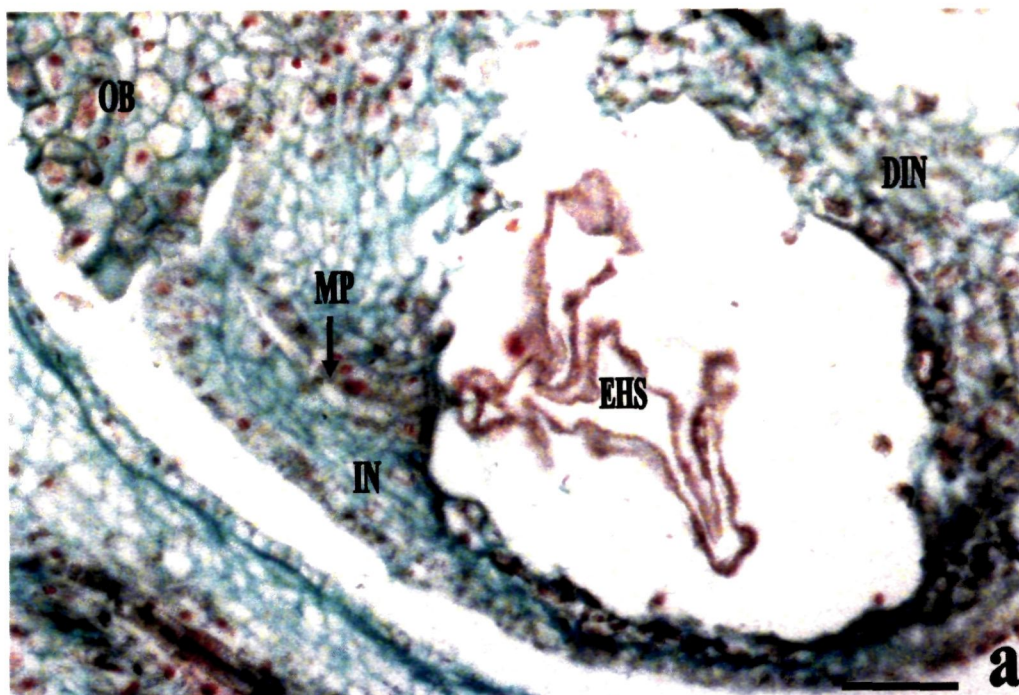


Plate – 7.5

- a** Embryo sac showing degenerated synergids after the entry of pollen tube in both the synergids. Bar = 60 μm .
- b** An enlarge view of degenerated synergid (DS). (PT = Pollen tube; PN = polar nuclei and ECN = egg cell nucleus). Bar = 60 μm .

Plate – 7.5

Panax wangianus S. C. Sun

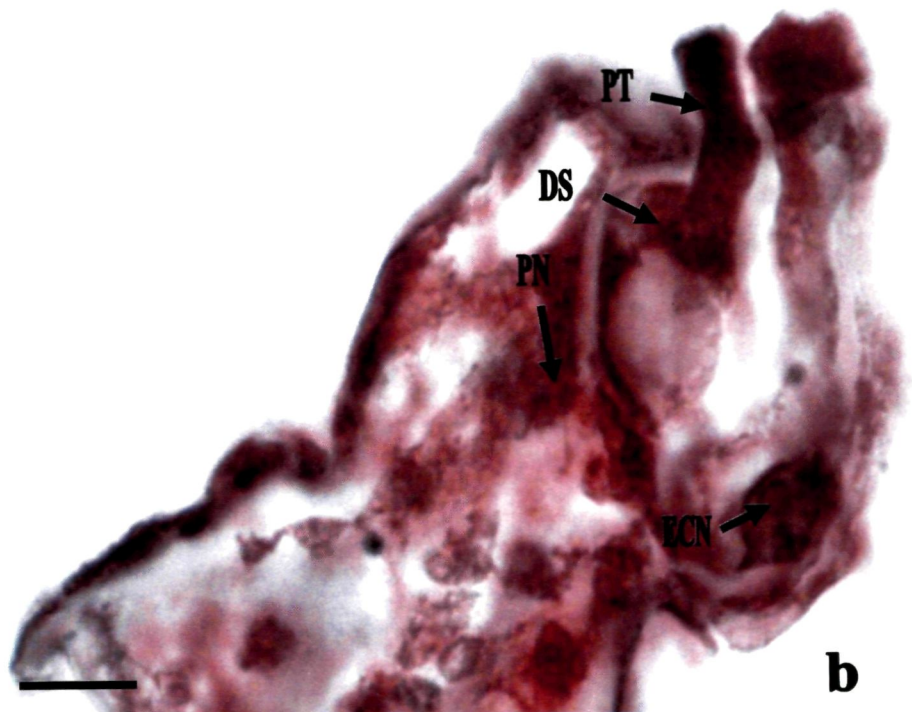
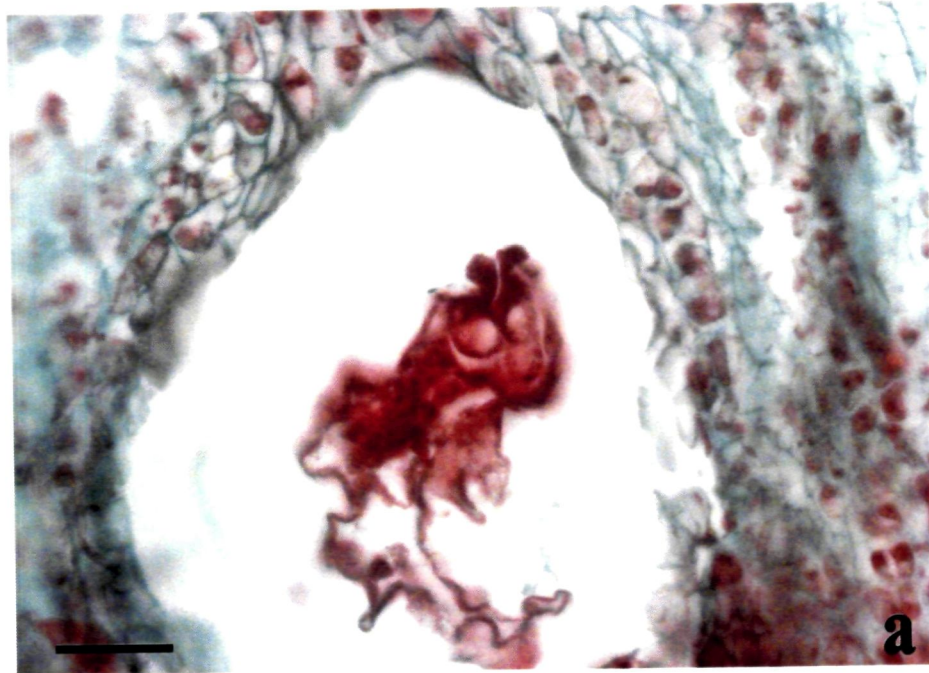


Plate – 7.6

- a** **An enlarge view of zygote with prominently stained nucleus and nuceolus as well as dense cytoplasm. Note increase in size of the zygote after fertilization. Bar = 50 μm .**
- b** **An enlarge view of primary endosperm nucleus (PEN). Bar = 48 μm .**
- c ,d** **Longitudinal section of ovule showing the cellularization of endosperm. (CE = Cellular endosperm and OV = ovule). Bar; c = 55 μm ; d = 60 μm .**

Plate – 7.6

Panax wangianus S. C. Sun

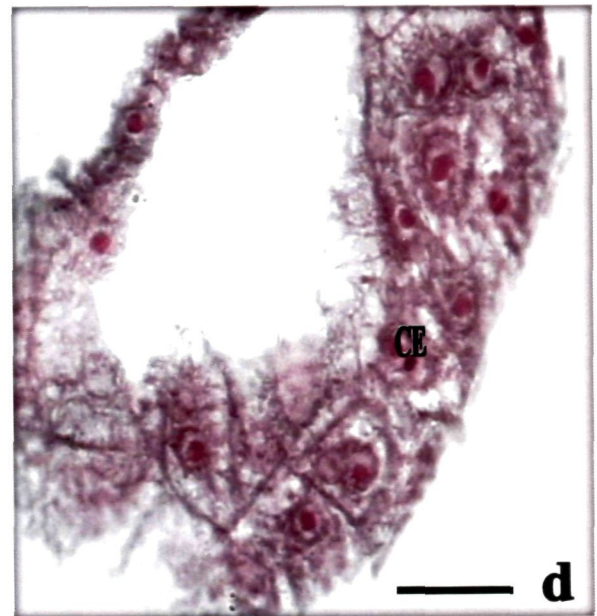
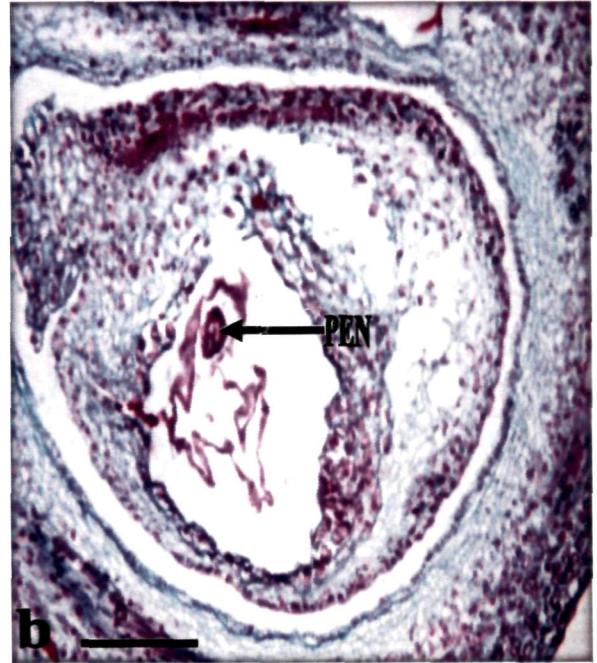
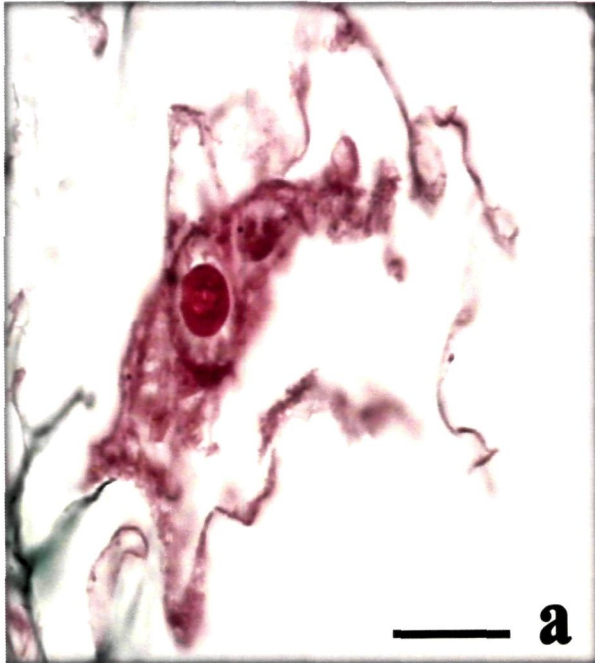


Plate – 7.7

- a** Longitudinal section of ovule (OV) showing the differentiating endosperm proper with two highly folded haustoria (HS). (CE = cellular endosperm). Bar = 60 μm .
- b** An enlarged view of haustorium with several nuclei suspended within the haustorium. Bar = 40 μm .

Plate – 7.7

Panax wangianus S. C. Sun

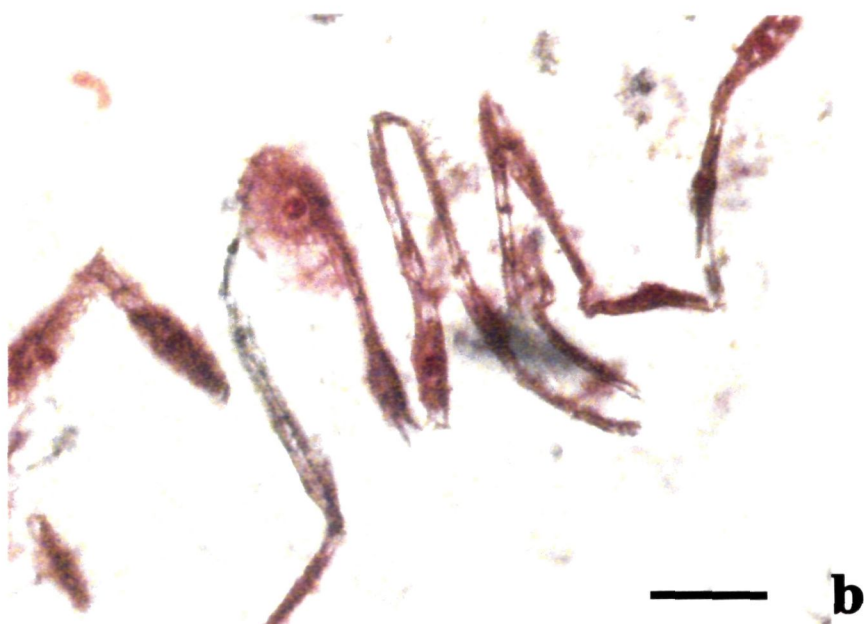
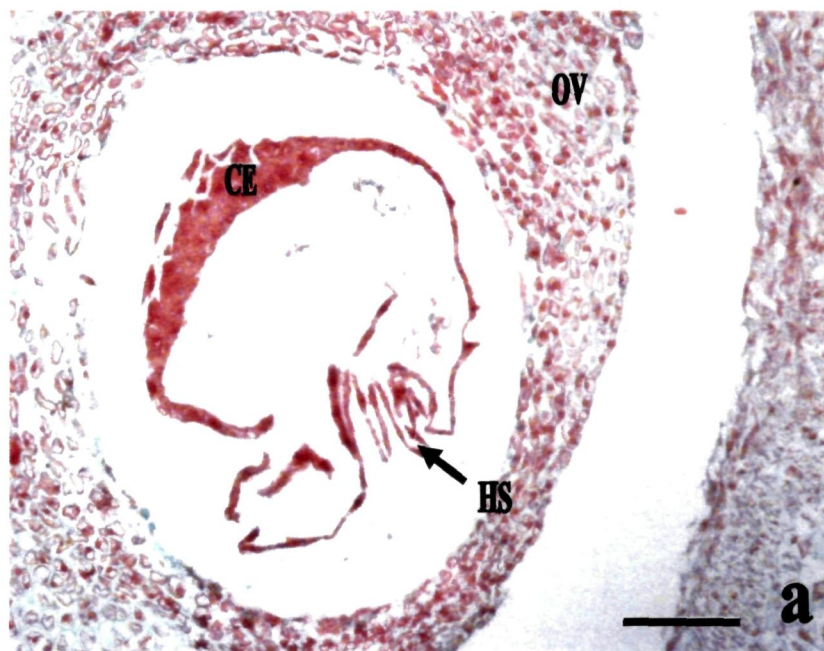
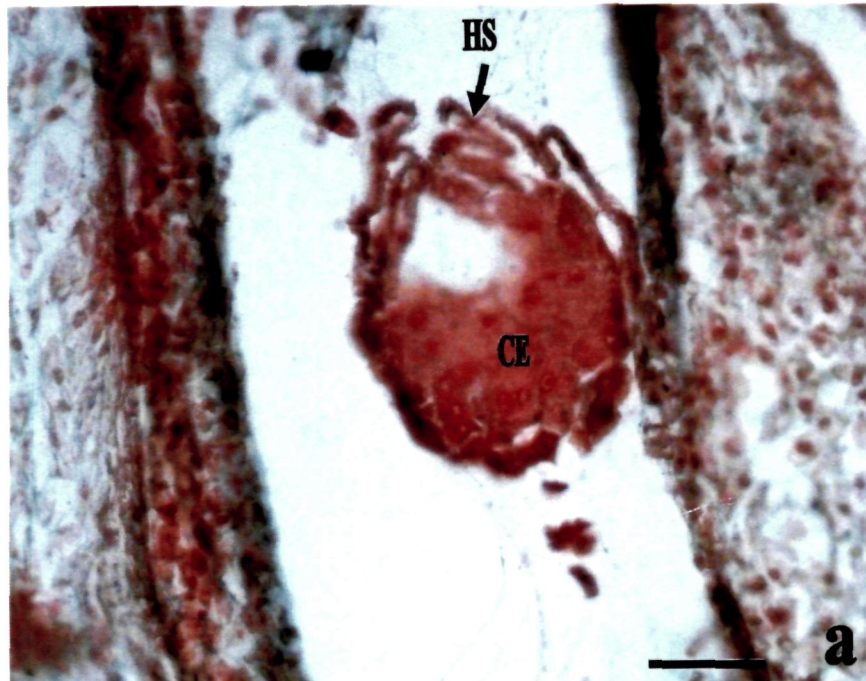


Plate – 7.8

a, b Endosperm with two unfolded haustoria (HS) on its either sides. As the ovule (seed) enlarges the haustoria are unfolded and absorb nutrients from the ovary wall. (CE = cellular endosperm). Bar = 50 μ m.

Plate – 7.8

Panax wangianus S. C. Sun



Endosperm proper with two unfolded haustoria on its either sides

Plate – 7.9

- a, b** Endosperm proper with starch grains after PAS staining in the cellular endosperm. Bar = 30 μm .
- c** Endosperm proper with protein bodies after staining with mercuric bromophenol blue in the cellular endosperm. Bar = 30 μm .

Plate – 7.9

Panax wangianus S. C. Sun

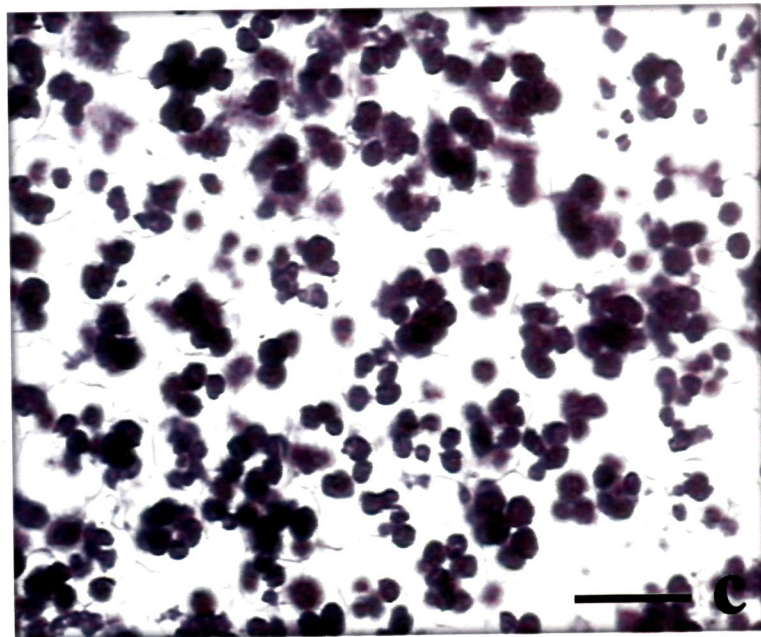
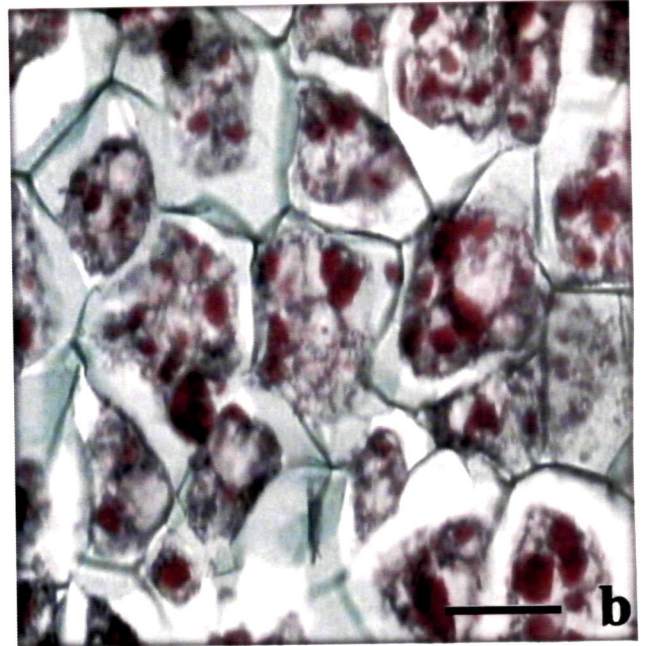
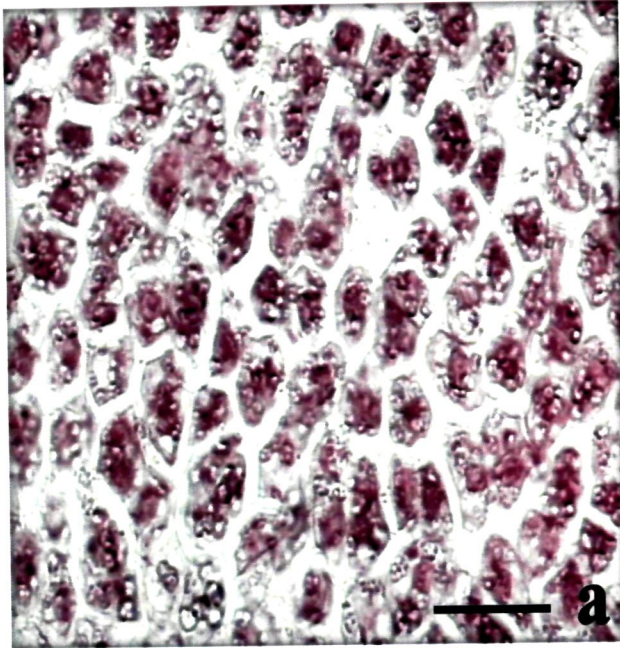


Plate – 7.10

- a** **A portion of cellular endosperm showing the haustoria (HS) in the cellular region. (CE = cellular endosperm). Bar = 50 μm .**

- b** **Mature endosperm, characteristic of both the nuclear and cellular features. (NE = Nuclear endosperm and CE = cellular endosperm). Bar = 50 μm .**

Plate – 7.10

Panax wangianus S. C. Sun

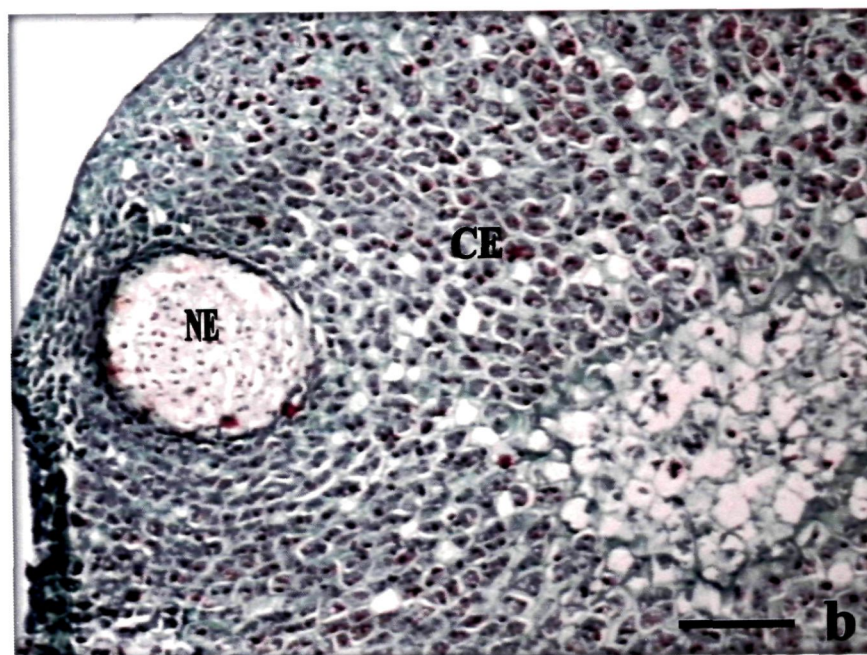
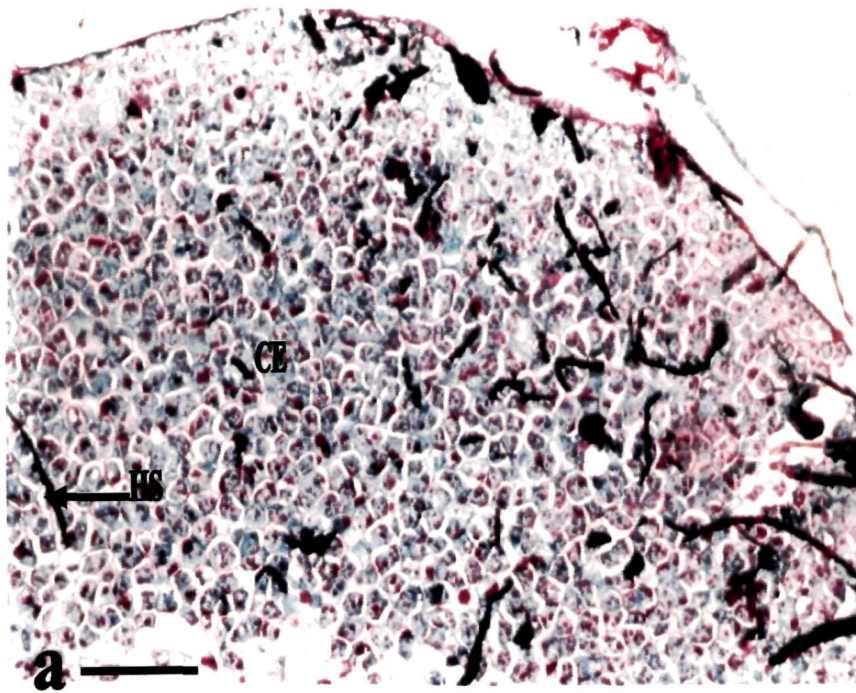


Plate – 7.11

a, b, c, d **Different stages of embryo development within the nuclear endosperm (NE). (CE = Cellular endosperm; EM = embryo; GEM = globular embryo and SP = suspensor). Bar; a = 50 μm ; b = 55 μm ; c = 65 μm and d = 70 μm .**

Plate – 7.11

Panax wangianus S. C. Sun

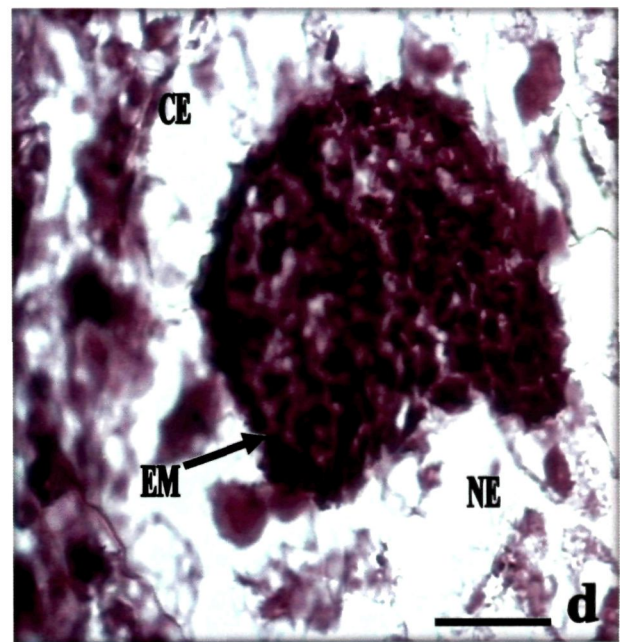
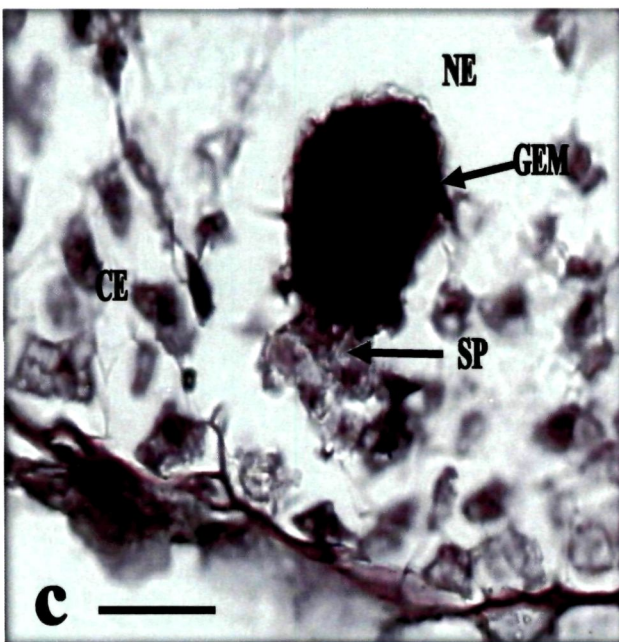
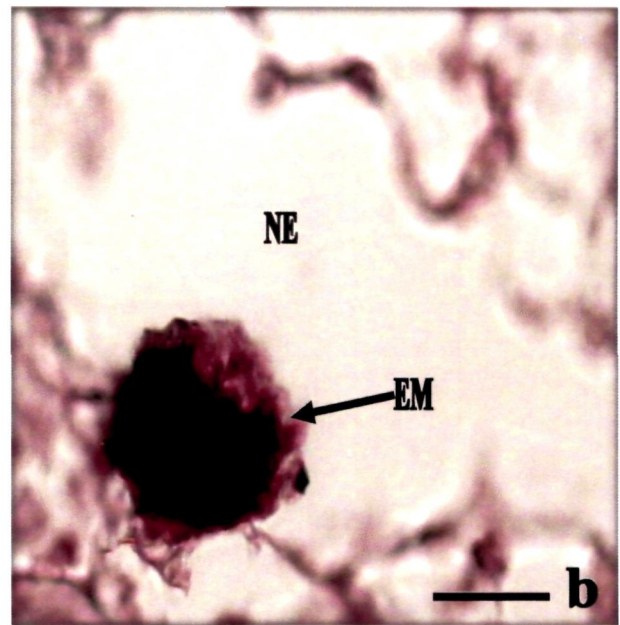
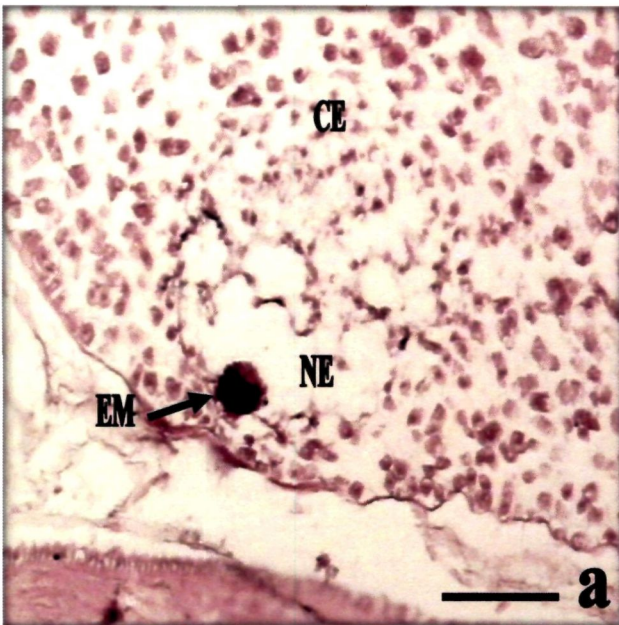
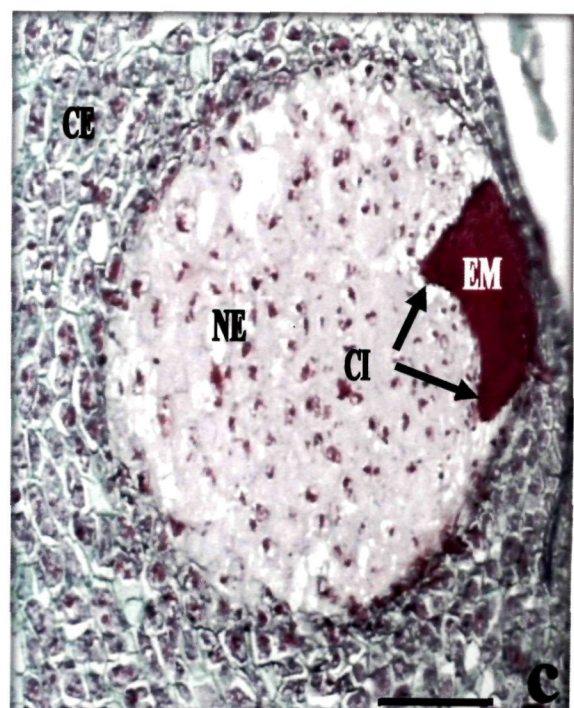
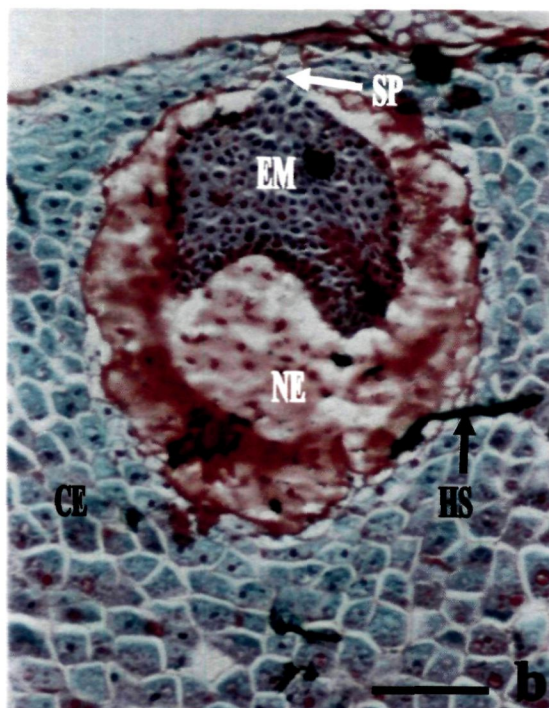
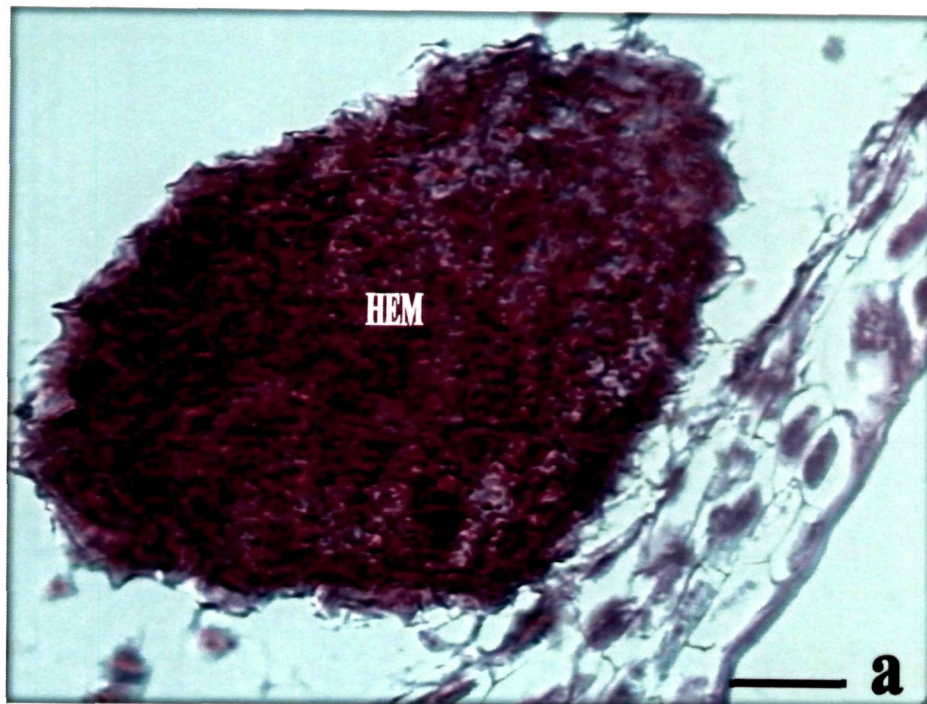


Plate – 7.12

- a** **Showing Heart-shaped embryo (HEM). Bar = 70 μ m.**
- b, c** **Longitudinal section of seed showing endosperm with both the nuclear and cellular nature. Note the embryo is present in the nuclear part of endosperm. (NE = nuclear endosperm; CE = cellular endosperm; HS = haustoria; EM = embryo; SP = suspensor; CI = cotyledonary initials). Bar = 65 μ m.**

Plate – 7.12

Panax wangianus S. C. Sun



Chapter-8

Seed Germination in *Panax wangianus*

8.1 Introduction

The seed functions as the reproductive unit of the Spermatophyta (seed plants), and links the successive generations. It is the dispersal unit of the plant, which is able to survive the period between seed maturation and the establishment of the next generation as a seedling after it has germinated (Bentsink and Koornneef 2008). Seed germination is the resumption of active growth of the embryo that results in the rupture of the seed coat and the emergence of the young plant (Copeland 1976). Germination begins with water uptake by the inactive, dry seed causing an abrupt renewal of metabolic activity. This results in embryo expansion, elongation of the radicle, and is concluded when the radicle punctures the surrounding tissues.

Seed germination depends on both internal and external conditions. The most important external factors include temperature, water and oxygen (Ungar 1995; Huang *et al.* 2003; El-Keblawy and

Al-Rawai 2005, 2006; Gorai and Neffati 2007) though light is not usually essential e.g. *Metrosideros excels* (Gabriele *et al.* 1999), *Carmichaelia* sp. (Ingrid and Heenan 2001), it may be required for some kind of seed e.g. *Lactuca sativa* (Agarwal and Dadlani 1992), *Dysoxylum spectabile* and *Alseusmia macrophylla* (Burrows 1999). 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).

Seeds of most *Panax* species are known to exhibit morphophysiological dormancy (Baskin *et al.* 1995), a type of seed dormancy where warm-cold sequence of seasonal temperature changes are needed to complete germination process. Thus, seeds of *Panax* species are known to have a complicated germination process and both the warm and cold stratification may take up to 18 to 22 months to fully germinate (Xiao *et al.* 1987; Anderson *et al.* 1993, 2002). This type of seed dormancy has been reported in *P. quinquefolius* (Anderson *et al.* 1993, 2002; Baskin *et al.* 1995; Lewis and Zenger 1982; Charron and Gagnon 1991; Schlessman 1985; Hovius 1996; Stoltz and Synder 1985; Yu and Kim 1992; Proctor and Bailey 1987; Xiao *et al.* 1987),

P. ginseng (Grushvitzky 1956; Li *et al.* 2000; Li 2001; Kim *et al.* 2008) and *P. stipuleanatus* (Chen *et al.* 2010). The seeds of *Aralia nudicaulis* also shows seed dormancy which can be overcome by three months of stratification at 5°C temperature (Blum 1974; Dirr and Heuser 1987). While in *Polyscias kikuyuensis*, there is no seed dormancy and seed germinates within 35-45 days (Rojo 1999).

It is well known that, exogenous applications of gibberellic acid (GA₃) had enhanced the germination of seeds (Wareing and Philips 1983; Taiz and Zeiger 1991; El-Bakkosh 2001; El-Barghathi and El-Bakosh 2005). 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 1991). Not much information are available on the seed viability of *Panax wangianus* distributed in Meghalaya, an attempt was made to find out the percentage of seed viability by germination test which may be helpful in seed quality programme.

8.2 Results

The fruits of *P. wangianus* are berry. Young fruits were green in color. As fruit mature, it became bright red at the bottom and black on

the top. In *P. wangianus* fruits are matured centripetally in umbel inflorescence. The color of pedicels also changed from green to crimson red. The number of fruits produced in *P. wangianus* was ranged from 1–30 per inflorescence. These fruits have 1–4 seeds or less frequently 5 seeds. In *P. wangianus*, seeds are bean-shaped, more or less globular in outline with minute strations (Plate – 8.1 a), 6-6.8 mm long, 4.5-5 mm wide and 3 mm thick. The percentage of one-seeded fruit is 26%; two-seeded fruit 31%, three-seeded fruit 22%, four-seeded fruit 12% and five-seeded fruits are 9% in the population of 2016 individuals. But the ability to produce flowers that mature into fruit increased with the age of the plant.

In *P. wangianus* seed germination were started after 17 days in the soil at 25°C temperature (Plate – 8.1 b) and the seedling emergence with two cotyledonary leaves was observed after 40 days (Plate – 8.2 a). The development of mature seedling with first leaves occurred after 52 days (Plate – 8.2 b). After 90 days of seeds sown, one-pronged plants grew in the soil collected from the natural condition (Plate – 8.2 c). The percentage of seed germination was 55% (Table 8.1). Seed germination is hypogeal. Whereas, seeds were germinated after 19 days in soil at 15°C temperature in BOD at 60%–80% relative

humidity and the fully developed seedlings were observed after 62 days. The percentage of seed germination was 20% in above mentioned experimental setup. The temperature at 4°C in BOD did not show any seed germination in *P. wanganus* (Table 8.1).

The percentage of seed germination would be increased by 0.1 % GA₃ treatment and lime after water soaking for 24 hours followed by scarification. After treating with GA₃, the emergence of radicle started after 15 days at 25°C temperature and seedling emergence observed after 48 days. The percentage of germination was 60% in above mentioned experiment setup. Whereas, seed germination took 12 days in lime mixed soil and seedling emergence was observed after 42 days at 25°C. The seed germination capacity increased upto 64% in lime mixed soil. The emergence of radicle from seeds was noticed five days delayed in soil (without any treatment) when compared to lime mixed soil. In otherwords, the seed germination in lime mixed soil was started 5 days before the soil one (Table 8.1). Lime mixed soil reduced the period of seed germination in *P. wanganus*.

Therefore, temperature and relative humidity plays an important role for seed germination in *P. wanganus*. Temperature between 15°C– 25°C and relative humidity 60% to 80% would be favorable for

the germination of this species. The highest germination percentage was obtained in lime mixed soil at 25°C. However, low temperature and sand did not show seed germination in *P. wangianus* (Table 8.1).

The moisture content of the seed was ~55%. The seeds were found to be recalcitrant in nature. The viability of seeds last only for two to three days after the removal of fleshy, fibrous fruit pulp. However, when the fruit pulp is intact, the seed viability was found to last for approximately seven to ten days. This was quite obvious that as long as the fruit pulp retains moisture, the seed viability persist. Once the outer moisture content dries off, the endosperm begins to shrink and eventually both embryo and endosperm losses their water content and became nonviable. Nevertheless, continuous soaking of seeds for more than two days in water also found deleterious to seed germination. It is therefore, presumed that fresher the seeds are, the more the rate of germination in *P. wangianus*.

Fruits of *P. wangianus* either eaten by birds or abscise staying near the parent plant (i.e. gravity seed dispersal). Seedlings were found to be clustered within a meter or less of the parent plant and fruits dispersed less than 50 cm or < 1 m from the parent plant because after senescence, aerial stem bent naturally.

8.3 Discussion

P. wangianus takes approximately 16 years to reach flowering and seed-producing stage (Chapter-2). Seed production in *P. wangianus* is positively correlated with age and size of the plant as similar in *P. quinquefolius* (Carpenter and Cottam 1982; Lewis and Zenger 1983; Schlessman 1985; Venugopal and Ahuja 2011). In *P. wangianus* mature fruits are bright red on the bottom and black on the top which is similar to *P. japonicus*, *P. vietnamensis*, *P. stipuleanatus* and *P. assamicus* whereas, in other species of *Panax*, mature fruits are completely red in color e.g. *P. ginseng*, *P. quinquefolius*, *P. notoginseng* and *P. pseudoginseng* except yellow color fruits in *P. trifolius* and black color fruits in *P. zingiberensis* (Wen 2001; Basnet and Dey 2008; Shu 2007; Chen *et al.* 2010). In *P. wangianus* fruits are developed in the outermost flowers of the umbel inflorescence because anthesis and pollination was affected earlier in outermost flowers. The position of developing flowers within the inflorescence affects fruit development (Bawa and Webb 1984).

In *P. wangianus*, seeds are bean-shaped, 6-6.8 mm long, 4.5-5 mm wide, 3 mm thick while in other species of *Panax* it is kidney-shaped; ovately round or ovately subglobose, 2.5-7.5 mm long, 2.0-6.5

mm wide and 1.5-6 mm thick. The single fruit contained 1-5 seeds in *P. wangianus* while 1-4 seeds in *P. quinquefolium* and *P. assamicus* and 1-3 seeds in *P. trifolium* and in *P. stipuleanatus* (Stoltz and Garland 1980; Basnet and Dey 2008; Schlessman 1985; Chen *et al.* 2010). In *P. wangianus* the percentage of one-seeded fruit is 26%, two-seeded fruit is 31%, three seeded fruit is 22%, four-seeded fruit is 12% and five seeded fruits are 9%. Stoltz and Garland (1980) also reported the percentage of one-seeded (16.3%), two-seeded (77.0%), three-seeded (6.5%) and four-seeded (0.2%) fruits in *P. quinquefolium*. Schlessman (1985) reported that flowers containing one, two and three ovaries produced one, two and three-seeded fruit, respectively in *P. trifolium*. In *P. stipuleanatus*, the proportion of mono-seed type, bi-seeds type and triple-seeds type is 9.53%, 58.66%, 1.81%, respectively (Chen *et al.* 2010). The percentage of single seeded fruits is 55%; two seeded fruits 25%, three seeded 18% and four seeded fruits 2% in *P. assamicus* (Basnet and Dey 2008).

Although *P. wangianus* has a life cycle pattern in respect to phenology, pollination, breeding behaviour and seed dispersal mechanism similar to other members of the genus *Panax* (Koren *et al.* 1998; Carpenter and Cottam 1982; Lewis and Zenger 1982, 1983;

Schlessman 1985; Duke 1980a, b; Catling and Spicer 1995). But germination test of seeds of *P. wangianus* revealed that there is no seed dormancy. While the seeds of *P. quinquefolius*, *P. ginseng* and *P. stipuleanatus* exhibited morpho-physiological dormancy (Anderson *et al.* 1993, 2002; Baskin *et al.* 1995; Charron and Gagnon 1991; Chen *et al.* 2010; Grushvitzky 1956; Kim *et al.* 2008; Lewis and Zenger 1982; Li *et al.* 2000; Li 2001; Proctor and Bailey 1987; Xiao *et al.* 1987).

The seed germination in *P. wangianus* starts after 17 days in the soil at 25°C and it takes 19 days at 15°C (Table 8.1) while in other species of *Panax* seed germination varies with respect to dormancy periods. For e.g. in *P. stipuleanatus* seed germination starts after 6 months and 15 days and in *P. assamicus* it takes 226.25 days (Chen *et al.* 2010; Basnet and Dey 2008). *P. ginseng* and *P. quinquefolius* has required two-stage treatment to overcome endogenous seed dormancy. During the first stratification period under warm temperatures of 15°C to 20°C for three to five months (Jo *et al.* 1988), cotyledons, hypocotyls, radicles, and epicotyls become visible and the embryos continue to develop and reach a length of 3 to 3.5 mm (Yu and Kim 1992; Lewis and Zenger 1982; Schlessman 1985). Seeds with fully developed embryos need a second period at a cold temperature of 1°C

to 2°C for five to nine months to overcome endogenous dormancy (Proctor and Bailey 1987; Xiao *et al.* 1987; Anderson *et al.* 1993, 2002; Charron and Gagnon 1991). However, such type of dormancy has not been noticed in *P. wangianus*.

Effect of GA₃ was found to be significant on seed germination in *P. wangianus*. After treating with GA₃, the emergence of radicle started after 15 days at 25°C temperature and seedling emergence observed after 48 days. The percentage of germination was 60% in above mentioned experiment setup (Table 8.1). Similarly in *P. ginseng* the seeds treated with gibberellic acid shortens the length of the first stage of after-ripening period from four to two months; consequently, the whole period of preparation of the seeds for sowing is reduced from eight to six months (Grushvitzky and Limarj 1965; Baranov 1966; Hobson 1999).

In Nongkrem sacred grove, the soil type is oxisols and acidic (pH 5.5) in which exchangeable calcium and exchangeable magnesium concentration is 1.26 meq 100g⁻¹ and 4.24 meq 100g⁻¹ respectively (Arunachalam 2002). It means Ca⁺⁺ and Mg⁺⁺ play an important role in seed germination of *P. wangianus*. The germination percentage was slightly higher in lime mixed soil i.e. 64% at 25°C. Calcium limestone

(CaCO₃) also increases the soil pH and Ca⁺⁺ concentration which has been found to significantly increase the seed germination rate of *P. wangianus* and *P. quinquefolius* (Beyfuss 1998; Hankins 2000; Nadeau *et al.* 2003; Slak 2004). The seed germination capacity increased upto 64% in lime mixed soil in *P. wangianus* (Table 8.1). In *P. wangianus* seed germination does not occur in sand while *P. ginseng* and *P. assamicus* shows seed germination in sand (Kim *et al.* 2008; Basnet and Dey 2008).

Temperatures between 15–25°C seem to be favorable for the germination of this species. Germination was inhibited by either an increase or decrease in temperature from the range given above. The highest germination percentage was obtained in lime mixed soil at 25°C (Table 8.1). The moisture content of seeds of *P. wangianus* was 55% while in seeds of *P. ginseng* it was around 7.2%, which is lower than that of recalcitrant or intermediate seeds (Kim *et al.* 2008).

In *P. wangianus* seed dispersal generally occurred by birds because bright color of fruits attracts the birds and fruit pulp provides food to them. In larger plants (five-pronged and six-pronged) fruits of *P. wangianus* abscise staying near the parent plant i.e. gravity seed dispersal. Seedlings were found to be clustered within a meter or less

of the parent plant and fruits dispersed less than 50 cm or < 1 m from the parent plant because after senescence, aerial stem bent naturally. The same mechanism of seed dispersal has been found in *P. quinquefolius* (Cruse-Sanders and Hamrick 2004).

Table- 8.1 Germination of seeds of *Panax wangianus* under different conditions

Culturing method	Temperature	Total number of seeds/petridish	Days to start germination	Days to complete germination	Germination (%) after 107 days
Soil	25°C	50	17	52	55
Soil	15°C	50	19	62	20
Soil	4°C	50	0	0	0
Soil + lime	25 °C	50	12	42	64
Soil + lime	15°C	50	0	0	0
Soil + lime	4°C	50	0	0	0
Soil + GA3	25°C	50	15	48	60
Soil + GA3	15°C	50	0	0	0
Soil + GA3	4°C	50	0	0	0
Sand	25°C	50	0	0	0
Sand	15°C	50	0	0	0
Sand	4°C	50	0	0	0
Sand + GA3	25°C	50	0	0	0
Sand + GA3	15°C	50	0	0	0
Sand + GA3	4°C	50	0	0	0

Plate – 8.1

- a SEM photograph of a seed view opposite to the hilum region which is more or less globular in outline with minute strations in *P. wangianus*.**
- b Emergence of radicle from the seeds sowing after 17 days in the soil collected from the natural condition. Bar = 0.25 cm.**

Plate – 8.1

Panax wangianus S. C. Sun

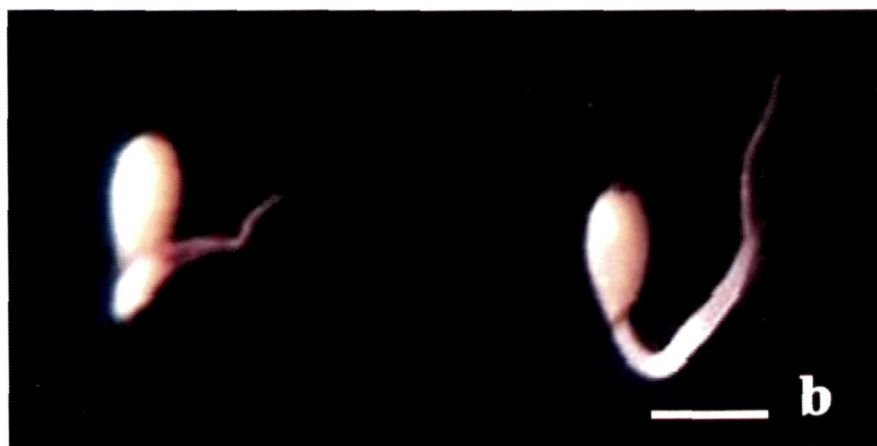
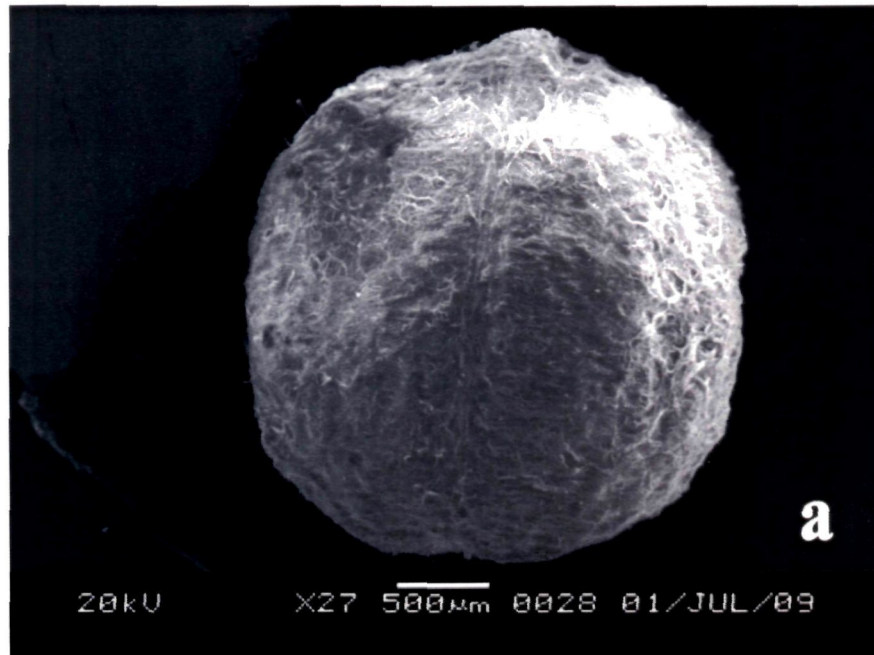


Plate – 8.2

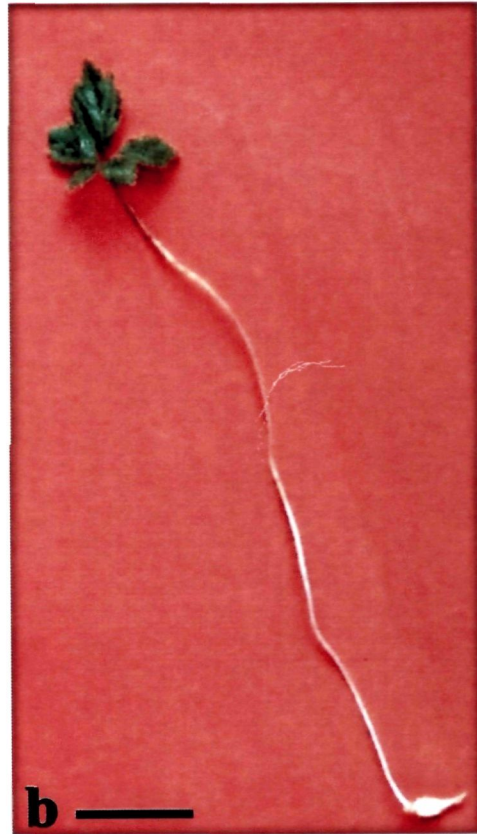
- a** A young 40 days old seedling with two cotyledonary leaves
(in the soil collected from the natural condition). Bar = 1.3
cm.

- b** A mature seedling of *P. wangianus* with first leaves after 52
days (in the soil collected from the natural condition).
Note the young minute tuber in the root. Bar = 2.0 cm.

- c** One-pronged plants after 90 days seed sowing in the soil
collected from the natural condition. Bar = 4.0 cm.

Plate – 8.2

Panax wangianus S. C. Sun



Chapter-9

Summary and Conclusion

Understanding the reproductive biology of species is essential to conservation efforts (Falk and Holsinger 1991; Anderson 1995), particularly of endangered species where there are only a very few populations with limited number of individuals. Therefore, in this thesis, reproductive biology of *Panax wangianus* S. C. Sun growing in North-East region of our country was studied with reference to the following aspects:

- ❖ To determine the interrelationship between the age, size, fecundity and climatic factors.
- ❖ To investigate breeding system and pollination mechanisms (It includes floral rewards, pollinators, pollen viability and pollen germination; stigma receptivity, pollen load on the stigma, ratio between pollen and ovule, number of viable seed in a fruit and seed dispersal).
- ❖ Developmental aspects of reproductive organs.
- ❖ Post-fertilization Development: endosperm and embryo formation.

❖ Seed germination.

P. wangianus (syn. *P. pseudoginseng*) S. C. Sun is a perennial, critically endangered, herb native to sub-tropical wet forests of North-East Himalayan regions especially in the Law Lyngdoh, Smit sacred grove in Nongkrem (east khasi hills) 25° 31' N, 91° 52' E at an altitude 1833 m AMSL, Law Lyngdoh (Mawphlang) 25° 26' N, 91° 44' E at an altitude 1796 m AMSL and Shillong peak 25° 32' N, 91° 49' E at an altitude 1965 m AMSL in Shillong, Meghalaya (Pushpangadan and Nair 2005). The sprouting of aerial shoot from the rhizome starts from March. Flowering was noticed in the months of April to May. Anthesis occurs in the month of June. Young fruits was observed in the month of July and August and matured during September to October. The dispersal of seeds took place in the end of October to early November.

P. wangianus had a whorl of digitately compound leaf at the summit of aerial shoot; the leaf (prong) consists of three to eight palmately compound leaflets with a petiole, of which the terminal three to five leaflets are larger than the lateral two to three leaflets. *P. wangianus* was classified into six different age classes based on the number of prongs. The umbel inflorescence consists of 13–75 flowers which arising from the centre of the leaf attachment at the top of the

which arising from the centre of the leaf attachment at the top of the aerial stem. Flowers are small, bisexual, odor-less, protandrous with five (or rarely four) calyx teeth, five petals, five stamens and an inferior ovary with one to five styles. Each ovary consists of one to five carpels which would be readily observed from the exterior by counting the number of furrows and ridges on the ovary. Each carpel has single pendulous, anatropous and unitegmic ovule. The stigma is dry with unicellular papillate outgrowths (Heslop-Harrison and Shivanna 1977). The number of stigmatic lobes ranges from one to five. The flowers have nectariferous epigynous disc which cover the ovary top and usually confluent with style bases. The length of stigma is 2 mm.

Pollen grains were tricolpate with reticulate sculpturing. The large cluster of pollen grains with viscin threads and pollenkit in *P. wangianus* is the first report for family Araliaceae. The mean number of pollen grains per flower was 1895, and the mean number of ovules was 3. Thus, the pollen to ovule ratio was 1895:3. Pollen viability by fluorochromatic reaction test confirmed that 43% of pollen grains were viable on the day of anthesis. Though 43% of viable pollen grains are available, sufficient viable pollens are not landed on the stigma through

visitors because of difference in height of anther and receptive surface of stigma as well as recurvature of stigma at 180°.

P. wangianus exhibits reverse herkogamy which enhances the chances of cross-pollination by the insects belonging to the order Diptera. The species has a mixed breeding system of autogamy and xenogamy, suggesting self-compatibility. Phenological status revealed that five and six-pronged plants contributed the most to flowering, fruiting and seed production through xenogamy and geitonogamy breeding mechanism.

The fruits are berry. Young fruits were green in color. As fruit mature, it became bright red on the bottom and black on the top. These fruits can have 1–4 seeds or less frequently 5 seeds. Fruits of *P. wangianus* abscise staying near the parent plant (i.e. gravity seed dispersal). The dispersal of seed is by animals (birds). Seed dispersal is passive.

The number of stamens in *P. wangianus* is five. The development of the five stamens is synchronous in *P. wangianus*. The anther is tetra-locular. The development of anther wall is dicotyledon type. The young anther wall comprises of an epidermis, endothecium,

two middle layers and a tapetum. In *P. wangianus*, a part of tapetum is derived from the single layer primary parietal cell, whereas another part is derived from the multilayered connective tissue which proves dual origin of tapetum. Tapetal cells are bi- and tetranucleate and sometimes contain polyploidy nuclei with dense cytoplasm and prominent nucleus and nucleolus. In *P. wangianus* meiosis in MMCs is accompanied by simultaneous cytokinesis which leads to the formation of tetrahedral and isobilateral tetrads.

Ovary is inferior with each locule uniovulate. Nectariferous epigynous disc cover the ovary top. The number of stigmatic lobes always corresponds to the number of carpels in each ovary. The number of carpels varied from one to five, which would be readily observed from the exterior by counting the number of lobes in fruits. The stigma is spatulate, dry, receptive surface hollow covered with unicellular papillate outgrowths. In *P. wangianus* the number of locules ranges from one to five in the gynoecium. Each locule contains single, pendulous and anatropous ovule. The ovules are unitegmic and tenuinucellate with distinct funicular obturator. The archesporial directly function as the megaspore mother cell. The development of the embryo sac confirms to the Polygonum or Normal type.

The zygote is characterized by prominent nucleus, nucleolus and dense cytoplasm. The PEN shows precocious development than the zygote. When the ovule is young, the endosperm is initially nuclear with two distinct highly folded haustoria in which several nuclei are suspended. Subsequently, centripetal wall formation sets in and the endosperm becomes cellular. A characteristic feature has been observed in the endosperm in *P. wangianus* in which both the nuclear and cellular endosperm remains together in the mature seeds. The embryo is present in the nuclear part of endosperm so that it can draw the nutrition easily. The embryo probably exerts some physical and physiological forces that prevent the nuclear endosperm to become cellularization.

The seeds are bean-shaped, more or less globular in outline with minute strations. Seeds of most *Panax* species are known to exhibit morpho-physiological dormancy (Baskin *et al.* 1995), a type of seed dormancy where warm-cold sequence of seasonal temperature changes are needed to complete germination process. Thus, seeds of *Panax* species are known to have a complicated germination process and both the warm and cold stratification may take up to 18 to 22 months to fully germinate (Xiao *et al.* 1987; Anderson *et al.* 1993, 2002). But

germination test of seeds of *P. wangianus* revealed that there is no seed dormancy. The seed germination in *P. wangianus* starts after 17 days in the soil at 25°C and the seedling emergence observed after 40 days. The percentage of seed germination was 55%. Effect of GA₃ was found to be significant on seed germination in *P. wangianus*. After treating with GA₃, the emergence of radicle started after 15 days at 25°C temperature and seedling emergence observed after 48 days. The percentage of germination was 60% in above mentioned experiment setup. The seed germination capacity increased up to 64% in lime mixed soil in *P. wangianus*.

Conclusions

- ❖ Morphological variations were observed in natural conditions on the basis of the number of prong and carpellate conditions.
- ❖ The color of flowers of *P. wangianus* also varied depending upon the sunlight intensity. Those plants grow in bright sunlight; the flowers would appear pinkish which has not been reported in other species of *Panax*. These pink colour flowers invite more pollinators than the greenish-white flowers produced by the shade growing plants.

- ❖ In Nongkrem sacred grove, only one to two percent one-pronged and two-pronged plants of *P. wangianus* exhibited neoteny (retention of ancestral juvenile characters by mature plants) and their age was above 40 years.
- ❖ Age class was significant to predict the size of the plant and its reproductive capacity.
- ❖ Phenological status revealed that larger plants (in terms of age class 35–50 years and above 50 years) are reproductively more successful due to its vegetative growth and flowering phenology which influences the more production of fruits and seed set in Nongkrem sacred grove. Therefore, in the view of conservation and management, the age class of 35–50 years and above 50 years is the most important for population sustainability.
- ❖ Statistical analysis revealed that a strong positive correlation was observed between the age versus vegetative and reproductive characters.
- ❖ Climatic factors such as temperature, precipitation and relative humidity show synergistic effect on both the vegetative and reproductive phases in *Panax wangianus* in the undisturbed Nongkrem sacred grove.

- ❖ The number of ovules are less when compare to viable pollen grains.
- ❖ Though 43% of viable pollen grains are available, sufficient viable pollens are not landed on the stigma through visitors.
- ❖ The frequency of pollinators visit is very low.
- ❖ The number of individuals in a population is also very low.
- ❖ It indicates that the number of visitation of pollinators and seed production is interlinked which in turn determines the population size.
- ❖ Plants of age class 35–50 years may facilitate the floral rewards to the pollinators.
- ❖ The large cluster of pollen grains with viscin threads and pollenkitt in *P. wangianus* is the first report for family Araliaceae.
- ❖ *P. wangianus* exhibits reverse herkogamy which enhances the chances of cross-pollination by the bees and flies belonging to family Halictidae and Syrphidae respectively.
- ❖ The species has a mixed breeding system of autogamy and xenogamy, suggesting self-compatibility.

- ❖ The development of anther wall is dicotyledon type. The young anther wall comprises of an epidermis, endothecium, two middle layers and a tapetum.
- ❖ In *P. wangianus*, a part of tapetum is derived from the single layer primary parietal cell, whereas another part is derived from the multilayered connective tissue which proves dual origin of tapetum.
- ❖ Tapetal cells are bi- and tetranucleate and sometimes contain polyploidy nuclei with dense cytoplasm and prominent nucleus and nucleolus.
- ❖ In *P. wangianus* meiosis in MMCs is accompanied by simultaneous cytokinesis which leads to the formation of tetrahedral and isobilateral tetrads.
- ❖ In *P. wangianus* invariably only one ovule is fertilized, out of three carpels, to form the seed.
- ❖ The Primary endosperm nucleus (PEN) shows precocious development than the zygote.
- ❖ The co-existence of nuclear and cellular type of endosperm in the mature seeds.

- ❖ The embryo is present in the nuclear part of endosperm so that it can draw the nutrition easily. The embryo probably exerts some physical and physiological forces that prevent the nuclear endosperm to become cellularization.
- ❖ Seed germination is hypogeal and the germination percentages ranged from 20% to 64%. It shows higher seed germination percentages when compared with field-based germination studies in the remaining *Panax* species.
- ❖ Our findings suggest that morpho-physiological dormancy in seeds may not be as widespread as previously reported within the genus *Panax*.

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Curriculum-Vitae

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Examination Passed	Division	% of marks	Subjects	Year	Board/ University
M.Sc.	Ist Div	68.21%	Botany	2005	C.C.S.University, Meerut
B.Sc.	Ist Div	61%	Botany, Chemistry, Zoology	2003	C.C.S.University, Meerut
Intermediate	IInd Div	59%	Hindi, English, Physics, Biology, Chemistry	2000	U.P.Board
High School	Ist Div	67%	Hindi, English, Maths, Science, S.Studies,Biology	1998	U.P.Board

Workshop and Trainings

- Participated in a training on **“Bioinformatics: A practical approach”** organized by North-Eastern Hill University, Shillong, during 19th-24th April, 2010.
- Participated in a workshop on **“Basic principles, preparatory methods and applications in Scanning electron**

microscope” organized by North-Eastern Hill University, Shillong, during 3rd – 5th Nov., 2009.

➤ Participated in a workshop on **“High performance computing”** organized by **Centre for Development of Advanced Computing (C-DAC), Pune, during 3rd-6th March, 2009.**

➤ Participated in a workshop on **“Capacity building training in plant taxonomy”** organized by **Botanical Survey of India, Eastern Circle, Shillong, during 3rd-15th Nov, 2008.**

➤ Passed 100 days certificate course on **“Medicinal and Aromatic Plants”** from **Central Institute of Medicinal and Aromatic Plants, Lucknow, India, during Feb 22nd - June 1st, 2007.**

➤ Passed a short term certificate course in computer software (MS Office, Photoshop, Web Designing, Internet, and HTML) from STG (Software Technology Group) in 2002.

Presentations and Conferences

➤ **Ahuja, P. and Venugopal, N., “Relationship between age, size and reproduction in *Panax wangianus* S. C. Sun (Araliaceae) an endangered medicinal plant in the sacred grove forest of Meghalaya, North-East India” in First Global Congress on plant reproductive biology held at Agra from 22nd – 24th October, 2010 (Poster Presentation).**

➤ **Ahuja, P. and Venugopal, N., “Allometric variation and reproduction in *Panax wangianus* S. C. Sun (Araliaceae) an endangered medicinal plant in the sacred grove forest of Meghalaya, North-East India” in National Conference on “Biodiversity for sustainable development: threats, indicators, climate change, poverty alleviation and targets for safeguarding biodiversity” held at Madurai Kamraj University, Madurai from 25th - 28th August, 2010 (Oral Presentation; got second prize).**

➤ **Ahuja P. and Venugopal N., “Pollinators, Pollen to ovule ratio, Pollen germination and Pollen viability in the endangered and medicinally important *Panax wangianus* S. C. Sun of North-East India” in the UPE-Bioscience Seminar on Assessment, Conservation and Value Addition of Biodiversity on 22nd - 23th April, 2010, held at NEHU, Shillong (Oral Presentation).**

- **Ahuja, P. and Venugopal, N.,** “Pollination mechanism, pollen viability and pollen germination in *Panax wangianus* S. C. Sun” in 97th Indian science congress held at University of Kerala, Thiruvananthapuram, Kerala from 3rd - 7th January, 2010 (**Oral Presentation**).
- **Ahuja, P. and Venugopal, N.,** “Reproductive biology of endemic and endangered medicinal plant *P. pseudo-ginseng*: differentiation of embryo sac, endosperm and pollen viability” in 96th Indian science congress held at North-Eastern Hill University, Shillong, Meghalaya from 3rd - 7th January, 2009 (**Poster Presentation**).
- **Ahuja, P. and Venugopal, N.,** “Reproductive biology of *Panax pseudoginseng*: organization of embryo sac and endosperm” in the “National Symposium on Frontiers in Biocomplexity and Biodiversity of Plants” held at North-Eastern Hill University, Shillong, Meghalaya from 14th - 15th March, 2008 (**Oral Presentation**).

Publications

- **Venugopal, N. and Ahuja P. (2011)** “Relationship between age, size, fecundity and climatic factors in *Panax wangianus* an endangered medicinal plant in the sacred grove forest of North-East India”. Journal of Forestry Research 22(3): 427–435.
- **Venugopal, N. and Ahuja P. (in Press)** “Allometric variation and reproduction in *Panax wangianus* S. C. Sun (Araliaceae) an endangered medicinal plant in the sacred grove forest of Meghalaya, North-East India”, In: Proceedings of the National Conference on “Biodiversity for sustainable development: threats, indicators, climate change, poverty alleviation and targets for safeguarding biodiversity”. Narosa publication, New Delhi.
- **Venugopal, N. and Ahuja P. (in Press)** “Phenology, Pollination and Breeding behavior of *Panax wangianus* S. C. Sun (Araliaceae) a vulnerable medicinal plant in the sub-tropical forest of Meghalaya, a North-East India”. In: Proceedings of the National Seminar on Tropical Ecosystems: Structure, Function and Services (TESFS-2010). Published by Institute of Forest Genetics and Tree Breeding, Coimbatore.
- **Venugopal, N., Ahuja P. and Lalchhanhimi (communicated)** “A unique type of endosperm in *Panax wangianus* S. C. Sun”. Current Science.

Relationship between age, size, fecundity and climatic factors in *Panax wangianus* an endangered medicinal plant in the sacred grove forest of North-East India

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Abstract: *Panax wangianus* (Syn. *Panax pseudoginseng*) S. C. Sun (Araliaceae) is a critically endangered, medicinal plant of North-East India. The objective of this study was to determine how plant size affects flowering phenology and to evaluate the effect of climatic factors on flowering, fruiting and seed production. Data on vegetative and reproductive characters were monitored from 2016 individuals of *Panax wangianus* population in Law Lyngdoh, Smit sacred grove in Nongkrem, Shillong, India. Leaflet area was measured by a planimeter. Size variables of both vegetative and reproductive traits in different age classes were measured. Climatic factors were recorded from 2007 to 2009. Age was recorded by counting the number of bud scale scars on the rhizome. Light intensity and relative humidity were measured using a photometer, LiCor Model LI-189 and thermohygrometer respectively. Different climatic variables are correlated with vegetative and reproductive phenological events. Statistical analysis revealed that a strong positive correlation was observed between the age versus vegetative and reproductive characters, except 1%–2% plants showed neoteny. Morphological variations were observed in natural conditions on the basis of the number of prong and carpellate conditions. Phenological status revealed that most of the individuals of the age class 35–50 years and above 50 years contributed the most to flowering, fruiting and seed production. Age class was significant to predict the size of the plant and its reproductive capacity. Climatic factors such as temperature, precipitation and relative humidity show synergistic effect on both the vegetative and reproductive phases in *Panax wangianus* in the undisturbed Nongkrem sacred grove. The color of flowers of *P. wangianus* also varied depending upon the sunlight intensity. Therefore, in the view of conservation

and management, the age class of 35–50 years and above 50 years is the most important for population sustainability.

Keywords: *Panax wangianus*; medicinal plant; North-East India; Nongkrem sacred grove; climatic factors; vegetative and reproductive relationship with age

Introduction

The Araliaceae family consists of approximately 55 genera and 1500 species which include a number of important medicinal and ornamental plants (Wen et al. 2001b). The members of this family are mostly distributed in the tropics and subtropics, especially in southern and Southeast Asia and the Pacific islands. There are several well-known genera from the temperate zones as well (e.g. *Aralia*, *Hedera*, *Oplopanax* and *Panax*). In India, Araliaceae is represented by 16 genera distributed mostly in the northern and north-eastern regions of Himalayas. *Panax* consists of approximately 18 species, of which 16 are from eastern Asia and two from eastern North America (Lee et al. 2004; Reunov et al., 2008). *Panax*, the generic name, from Greek term means “cure all” for its reputed medicinal use in China (Anderson et al. 2002). The Chinese have been using ginseng for over 2000 years as a tonic, a stimulant and a fatigue-resistance medicine (Wen et al. 1999).

Two American species, *Panax trifolius* and *Panax quinquefolius*, were defined on the basis of their morphology, molecular and pollen ultrastructure. Numerous studies of *P. quinquefolium* and *Panax ginseng* examined various ecological relationships (Lewis et al. 1982; Lewis 1984; Anderson et al. 1984; Anderson 1996; Hackney 1999; Shahi 2007; Van der Voort 2005; Stathers et al. 1986), reproductive biology (Carpenter et al. 1982; Lewis et al. 1982; Schlessman 1985 1987), chemical composition (Shim et al. 1983; Tomoda et al. 1985; Hansen et al. 1986; Hikino et al. 1986; Oshima et al. 1987), medicinal properties (Hu 1976; Carlson 1986; Dubrick 1983) and growth, yield and rates

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of photosynthesis, respiration and transpiration under varied solar radiation and/or temperature conditions (Hu 1976; Lee et al. 1980; Konsler 1986; Proctor et al. 1986; Stoltz 1982; Strick et al. 1985).

Yoo et al. (2001) reported that several Himalayan species of *Panax* were taxonomically problematic due to sympatry of morphologically distinct taxa and the existence of intermediates. With the advent of molecular techniques efforts were made to resolve various taxonomic disputes by using nuclear ribosomal DNA, ITS sequences, AFLP and cpDNA restriction site variations (Wen et al. 1996; Zhou et al. 2005; Choi et al. 2000).

Panax wangianus (syn. *Panax pseudoginseng*) S. C. Sun is a perennial, critically endangered, herb native to sub-tropical wet forests of North-East Himalayan regions especially in Meghalaya (Pushpangadan et al. 2005). Its rhizome was used as a blood-regulating medicine a tonic (Wen 2007). *P. wangianus* was abundantly distributed about a century ago. However, at present their population is decreasing alarmingly because of various human impacts such as urbanization, over exploitation of natural resources, pollution of soil, water and atmosphere due to coalmine activities which contribute to the global climatic change (Pushpangadan et al. 2005). Relationship between age, size, reproduction and demographic studies of *P. quinquefolius* was extensively studied in North America (Mooney et al. 2009; Charron et al. 1991). *P. wangianus* growing in sacred grooves of Meghalaya in wild conditions has not been thoroughly investigated on its growth, behavior and reproductive biology. The present investigation reveals the morphological and reproductive changes in relation to the age of the plant and the effect of climatic factors on flowering, fruiting and seed production. This study will be helpful to understand the reproductive biology for its conservation and sustainable utilization.

Materials and methods

Study site and climate

Populations of *P. wangianus* were located in the Law Lyngdoh, Smit sacred grove in Nongkrem (east khasi hills) 25°31'N, 91°52'E at an altitude 1 833 m AMSL Law Lyngdoh (Mawphlong) 25°26'N, 91°44'E at an altitude 1 796 m AMSL and Shillong peak 25°32'N, 91°49'E at an altitude 1 965 m AMSL in Shillong, Meghalaya. There are no anthropogenic activities like cutting, grazing etc. in Nongkrem sacred grove because of various religious beliefs of tribal communities of Meghalaya in North-East India. Due to these beliefs, certain patches of forests were designated as sacred grove under customary law and were protected from any product extraction by the community (Tiwari et al. 2008). Therefore, *P. wangianus* was grown well and 4.16 % plants reached up to the age of 50 years or above. It was grown in colonies of a few plants in rich, shady, deciduous forests and in deep leaf litter.

Climatologically, this study area belongs to the sub-tropical wet climatic region (Champion et al. 1968). On the basis of variation of temperature, rainfall and wind, one year in the region

may be divided into four distinct seasons: (1) winter (December to February), (2) pre-monsoon or summer (March–May), (3) monsoon (June–September) and (4) retreating monsoon (October and November) (Porwal et al. 2000; Dhirendra et al. 2010; Tripathi et al. 2004). This region receives abundant southwest monsoon from June to October. Highest rainfall was recorded in July and August. The mean temperature ranges from 6 to 17°C in winter and from 15 to 24°C during summer. The data on the climatic factors were collected from Central Seismological and Meteorological observatory, Shillong station, Government of India. The average monthly mean, mean maximum, mean minimum temperature, rainfall and relative humidity for the years 2007, 2008 and 2009 were chosen for this study (Fig. 5). Light intensity and relative humidity were measured using a Photometer, LiCor Model LI-189 and thermohygrometer (TFA, Germany) respectively. Soil pH was determined immediately within 24 h of collection following standard methods outlined by Anderson and Ingram (1993). The soil type is oxisols which can retain moisture content (Brady et al. 2002).

Species description

P. wangianus has a whorl of digitately compound leaf at the summit of aerial shoot; the leaf (prong) consists of three to eight palmately compound leaflets with a petiole, of which the terminal three to five leaflets are larger than the lateral two to three leaflets. The flowers were arranged in an umbel inflorescence on a long peduncle arising from the centre of the leaf attachment at the top of the aerial stem. The flowers were bisexual and pentamerous with an inferior ovary. The fruits were berry. The population was monitored for three consecutive years (2007, 2008 and 2009), with an interval of two or three weeks duration during the growing season of *P. wangianus* from April to September. In each visit both the vegetative and reproductive statuses were recorded (Table 1 and Table 2). The vegetative and reproductive characters were taken from the juvenile stage to fruit formation. After full expansion the length and width of leaflets were measured and their leaflet area was measured by a Planimeter (Anderson et al. 1993). Age was recorded by counting the number of bud scale scars on the rhizome that form as a result of the annual abscission of the aerial stem (Fig. 1). The aerial stem is produced only once in a year per rhizome. The relationship between the age of the plant and morphological characters (height of the plant, the number of prongs and leaflets and average leaflet area) and reproductive characters (length of peduncle, initiation of flower buds, mature flowers, anthesis of flowers, numbers of axillary inflorescence, young and mature fruit formation and seed dispersal) were calculated statistically by using Pearson's correlation coefficient. The mean value and standard deviation were calculated from 2 016 individuals of the population. Multiple regression analysis was calculated to determine the influence of age on morphological and reproductive characters by using Statistica version 5.0 (Zar 1974).

For scanning electron microscope (SEM), glutaraldehyde fixed (post-fixed with osmium tetroxide) and dehydrated flowers were critical point dried in a Jeol JCPD-5 critical point dryer,

3-methyl butyl acetate solution as the exchange liquid. Dried flowers were dissected with razor blades, sputter coated with gold in an Eiko ion coater and examined with a Jeol JSM 6360 at 20 kV.

Results

Morphological and reproductive variations in relation to the age of the plant

The height of aerial shoot ranges from 30 cm to 112 cm depending upon the age of the plant. Similarly the aerial shoot diameter as well as rhizome diameter also varies from 0.3 cm to 1.5 cm and 0.5 cm to 3.5 cm respectively in different age classes (Table 1). Morphological variations were observed in natural conditions on the basis of prong number. Leaflet area increased concurrently

with age. Plants of age class 1–10 years had one prong with three to five leaflets and those of 10–15 years had two prongs with six to ten leaflets respectively. The average leaflet area of one-pronged and two-pronged plants was 2 cm² and 4 cm² respectively. Plants of age class 16–24 years had three prongs with 9–15 leaflets and those of 25–34 years age class contained four prongs with 12–20 leaflets. The average leaflet area of three-pronged and four-pronged plants was 8 cm² and 16 cm² respectively. While plants of age class 35–50 years and above 50 years had five prongs with 22–36 leaflets and six prongs with 30–42 leaflets respectively. The average leaflet area of five-pronged and six-pronged plants was 32.5 cm² and 37.5 cm² respectively. Petiole length also increases with age of the plant. It ranges from 3 cm to 32 cm depending upon age and the number of prongs of the plant (Table 1). Occasionally only 1%–2% one-pronged and two-pronged plants were of more than 40 years old. It indicated that neoteny occurs in *P. wangianus* (Fig. 1).

Table 1. Morphological features of *Panax wangianus* growing in Nongkrem sacred grove

Age Class (year)	Number of prongs	Number of individuals (n) and of population percentage (%)	Height of aerial shoot (cm)	Aerial shoot diameter (cm)	Range of rhizome diameter (cm)	Total number of leaflets	Breadth of leaflet (cm)	Average leaflet area (cm ²)	Range of petiole length (cm)
1–10	1	187=9.27%	20.00 ± 6.20	0.3 ± 0.02	0.5–0.7	3–5	0.4–0.8	2.00 ± 0.12	3–4
10–15	2	243=12.05%	20.00 ± 6.20	0.4 ± 0.03	0.7–1.5	6–10	0.4–1.0	4.00 ± 0.02	4–7
16–24	3	482=23.90%	30.00 ± 6.20	0.5 ± 0.02	0.8–1.5	9–15	1.0–2.0	8.00 ± 0.58	6–10
25–34	4	602=29.86%	80.00 ± 6.20	0.7 ± 0.06	1.0–2.0	12–20	2.0–3.0	16.00 ± 1.73	10–15
35–50	5	418=20.73%	95.00 ± 9.09	1.0 ± 0.01	1.0–3.0	22–36	3.0–4.0	32.5 ± 3.76	26
50 and above	6	84=4.16%	110.00 ± 6.20	1.5 ± 0.12	2.0–3.5	30–42	3.5–4.5	37.5 ± 3.25	28–32

“±” indicates standard deviation.

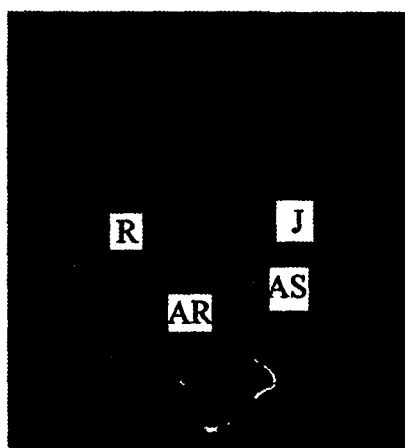


Fig. 1 An entire 48 years old neoteny plant of *Panax wangianus* with one-prong consists of five leaflets. Aerial shoot (as); joint (j) between petiole and aerial shoot; Rhizome (r) with adventitious root (ar). Bar = 4.6 cm.

In Nongkrem sacred grove, plants of age class 1–10 years and 10–15 years were either juvenile or vegetative stages only, that is, they were non-fecundity. During 2007 and 2009, the plants did

not flower until they were grown and attained the minimum age of 16 years. Plants older than 16 years with more than three prongs were producing flowers from April to early May. The length of peduncle of terminal inflorescence also varies from 15 cm to 40 cm in different age classes. The number of flowers in an umbel was 13–56 and 25–60 flowers in the age class of 16–24 and 25–34 years respectively (Table 2). Plants older than 35 years had two to three axillary inflorescences in addition to the terminal inflorescence (Fig. 3). The number of flowers in an umbel varied from 25 to 73 flowers. The numbers of axillary inflorescence were five to eight. The number of flowers ranged from 45 to 75 in the 50 years old plant (Fig. 2). The length of peduncle of axillary inflorescence was 7 cm in age class of 35–50 years and 9 cm in age class of 50 years and above (Table 2).

The number of style and stigmatic lobes varies in each inflorescence (Table 2). Generally, each ovary consists of three carpels. In the age class of 35–50 years and above 50 years, the number of carpels varied from three to five, which would be readily observed from the exterior by counting the number of lobes in fruits (Fig. 3). Each carpel had single pendulous, anatropous and unitegmatic ovule. The stigma was dry with unicellular papillate outgrowths. In the age class of 16–24 years, the flowers had one to two stigmatic lobes. The flowers of age class

of 25–34 years had one to three numbers of stigmatic lobes. While plants of age class of 35–50 years and above, the number of stigmatic lobes in each inflorescence ranged from one to five (Fig. 3). Flowers with penta and tetra stigmatic condition have

five and four carpels respectively. The number of stigmatic lobes always corresponds to the number of carpels in each ovary. (Fig. 4a and b). The terminal inflorescence matured first followed by axillary inflorescence (Fig. 3).

Table 2. Reproductive features of *Panax wangianus*

Age (year)	Class	Number of prongs	Length of peduncle of terminal inflorescence (cm)	Length of peduncle of axillary inflorescence (cm)	Number of carpels/plant	Range of number of stigmatic lobes/plant	Range of number of flowers/plant	Range of number of axillary inflorescence/plant	Range of number of fruits/plant
1–10		1	–	–	–	–	–	–	–
10–15		2	–	–	–	–	–	–	–
16–24		3	15	–	3	1–2	13–56	–	1–3
25–34		4	27	–	3	1–3	25–60	–	4–9
35–50		5	38	7	3–5	1–5	25–73	2–3	6–17
50 and above		6	40	9	3–5	1–5	45–75	5–8	7–30

“–” indicates no flowering.

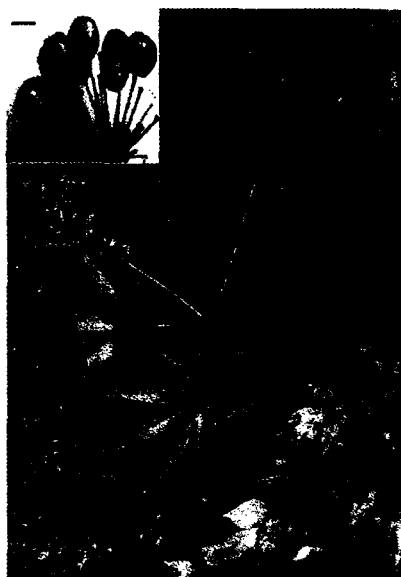


Fig. 2 Above 50 years old plant with six prongs (38 leaflets), seven axillary inflorescence and one terminal inflorescence with fruits. Mature fruits are bright red on the bottom and black on the top. Bar = 11.40 cm. (Inset An enlarged view of mature fruits. Bar = 5.5cm).



Fig. 3 An enlarge view of terminal (ti) and axillary inflorescences (ai) showing unistigmatic to pentastigmatic as well as tri-pentacarpellary condition. Terminal inflorescence bearing green young fruits. Unistigmatic (us); bistigmatic (bs); terminal inflorescence (ti); axillary inflorescence (ai) and pentacarpellate (pc). Note the terminal inflorescence mature earlier than the axillary one. Bar = 10 cm. (Inset uni-pentacarpellate fruits; Bar = 5cm).

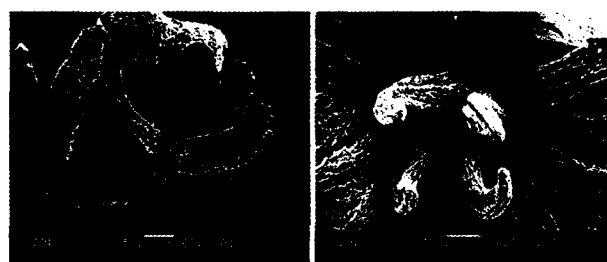


Fig. 4 (a) Scanning electron microscope of pentastigmatic flower. (b) Scanning electron microscope of tetrastigmatic flower. FI= anther filament.

Young fruits were green in color which appeared in July and matured during October. As fruits mature, it became bright red on the bottom and black on the top (Fig. 2). The color of pedicel also changed from green to crimson red. Fruits contained one to five seeds. The number of fruits produced by the age class 16–24 years and age class 25–34 years was one to three and four to nine respectively. Age class of 35–50 years and above produced 6–17 and 7–30 number of fruits respectively. In *P. wangianus* the percentage of one-seeded fruit is 26%, two-seeded fruit 31%, three seeded fruit 22%, four-seeded fruit 12% and five seeded fruits are 9%. But the ability to produce flowers that mature into fruit increased with the age of the plant. Phenological and reproductive characters revealed that most of the individuals of age class 35–50 and 50 years above plants contributed most of the flowering, fruiting and seed production.

Effect of different climatic factors on the phenology of *P. wangianus* in Nongkrem sacred grove

The Nongkrem sacred grove is spread over an area of about 6 ha. The temperature increasing about 4°C favoured the sprouting of aerial shoot from the rhizome during March (spring season) after dormancy. The vegetative growth continues up to the end of May

(pre-monsoon or summer season). Flower buds appear from late April to May. Anthesis occurs in the month of June (i.e. beginning of the monsoon season), during which period the monthly mean temperature is 20.77°C, monthly mean precipitation is 299 mm and monthly mean relative humidity is 82%. Small plants (three-prong and four-prong) have a shorter flowering season, while large individuals (five-prong and six-prong) extend their blooming. The young fruits are observed in July–August (i.e. peak monsoon season). During this period monthly mean temperature, monthly mean precipitation and monthly mean relative humidity was maximum, they were 20.47°C, 388 mm and 89%, respectively. However, the mature fruits were produced in the month of September to October, during these months monthly mean temperature, monthly mean precipitation and monthly mean relative humidity were recorded, they were 19.26°C, 177.85 mm and 78.75%, respectively. The dispersal of seeds take place in the month of October–November (retreating monsoon period to the beginning of winter), during this period there is decrease in monthly mean temperature (16.55°C), monthly mean precipitation (79.45 mm) as well as in monthly mean relative humidity (89%) (Fig. 5). Light level ranges from 5.18–100.48 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ with an average of 33.79 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ during the life span of *P. wanganus* from March–October. The color of corolla of *P. wanganus* also varied depending upon the intensity of sunlight. Those plants grow in bright sun-light, the flowers would appear pinkish. Flowers' color of the plants, which are growing in shade, is greenish-white. The soil of the Nongkrem sacred grove is loamy, reddish brown in color, lateritic in origin and acidic (pH 4.5–4.62). The soil type is oxisols, where moisture content is 30%–40% even during the dry season. Humus

increases its water holding capacity up to 50%–60%.

Statistical correlation between the age, morphological and reproductive traits of *P. wanganus*

In *P. wanganus*, during the study period from 2007 to 2009, a strong positive correlation was observed between the age versus height of the plant ($r^2 = 0.93$), aerial shoot diameter ($r^2 = 0.92$), rhizome diameter ($r^2 = 0.94$), total number of leaflets ($r^2 = 0.97$), breadth of leaflet ($r^2 = 0.98$), average leaflet area ($r^2 = 0.96$) and petiole length ($r^2 = 0.94$) (Table 3).

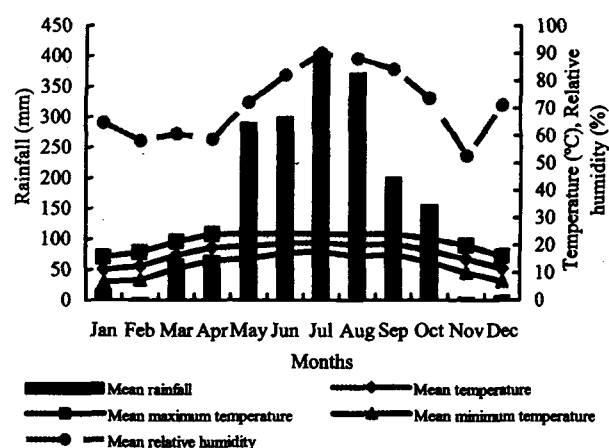


Fig. 5 Different climatic factors in the study area of *P. wanganus* during 2007–2009.

Table 3. r^2 value of different morphological features versus age of the plant

Dependent variable	Height of the plant	Aerial shoot diameter	Rhizome diameter	Total number of leaflets	Breadth of leaflet	Average leaflet area	Petiole length
Age of the plant	0.93	0.92	0.94	0.97	0.98	0.96	0.94

Similarly, the reproductive parameters also showed positive correlation with the age of the plant. The value of correlation coefficient of length of peduncle ($r^2 = 0.95$), length of peduncle of axillary inflorescence ($r^2 = 0.78$), number of carpels ($r^2 =$

0.81), number of stigmatic lobes ($r^2 = 0.92$), number of flowers ($r^2 = 0.88$), number of axillary inflorescence ($r^2 = 0.69$) and number of fruits ($r^2 = 0.93$) were also very high (Table 4).

Table 4. r^2 value of different reproductive features versus age of the plant

Dependent variable	Length of peduncle of terminal inflorescence	Length of peduncle of axillary inflorescence	Number of carpels	Number of stigmatic lobes	Number of flowers	Number of axillary inflorescence	Number of fruits
Age of the plant	0.95	0.78	0.81	0.92	0.88	0.69	0.93

Discussion

The vegetative as well as reproductive, phenological relationships in *P. wanganus* showed that nearly 90% of the features correlate with the age of the plant. Wen (2001a) and Shu (2007)

demonstrated the high level of morphological and reproductive plasticity in eleven species of *Panax* with respect to the shape and size of rhizome, number of leaves, leaflet shape, pubescence of leaves and number of flowers in an umbel inflorescence. Ghou et al. (2010) reported the morphological variations in stem, leaf, root, flower and fruit in the cultivated populations of *Panax notoginseng*. However, in population of *P. wanganus* growing in

Nongkrem sacred grove, there is a clear-cut correlation between the different age classes with respect to the height of the plant, aerial shoot diameter, rhizome diameter, total number of leaflets, breadth of leaflet, average leaflet area, petiole length, length of peduncle, length of peduncle of axillary inflorescence, the number of carpels, stigmatic lobes, lowers, axillary inflorescences and fruits (Table 1 and 2).

With respect to the age classes in *P. wanganus*, the height of the aerial shoot did not vary much in age class 1–10 years and 10–15 years with one and two-pronged plants respectively. However, in the age class of 16–24 years, 25–34 years, 35–50 years, 50 years and above, there was a steady increase in the height of aerial shoot, aerial shoot diameter, rhizome diameter, petiole length and breadth of the leaflet (Table 1). The height of aerial shoot of *P. wanganus* showed the similar growth trend when compared to *P. japonicus* and *P. vietnamensis*. While, in the remaining six species, *P. ginseng*, *P. zingiberensis*, *P. notoginseng*, *P. pseudoginseng*, *P. quinquefolius* and *P. trifolius*, the height of aerial shoot ranged from 5 to 60 cm (Wen 2001a; Shu 2007).

The number of prongs in *P. wanganus* was 1–6, while Wen (2001a) and Shu (2007) reported that the number of prongs was 1–5 in *P. quinquefolius* and in *P. ginseng*, *P. japonicus*, *P. zingiberensis*, *P. notoginseng*, *P. pseudoginseng*, *P. vietnamensis*, *P. stipuleanatus*, *P. bipinnatifidus*, *P. quinquefolius* and *P. trifolius* it ranged from 3–7.

Anderson et al. (1993) mentioned that in *P. quinquefolius* one-prong plant of two years of age possessed three to five leaflets, two-prong plants (with 10 leaflets) ranged from three to six years of age, three-prong plants (with 15 leaflets) ranged from seven to nine years of age, and four-prong plants (with 20 leaflets) ranged from 10 to 11 years of age. In *P. wanganus* plants of age class 1–10 years had one prong with three to five leaflets and 10–15 years had two prongs with six to ten leaflets, respectively. Plants of age class 16–24 years had three prongs with 9–15 leaflets and 25–34 years age class contained four prongs with 12–20 leaflets. Plants of age class 35–50 years had five prongs with 22–36 leaflets. Lewis et al. (1983) observed that four-prong and five-prong plants represented the oldest individual of a population of *P. quinquefolius*. But in *P. wanganus*, six-prong plants with 30–42 leaflets fell under the category of 50 years and above age class. Similar observations were reported in *P. quinquefolius* by using the combination of leaf area and leaf number which are the good indicators of age (Furedi 2004; McGraw et al. 2005). In *P. quinquefolius* the maximum number of leaflets was 20 (Anderson et al. 1993). While, in *P. ginseng*, *P. japonicus*, *P. zingiberensis*, *P. notoginseng* and *P. pseudoginseng*, it ranged from 9–42 leaflets (Shu 2007; Wen, 2001a).

In Nongkrem sacred grove, only 1%–2% one-pronged and two-pronged plants of *P. wanganus* exhibited neoteny and their age was above 40 years. The retention of ancestral juvenile characters by mature plants was reported in few members of family Araliaceae such as *Schefflera racemifera* and *S. longipetiolata* by Fiasch et al. (2006).

Lewis et al. (1982), Carpenter et al. (1982); Lewis (1984); Anderson et al. (1993) and Gagnon (1999) reported that the

height of aerial shoot, the number of leaves and leaflet and leaflet area increased, they were the good predictors of age in *P. quinquefolius* as estimated by the number of bud scars on the rhizome. Similar observations have occurred in *P. wanganus* (Table 1). The maximum age of *P. quinquefolius* estimated by Anderson et al. (1993) was 25–30 years, and in *P. ginseng* it was 140–150 years (Grushvitskii 1961; Khrolenko et al. 2007). In *P. wanganus* it was above 50 years. Rhizome damage could make the plant appear younger than its actual age, but growth probably would continue with adventitious roots assuming the storage and absorption functions (Anderson et al. 1993).

Similarly the reproductive features were also positively correlated with length of peduncle, length of peduncle of axillary inflorescence, the number of carpels, stigmatic lobes, flowers, axillary inflorescences and fruits, and flowering season, age versus fecundity of the plant and ripening of fruits etc. In *P. wanganus*, the length of peduncle of terminal inflorescence ranged from 15 cm to 40 cm. While, in *P. ginseng*, *P. japonicus*, *P. zingiberensis*, *P. notoginseng* and *P. pseudoginseng* the length of peduncle of terminal inflorescence ranged from 7 cm to 30 cm (Shu 2007; Wen 2001a).

Farrington (2006) reported two pronged plants showed fecundity in *P. quinquefolius*. However, in *P. wanganus* the fecundity was observed from three-pronged plants and onwards.

Schlessman (1985) reported three and four-pronged plants tended to flower earlier compared to one and two-pronged plants. In *P. quinquefolius*, 20%–50% of two-year-old plants have inflorescences (Proctor et al. 2003). Fiebig et al. (2001) reported that there were 32–79 flowers in an inflorescence in a three-year-old plant of *P. quinquefolius*. Shu (2007) and Wen (2001a) reported that the numbers of flowers per plant ranged from 20 to 100 in *P. ginseng*, *P. japonicus*, *P. notoginseng* and *P. pseudoginseng*. Out of 11 literature surveyed species of *Panax*, the only species *P. trifolius* is a dioecious plant in which male and female umbel consists of 15–30 flowers and 3–14 flowers respectively (Wen 2001a). But in the case of *P. wanganus*, all the flowers in an umbel were hermaphrodite. Only in the three-pronged (more than six leaflets) plants, the inflorescence consisted of 13–56 number of flowers. The earliest flowering plants were 16 years of age, they had few flowers and only rarely formed fruit, but after 24 years of age all plants developed flowers and most had fruits. Five and six-pronged plants produced more flowers and fruits. One and two-pronged plants remained vegetative or in juvenile stage (Table 2).

Anderson et al. (1993) reported there was only one axillary inflorescence in *P. quinquefolius* and the length of peduncle of axillary inflorescence was 1 cm. It rarely produced fruits. Shu (2007) and Wen (2001a) reported there was 1–5 axillary inflorescences in *P. japonicus*, *P. pseudoginseng*, *P. vietnamensis* and *P. bipinnatifidus*. While in *P. wanganus* the number of axillary inflorescence was 2–8. The length of peduncle of axillary inflorescence was measured from 7 cm to 9 cm depending upon the age of the plant and all the axillary inflorescence also possessed fruits. The young fruits of *P. wanganus* collected in July remained greenish and turned into red bottom and black on the top after six to eight weeks (Fig. 2). But in *P. quinquefolius* all the

ripened fruits are reddish in color (Proctor et al. 2003).

The present and prior investigation results (Anderson et al. 1993; Carpenter et al. 1982; Lewis et al. 1982) showed that the fruit production is correlated with plant size. The initiation of flower and fruit production varies in different population of *P. quinquefolium*. Anderson et al. (1993) reported that the fruit production started in the fourth year old plants of *P. quinquefolium* in Illinois and the number of fruits was 30–40. While Carpenter et al. (1982) reported fruit production began after eight years in *P. quinquefolium* in Wisconsin. Farrington (2006) mentioned that all the plants irrespective to the age produce berries contained 1–3 seeds. In *P. wangianus* fruit production started after 16 years and it increased with the age of the plant. Plants of 50 years and above produced 7–30 fruits. Schlessman (1985) reported that flowers containing one, two and three ovaries produced one, two and three-seeded fruit, respectively. And the ratio of one-ovuled to two-ovuled flowers increased with plant size (age). In *P. wangianus* the percentage of one-seeded fruit is 26%, two-seeded fruit is 31%, three seeded fruit is 2%, four-seeded fruit is 12% and five seeded fruit is 9%. Stoltz et al. (1980) also reported the percentage of one-seeded (16.3%), two-seeded (77.0%), three-seeded (6.5%) and four-seeded (0.2%) fruit in *P. quinquefolium*.

Shu (2007) reported all the species of *Panax* in China were two carpellate and bistigmatic except *P. japonicas* which has two to five carpels and two to five growing styles in China. Wen (2001a) reported one to three carpellate conditions except in *P. trifolius*. Wen et al. (2001b) mentioned pentacarpellate condition was an evolutionarily liable and developmental abnormality in Araliaceae. Philipson (1970) and Eyde et al. (1971) also reported two to five carpellate is common in Araliaceae. The pluricarpellate condition (upto 20 carpels) is a primitive condition in Araliaceae. The American ginseng with one-styled flowers represents an intermediate step in the evolution of functional maleness. Asiatic ginseng is a functional hermaphrodite because all the individuals of *P. wangianus* in the population growing in Nongkrem sacred grove show one to five stigmatic with three to five carpellate conditions.

Several climatic variables such as temperature (Ashton et al. 1988; Pfeifer et al. 2006), precipitation (Borchert et al. 2004; Dominguez et al. 1995; Inouye et al. 2003; Opler et al. 1976; Pfeifer et al. 2006; Stevenson et al. 2008; Stiles 1977; Tyler 2001). The other variables such as soil nutrient concentration (Dahlgren et al. 2007) can trigger flowering. The biological advantages of extended blooming include: (a) a reduced risk of reproductive failure; (b) an increased chance of mating with more individuals; and (c) a better control of the relative investment in flowers and fruits (Bawa 1983). The color of corolla of *P. wangianus* also varied depending upon the intensity of sunlight. Those plants grow in bright sun-light, the flowers would appear pinkish. The plants which are growing in shade, the color of flowers is greenish-white. Guo et al. (2010) and Liu et al. (1992) proved that *P. notoginseng* is shade-loving plant with C_3 cycle, which is sensitive to sunshine and dislikes direct irradiation by sun. The favorable climatic conditions and protection over a long period of time play a major role in growth and reproduction of *P.*

wangianus in Nongkrem sacred grove. When the weather approaches the winter, the leaves and aerial stem will get senescence due to the low rainfall and moderate temperature. Venugopal et al. (2007) and Dhirendra et al. (2010) reported that relative humidity played a secondary role for the tree species in the sub-tropical wet climate. However, this was not applied for the understory shaded herbaceous plant growth. Therefore, increase in temperature, rainfall and relative humidity shows its effect on the sprouting of aerial shoot from the rhizome and further growth and reproduction in *P. wangianus*.

Larger plants (in terms of age class 35–50 years and above 50 years) are reproductively more successful due to its vegetative growth and flowering phenology which influences the more production of fruits and seed set in Nongkrem sacred grove. Morphological variations were observed in natural conditions on the basis of the number of prong number and carpellate conditions. Age class was significant to predict the size of the plant and its reproductive capacity. Therefore, in the view of conservation and management, the age class 35–50 years and above 50 years is the most important for population sustainability. Plants of age class 35–50 years may facilitate the floral rewards to the pollinators. Therefore, in *Panax wangianus*, there is a clear-cut correlation between the age of the plant and pentacarpellate condition, formation of axillary inflorescences with more productivity. The color of flowers of *P. wangianus* also varied depending upon the sunlight light intensity. The soil moisture content 30%–40% of oxisols is enough to trigger the vegetative growth after dormancy during March. The onset of flowering is primarily related to temperature and precipitation. Climatic factors such as temperature, precipitation and relative humidity show synergistic effect on both the vegetative and reproductive phases in *Panax wangianus*. Nongkrem sacred grove is undisturbed climatic climax forest, in which environment conditions are stable in terms of precipitation, temperature and relative humidity. Therefore, there is a relationship between the development of vegetative and reproductive features with environmental conditions.

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