K.R. Shivanna · Rajesh Tandon

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Foreword

Tropical countries, including India, are rich in biodiversity with a high incidence of endemism. For the sake of short-term gains, vast areas of wilderness have been destroyed. However, the sustainability of whatever biodiversity is still available in various ecosystems is seriously threatened by continuing human need. Unless effective measures are taken to salvage biodiversity, a large number of species would become irretrievably lost. According to some conservation biologists, we are soon heading towards the sixth mass extinction in evolutionary history. Successful reproduction is the basis not only for the stability of the species in their natural habitat but also for the productivity of our crop plants on which humans and their domesticated animals are dependent. Studies on reproductive ecology are, therefore, important for the effective management of our dwindling biodiversity and improvement of yield in the crop plants. Unfortunately, knowledge of reproductive ecology of wild and crop species in the developing countries is meagre. This constitutes a major limitation that hampers our efforts to conserve biodiversity and optimize crop yields and animal production.

Contrastingly, there is enormous information available on reproductive ecology of wild and cultivated species growing in countries with developed economies. Therefore, they have been able to manage their biodiversity and optimize crop yields in a substantial way than their counterparts in developing nations. Also because of the availability of baseline data on reproductive ecology on their plant resources, most of their present studies on reproductive ecology are aimed at formulating hypotheses, testing their validity and preparing mathematical models on reproductive events. Tropical countries do not have even the baseline data on most of the species. Therefore, there is urgent need to initiate and emphasize research on reproductive ecology in tropical countries, where population pressure is acute and malnutrition is rampant, for effective management of biodiversity and to improve the yield and nutritional quality of the crops.

One of the limitations of initiating research in this area is lack of mentors and more importantly authentic manuals to motivated and enterprising young plant biologists for conducting research on various aspects of reproductive ecology. *Reproductive Ecology of Flowering Plants: A Manual* is the first attempt in this direction. K.R. Shivanna and Rajesh Tandon have been working in this field throughout their research careers. This handbook covers the total range of reproductive ecology starting with a thorough study of plants and sites of their occurrence, phenology, breeding system, record of

reproductive events including dispersal of diaspores and ending with seed viability, dormancy and finally seedling recruitment, the ultimate step in the sustainability of populations. This manual will be extremely useful to those who are residing in areas with rich flora but lacking in essential laboratory facilities. I am confident that the manual would encourage the younger generation to take up research in this important discipline of production and utilization of plant resources.

Sriram Institute for Industrial Research
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6 June 2014

H.Y. Mohan Ram

Preface

The stability of any species in its natural habitat is dependent on its successful reproduction and recruitment of new individuals to sustain populations. Erosion of biological diversity is one of the most challenging issues of our times. Although overexploitation, habitat destruction and climate change are the proximate causes for erosion of biological diversity, the ultimate driving force for pushing any species to endangered category is its inability to produce enough number of seeds and to recruit new individuals to sustain populations. Tropical countries are rich in biodiversity with a high proportion of endemic elements, and presently many plant species in tropical rain forests are at maximum risk of reproductive failure. One of the major problems in conservation and management of tropical forests is lack of baseline data on their reproductive ecology. This is particularly true for a large number of endemic, endangered and economically important species. This lack of knowledge is largely because very few laboratories in tropical countries, particularly in south and south-east Asia, are working on reproductive ecology. In the absence of such data, any conservation efforts are arbitrary and remain ineffective. Therefore, it is necessary to initiate extensive studies on reproductive ecology of our plants as a requirement for their effective conservation and sustainable utilization. Also, in most of our crop plants, fruits and seeds are the economic products and any constraint in reproduction reduces their production. For sustenance and improvement of yield in the crop species, an understanding of reproductive ecology is an essential prerequisite. Thus, reproductive ecology has direct relevance to conservation biology, agri-horticulture, forestry and plant breeding.

Several workshops and training programmes conducted in India during the last few years on reproductive ecology have shown an enormous enthusiasm amongst young researchers to initiate research. A major limitation for the beginners to take up research is absence of simple work manuals that provide the basic steps used in studying reproductive ecology. A few manuals available so far deal largely with pollen biology and pollination biology, and there is no manual covering the whole spectrum of reproductive ecology including seed biology, seed dispersal and seedling recruitment which are important in sustaining the populations and species in their natural habitat.

The major aim of this manual is to fill this void and provide the essential working steps involved in studying all aspects of reproductive ecology. The intention is to keep the volume small, and therefore the methods described have been selective (based on the need and our own experience) and does not

aim to give a monographic coverage of all the available techniques. Each chapter gives a concise conceptual account of the topic before describing the protocols. Important relevant references are cited to help the user to get more theoretical information in the subject. The manual covers over 60 protocols. Each protocol lists special requirements followed by detailed working steps so that the researchers do not find any difficulty in conducting investigations. Appendices provide the details of preparation of fixatives, stains and a few other relevant requirements. Most of the protocols presented in the manual require minimum facilities which are generally available in colleges, university departments and research institutions. This is important since most of the biodiversity in developing countries in the tropics is located where only a limited number of well-established laboratories are present.

The manual caters to teachers, students and researchers who deal at any level of reproductive ecology of flowering plants – botanists, zoologists, entomologists, ecologists, agri-horticulturists, foresters, plant breeders and conservation biologists. We hope that this manual would stimulate youngsters to undertake studies on reproductive ecology and generate useful data over the years which would be invaluable for conservation and management of our plant diversity.

We are grateful to Prof. H. Y. Mohan Ram, for his collaboration in some of our studies on reproductive ecology over the years, encouragement throughout our career and also for writing the foreword for the manual. We sincerely thank Prof. N. S. Rangaswamy for encouragement and counsel, which have been invaluable. Palatty Allesh Sinu, Department of Animal Science, Central University of Kerala, Kasaragod, has critically gone through the chapters on seed dispersal and seedling recruitment. We express our indebtedness to him. Dr. Mamta Kapila, Publishing Editor, Springer (India) Pvt. Ltd., took special interest in the publication of this manual.

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KRS thanks the Indian National Science Academy for the award of positions as INSA Senior Research Fellow (2003–2008) and INSA Honorary Scientist (2009–to date), and the Ashoka Trust for Research in Ecology and the Environment (ATREE), Bengaluru, for providing facilities. It is a pleasure to thank Giby Kuriakose and Manju Vasudevan Sharma who were associated with research activities of KRS during the past 10 years.

Bengaluru, India — K.R. Shivanna
Delhi, India — Rajesh Tandon

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About the Authors

K.R. Shivanna formerly Professor and Head, Department of Botany, University of Delhi, is now associated with Ashoka Trust for Research in Ecology and the Environment, Bangalore, as INSA Honorary Scientist and Honorary Senior Fellow. He has been doing research on reproductive biology of flowering plants for over 50 years. He has published over 150 original research papers in refereed journals and authored/edited five books on pollen biology and biotechnology. He has worked at the Royal Botanic Gardens, Kew, UK, Welsh Plant Breeding Station, Wales, UK, University of Melbourne, Australia, University of Siena, Italy, and University of Saskatchewan, Saskatoon, Canada, as a Visiting Scientist/Professor. He is a recipient of many awards including Panchanan Maheshwari Medal, and a Fellow of the Indian National Science Academy, Indian Academy of Sciences, National Academy of Agricultural Sciences and National Academy of Sciences, India.

Rajesh Tandon is currently working as Associate Professor at the Department of Botany in University of Delhi. He did his Ph.D. in 1998 from the University of Delhi. Since then he has been teaching and conducting research at the Department of Botany, University of Delhi, and his research interests have been plant reproduction, developmental biology and conservation biology. He has published over 30 research papers in reputed international journals and contributed chapters to several books. He is a recipient of Dr. Y. S. Murthy Young Scientist Award by the Indian Botanical Society.

Introduction

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Reproductive ecology covers all aspects of reproductive events and their interactions with biotic and abiotic components of the environment. Although reproductive strategy of all organisms is to maximize reproductive success and genetic diversity, they differ in the means of achieving these goals. The main reproductive strategies in plants are largely dictated by their immobility. They have to use other agents particularly animals to perform some of their reproductive functions. A large number of flowering plants follow a dual strategy of reproduction through seeds as well as through vegetative propagules (asexual/vegetative reproduction); this dual strategy maximizes fitness by combining the advantages of both the types of reproduction. Important means of natural vegetative propagation are the production of: bulbs (onions, lily and tulip), corms (*Gladiolus*, *Crocus* and *Freesia*), stem tubers (potato), tuberous roots (sweet potato, dahlia, *Canna* and turmeric), rhizomes (iris and ginger), suckers (chrysanthemums), runners (strawberry) and bulbils (*Agave*). Vegetative reproduction facilitates colonization and local dominance of the population in well-adapted niches by rapid lateral expansion. However, it limits genetic variability due to lack of gene exchange and also dispersal benefits. Increased homozygosity in the species also becomes an impediment for long-time survival, as it hinders the competitive ability of the plants to adapt to new niches in changing environments and thereby restricting the populations to specialized habitats.

In many commercial crops, vegetative reproduction is induced through human intervention. Horticulturists have been able to propagate desired varieties through various methods such as stem cuttings, grafting, layering and budding in a number of crops (McKey et al. 2010). In micropropagation, tissue culture technology is used to produce a large number of plants by culturing practically any part of the plant body and inducing organogenesis (root and shoot formation) or embryogenesis (formation of somatic embryos). Micropropagation at a commercial scale is being practised in a number of species such as orchids and bananas (Kozai 1991; Chugh et al. 2009; McKey et al. 2010; Mudoi et al. 2013). In all the above methods of asexual reproduction, seeds are not the units of reproduction. However, in a few species, there is another unique method of asexual reproduction, termed apomixis, which results in seed formation without fertilization (Richards 1986, 2003; Koltunow 1993; Ozias-Akins and Van Dijk 2007; Barcaccia and Albertini 2013). The progeny resulting from all methods of asexual reproduction, including apomixis, are the clones of the parent plant.

Sexual reproduction is the only method which permits genetic recombination (through meiosis and fertilization) and results in heritable variations, the raw materials for evolution. Sexual reproduction in flowering plants is more complex than in the other groups of plants. Flowers are the units of sexual reproduction. Therefore, the first event in sexual reproduction is the initiation and

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development of the flower. Although they show a high degree of variability in structural features, flowers of all species essentially bear the stamens and/or the pistil, representing the male and female partners, respectively. Stamens are differentiated into the filament and the anther. Anthers produce pollen grains, the male partners in sexual reproduction. Pollen grains represent highly reduced male gametophytes. Mature pollen grains are shed from the anthers following their dehiscence. The pistil is divided into the stigma, style and ovary. The stigma is the recipient of pollen grains, the style is the conduit for the growth of pollen tubes carrying male gametes, and the ovary is the container of ovules which harbour the female gametophyte.

Pollination is the transfer of pollen grains from an anther to the stigma. Flowering plants have developed amazing adaptations to achieve pollination. Following compatible pollination, pollen grains germinate on the stigma, and the resulting pollen tubes grow through the tissues of the stigma and style and enter the ovary and eventually the ovule. These events from pollination up to the entry of pollen tubes into the ovule are termed pollen–pistil interaction. The pollen tube eventually enters the embryo sac, the female gametophyte, located in the ovule, and releases the two sperm cells. One of the sperms fuses with the egg to give rise to the zygote, and the other fuses with the fused polar nuclei (secondary nucleus) to give rise to the primary endosperm nucleus. Following incompatible pollinations (self-pollen in self-incompatible species and cross-pollen from the reproductively isolated species), pollen grains are unable to complete pollen–pistil interaction, and thus pollen tubes fail to reach the ovary resulting in the failure of fertilization. Some interspecific crosses show post-fertilization barriers; fertilization occurs, but the zygote or young embryo aborts and no viable hybrid is realized.

The zygote develops into an embryo, and the primary endosperm nucleus (resulting from the fusion of one of the sperms with secondary nucleus) produces the endosperm, a nutritive tissue for the developing embryo. The details of the development of the embryo and endosperm have been well investigated (Maheshwari 1950, 1963; Johri et al. 1992; Raghavan 1997; Ramawat et al. 2014). Fertilized ovules develop into the seeds and the ovary into the fruit. Following the maturation of the fruits, the seeds are dispersed by various agents. Eventually the seeds land on the soil and, under suitable conditions, germinate and produce seedlings. A large number of seedlings perish due to predation, competition or abiotic stress, and only a very small proportion of them eventually grow into new adult plants, completing the cycle of sexual reproduction.

In cultivated species, seed is generally considered as the end product of sexual reproduction. However, in wild species, sexual reproduction also includes events leading to natural recruitment (seed biology and seedling recruitment). Thus, sexual reproduction in flowering plants broadly involves the following sequential events:

- Flower initiation and development
- Production of functional pollen grains and ovules
- Transfer of pollen grains from the anther onto a receptive stigma (pollination)
- Pollen–pistil interaction and fertilization
- Development of seeds and fruits
- Dispersal of seeds and their germination leading to the establishment of viable offspring

Any break in these sequential events results in the termination of reproductive events. Various aspects of reproductive ecology may be pursued interdisciplinarily by integrating techniques employed in several major disciplines such as ecology, population biology, genetics, physiology, molecular biology, biotechnology and conservation biology. Depending on the rationale, relevant techniques from these areas may be appropriately used in studies on reproductive ecology for achieving both fundamental and applied objectives. However, for generating baseline data on reproductive ecology, field studies with limited laboratory facilities are satisfactory.

1.1 Reproductive Ecology and Conservation Biology

Effective management of biological diversity is going to be a major challenge in the coming decades (Sodhi and Ehrlich 2010; Shaanker et al.

2010; Anonymous 2011a; Bawa et al. 2011). Biodiversity includes all heritable variations at all levels of organization (Wilson 1997). It is generally considered at three levels: genetic diversity (all the genes and alleles present in the populations), species diversity (all the species present on earth: prokaryotes, monera, bacteria and cyanobacteria, and eukaryotes, protista, fungi, plants and animals) and community diversity (associated interactions with biotic and abiotic components of the environment) (Bawa et al. 2011). Biodiversity provides a range of vital ecosystem services which are essential for the sustainability of biota including humans. Plant genetic resources (PGRs) are an important component of plant biodiversity. They include all plant species being used and those that have potential use for human needs. Wild PGRs are also the repository of novel genes for nutrition, resistance to biotic and abiotic stresses and a range of raw materials needed for medicinal and industrial uses.

Habitat degradation/modification, overexploitation and climate change as a result of human activities have threatened the sustainability of our biodiversity (Reaka-Kudla et al. 1997; Lovejoy and Hannah 2005; Memmott et al. 2007; Primack et al. 2009; Potts et al. 2010; Sodhi and Ehrlich 2010; Anonymous 2011a; Bawa et al. 2011; Dunnell and Travers 2011; Hooper et al. 2012). The threat of species extinction is more pronounced in the tropical countries such as Brazil, India, Malaysia and Indonesia where population pressure on forests and other natural resources has increased enormously in recent years. A large number of species have been pushed into the vulnerable status (rare, endangered and threatened category; RET species) as their populations have declined markedly. Unless suitable measures are taken, many of the threatened species would soon be extinct. According to many conservation biologists, an accelerating decline in world's biodiversity as a result of human activity is leading to the sixth major extinction event on our planet (Sodhi and Ehrlich 2010; Lenzen et al. 2012). Developing countries have additional disadvantage in the management of biodiversity. Bioresources are overexploited not only to cater to the needs of local people but also to rapidly expanding international trade (to satisfy the consumer demands in the developed countries). According to an estimate, ~30 % of global threats to the red listed species are due to international trade (Lenzen et al. 2012). Recent progress in developing genetically modified plants and their commercialization is another threat to the sustainability of our biodiversity (Bawa et al. 2011).

Biodiversity management involves documentation of biodiversity, its conservation and sustainable utilization. Conservation biology is the scientific study of the maintenance, loss and restoration of biological diversity. Conservation efforts particularly in the developing countries have so far been arbitrary, and there are hardly any success stories. Although habitat degradation, overexploitation and climate change are the proximate causes for species extinction, the ultimate cause is the reproductive failure. The continued perpetuation and survival of the species depend on its effective reproduction and regular recruitment of new individuals to sustain populations. Reproductive failure results when there are constraints in one or several reproductive events (Corlett 2007). These constraints lead to a gradual reduction in population size. When this continues for a prolonged period, it leads to eventual extinction of the population. Often, the population resorts to selfing, when its size is reduced beyond a critical number, as a means of reproductive assurance. As pointed out earlier, inbreeding for many generations leads to the loss of genetic variability, inbreeding depression, progeny with reduced vigour and fitness and loss of evolutionary potential to cope with changed habitat (Richards 1986). Thus, in the final analysis, the species enter the threatened category when deaths exceed births for a prolonged period due to some constraints in their reproduction and/or recruitment. Knowledge on the details of reproductive ecology of the species becomes vital to their management, conservation and sustainable utilization. In the absence of data on reproductive ecology, any conservation efforts remain ineffective. The species that have been successfully restored generally happen to be the best studied species particularly on their reproductive ecology

(Kwak and Bekker 2006). The data on reproductive ecology of the species are also needed to monitor the success or failure of any conservation efforts.

Tropical rain forests are currently at the greatest risk from reproductive failure (Wilcock and Neiland 2002). Constraints in pollination, seed dispersal and seedling recruitment are the major limitations faced by the tropical species. Pollination is a prerequisite for seed set. Only a small proportion of species growing in natural habitats depend on abiotic agencies for pollination; a great majority of them (>80 %) depend on biotic pollinators. Biotic pollination has become highly vulnerable in recent years as a result of habitat loss and climate change. This has raised great concern on the sustainability of biodiversity (Biesmeijer et al. 2006; Potts et al. 2010; Cameron et al. 2011; Garibaldi et al. 2013; Tylianakis 2013).

Seed biology is another important aspect of reproductive ecology which has relevance to conservation. Only a very small proportion of seeds produced are able to germinate and establish as new adult plants. Therefore, sufficient seed production, their dispersal to suitable sites, retention of their viability in the soil seed bank until suitable conditions become available for their germination and seedling establishment are the critical events for successful recruitment. These events, individually or in combination, often act as limiting factor(s) for recruitment (Dalling et al. 2002; Corlett 2004; Norjhauer and Newberg 2010; *see* Fenner and Thompson 2005; Ghazoul and Sheil 2010).

Extensive data is available on the reproductive ecology of temperate species. The information available on tropical species, particularly of the Old World, is very limited (see Bawa and Hadley 1990; Corlett 2004; Roubik et al. 2005). It is, therefore, necessary to initiate studies on a large scale in the tropics with the main objective of collecting baseline data on reproductive ecology of our plant species and communities. Such a data is invaluable for effective management and conservation of our plant resources. Infrastructural facilities required to collect baseline information on reproductive ecology relevant to conservation are rather modest and do not require expensive gadgets or equipment.

1.2 Reproductive Ecology and Crop Productivity

Studies on reproductive ecology have direct bearing on the optimization of crop productivity. Reproductive success in cultivated species is assessed on the basis of economic yield. As seeds are the economic products in most of our crop plants, reproductive success is assessed on the basis of seed production. As pointed out earlier, seeds are the products of a series of sequential reproductive events. Pollination is one of the crucial events in reproductive success, and its efficacy contributes significantly to crop production. Except the cereals, in which pollination takes place by wind, a great majority of our crop plants are pollinated by a variety of animals, particularly insects. Amongst insects, managed bees as well as native unmanaged wild species of bees are the most important pollinators of crop species. Pollination limitation in crop species results in a marked reduction in crop productivity.

The following are the major causes for inadequate pollination in crop plants:

- Reduction in the density and abundance of native pollinators because of the loss of their habitat
- Extensive use of pesticides and herbicides and a marked increase in the level of pollutants to which pollinators are very susceptible
- Monoculture cropping system, particularly in the Western countries, in which the same crop is grown in a large area often extending to hundreds of hectares
- Climate change resulting in the disruption of precisely timed relationship between plants and pollinators

Apart from productivity, deficient pollination also affects the quality of fruits. In multi-seeded fruits such as apple, tomato, pumpkin and melon, pollination deficiency reduces the number of seeds in fruits and results in smaller and often deformed fruits. Under conditions of inadequate pollination, the application of additional fertilizers

or improved agronomic practices would not be effective in enhancing the yield.

Information on the details of pollination ecology of our orchard and seed crops is essential to develop methods to sustain pollination services. In Western countries, the problem of insufficient pollination is being effectively overcome by careful management of managed pollinators particularly honeybees and bumblebees for pollination services (*see* James and Pitts-Singer 2008). This approach has been used routinely in the USA since the 1940s and has grown steadily over the years into a well-organized multimillion dollar industry. The technology is being followed in many other countries. Managed honeybees are regularly used for pollination services of several crops such as almonds, apples, blackberries, blueberries, cherries, cranberries, pears, plums, squash, tomatoes, watermelons, canola and sunflower.

Managed honeybees are not efficient pollinators for all crop species. They have been in use for many field and greenhouse grown crops as they were the only managed pollinators available until recently and also the ease of their transportation. Several wild bees such as bumblebees and solitary bees such as species of *Megachile*, *Osmia* and *Nomia* are more efficient pollinators than honeybees for several crop species. In recent years, the technology has become available to manage some of these bee species and is being used for commercial pollination services (Slaa et al. 2006; James and Pitts-Singer 2008; Mader et al. 2010; Willmer 2011).

Another important outcome of recent investigations on pollination ecology of crop species has been the importance of native wild bees in increasing the yield even in the presence of managed pollinators (Garibaldi et al. 2013; Brittain et al. 2013a, b). In a recent study based on pollination of 41 crops from 600 field sites distributed throughout the world, Garibaldi et al. (2013) showed that pollination of crop species with wild insects is more effective than honeybees, and enhancement of fruit set by honeybees and wild insects is independent. Thus, managed honeybees supplement rather than substitute pollination services rendered by wild insects. These studies, apart from highlighting the importance of wild pollinators in crop species, clearly indicate that managed honeybees cannot compensate for the loss of wild pollinators. The trend is to develop integrated use of wild and managed pollinators by making the habitat pollinator friendly to increase the density and diversity of wild pollinators (James and Pitts-Singer 2008; Garibaldi et al. 2013).

In spite of enormous progress made in the developed countries on the management of pollinators to optimize crop yield, the use of managed pollinators is rather limited in the developing countries (Roubik 1995). They continue to depend largely on wild pollinators for pollination services. Several studies in the developing countries in general and India in particular have indicated that pollination is a constraint for seed and fruit set in a range of field and orchard crops (Deodikar and Suryanarayana 1977; Prasad et al. 1989; Sihag 1995; Partap et al. 2001). A recent study has indicated that a decline in pollinator populations has resulted in reduced vegetable yields in the country (Kinver 2010). In the Himalayas (covering India, Nepal, Bhutan, China, Pakistan and Afghanistan), a clear decline in the yield and quality of fruits in orchard crops such as apples, pears and almonds and seed crops such as buckwheat due to pollination constraints has been recorded (Dulta and Verma 1987; Partap et al. 2001; Ahmad et al. 2004). However, apple is the only crop in which managed honeybees are being used to some extent (Partap 2010). In some provinces of China, extensive manual pollination is being followed to sustain apple productivity (Partap and Partap 2000).

Lack of data on pollination ecology, particularly on the details of pollinators of different crop species and their competing species, density and diversity of the pollinators and their temporal and spatial variation, is the major constraint in making use of managed pollinators for pollination services. Except apple in which there is considerable information, there are hardly any in-depth studies on the pollination ecology of any other crop species. It is, therefore, important to study the role of wild pollinators in sustaining crop yield. In the absence of data on the diversity and

abundance of wild pollinators and also on long-term monitoring of pollinators, we do not know the fate of the populations of wild pollinators. For several crop species, we do not have even the basic information on their pollinators/pollinator guilds. This requires build-up of expertise and initiation of intensive studies on pollination ecology of crop species in the developing countries.

Another area of crop productivity which has a direct relevance to reproductive ecology is genetic improvement of our crop species. So far, hybridization has been the most important approach for genetic improvement of the crop species. One of the main objectives of the breeding programme in recent years is to transfer genes imparting tolerance/resistance to biotic and abiotic stresses from wild species to the cultivars. Most of them are incompatible. There are a range of techniques available to overcome crossability barriers (*see* Shivanna and Sawhney 1997). Information on the details of crossability barriers is required for an effective application of such techniques.

1.3 Reproductive Ecology and Release of Transgenics

There has been a major controversy throughout the world on the release of transgenics. One of the important concerns has been the possibility of pollen flow from the transgenic crop species to their wild relatives and their eventual introgression into wild populations (Armstrong et al. 2005). The introduction of transgenic crops particularly in the centres of origin of specific crop species where many of its wild relatives would be growing is likely to result in the transfer of transgenes into wild relatives and thus may affect the biodiversity (Bawa et al. 2011). Regulatory authorities need extensive data on various aspects of reproductive ecology of the transgenics and their wild relatives to rule out the possibility of the escape of engineered genes to the wild before permitting transgenics for field trials and their eventual release for cultivation. Thus, every new transgenic plant species requires a great deal of research in assessing the following four basic aspects (Marvier 2008):

- Potential for hybridization of transgenics with wild relatives
- Rate of hybridization
- Opportunities for backcrossing and introgression of transgenes into wild populations
- Ecological impacts of transgenes in the wild populations

The assessment of these areas of transgenics involves research into the following aspects of reproductive ecology:

- Spatial and temporal distribution of other cultivars and wild relatives in the area where the transgenics are grown
- Flowering phenology of transgenics and their wild relatives
- Temporal details of pollen viability of transgenic plants
- Temporal details of stigma receptivity of related wild species growing in the area
- Details of pollination ecology and pollen flow of transgenic plants through biotic and abiotic agents
- Compatibility relationships between transgenics and other cultivars/wild species
- Details of hybrid seed development, hybrid seedling establishment and the potential for the development of backcross seeds with wild populations and species

Expertise on reproductive ecology is required to collect data on all these aspects.

There are a number of books and reviews covering fundamental aspects of flower development (Greyson 1994), pollen biology and biotechnology, pollen–pistil interaction and seed development (Maheshwari 1950, 1963; Erdtman 1969; Stanley and Linskens 1974; Shivanna and Johri 1985; Johri et al. 1992; Raghavan 1997; Shivanna 2003; Ramawat et al. 2014) and details of pollination biology (McGregor 1976; Moore and Webb 1978; Faegri and van der Pijl 1979; Real 1983; Jones and Little 1983; Richards 1986; Free 1993; Roubik 1995; Harder and Barrett 2006; Waser and Ollerton 2006; Patiny 2012; Willmer 2011). Realizing the importance of biodiversity and reproductive biology, many international journals have brought out special issues on the importance

of biodiversity and its management, structural and functional aspects of reproductive biology, pollination biology and mating system (Anonymous 2004, 2009, 2010, 2011b, 2012). Seed biology and seedling recruitment have been covered in several volumes (Baskin and Baskin 1988; Turner 2001; Fenner and Thompson 2005; Ghazoul and Sheil 2010). When compared to a large number of compilations on the structural and functional aspects of reproductive ecology, there are hardly any manuals covering the practical aspects of reproductive ecology. A few which are available cover pollen biology and pollination biology (Shivanna and Rangaswamy 1992; Dafni 1992; Kearns and Inouye 1993; Dafni et al. 2005). However, there are no manuals/handbooks covering the whole spectrum of reproductive ecology. An attempt is made to fill this gap in this manual. The chapters cover all the major areas of reproductive ecology – the phenology, floral biology and sexuality, pollen and pistil biology, pollen–pistil interaction, pollination ecology and fruit and seed ecology and seedling establishment – which are fundamental to the studies on reproductive ecology. The format followed is essentially similar to the earlier manual on pollen biology (Shivanna and Rangaswamy 1992).

1.4 Some General Guidelines on Protocols

Some general guidelines on the protocols described in this manual are given in the following paragraphs.

1.4.1 Familiarity with Study Plants, Study Sites and Relevant Literature

An essential prerequisite for studying reproductive ecology of any species is to become familiar with the study plants and the study sites. One should become thorough with the taxonomy (correct name, synonyms, the authority and the family) and morphology of the plant, its flowers and fruits by careful observations and by reading the literature. Latest edition of Mabberley's *Plant-Book* (Mabberley 2008) is the best source for nomenclatural details and basic features of families and genera. Some journals that are based on taxonomic studies recommend online herbarium databases to validate the correct scientific name and authority (e.g. www.ipni.org) or the list of available published literature (e.g. Kew Bibliographic Database). The investigator has to become familiar about the distribution of the study species and also if the species is endangered or endemic. Important ecological features of the study sites also need to be recorded.

It is essential to become familiar with the available information on the biology of the species by reading relevant literature in a number of standard journals such as the *American Journal of Botany*, *Annals of Botany*, *Biotropica*, *Botanical Journal of the Linnean Society*, *Journal of Tropical Ecology*, *Plant Species Biology*, *Flora, Ecology* and *Oikos*. Additionally, the most recent studies on relevant topics may be gathered from websites such as www.pubmed.com, www.scopus.com, www.sciencedirect.com and www.jstor.org, which have huge collection of earlier literature on studies on reproductive biology. It is always better to read as many papers as possible on the scope of the selected topics and methodologies used by different investigators. As the abstracts of the papers are available for free online, there should be no difficulty in keeping track of the literature on reproductive ecology. If the investigator does not have access to the journal, one can request the author for a PDF (author's e-mail is generally given in the abstract). The best method to become familiar with the techniques needed is to read methodologies described in as many papers as possible covering the aspects of reproductive ecology selected for studies. This would enable the investigator to understand the scope of the study and apply suitable method(s) in his/her studies more rationally. Often, a given protocol may need modification. It is not practicable to give all possible modifications needed in the protocols. The familiarity with the system and the literature would help the investigator to modify the protocol suitably, if found necessary.

Another essential requirement, which is often ignored by the investigators, is to prepare herbarium specimen of the study species. The herbarium specimen should be properly labelled with all the relevant information and deposited in a nearest recognized herbarium as a permanent record of the species investigated. Herbarium sheets are very important for authenticating and clarifying any identification doubts of the species by future investigators. Even fixed materials of flowers and fruits of different stages would be very useful. Representative samples of floral visitors have to be prepared using standard entomological techniques.

1.4.2 Special Requirements

Routine laboratory items such as microscopes, slides and cover glasses and glassware such as beakers, test tubes and pipettes are not included under the requirements. Only those which are specific to the protocol or may not be readily available in a general laboratory are included. While some of the reagents need to be prepared fresh, several of them, particularly stains, can be stored under refrigeration for many weeks or even months.

1.4.3 Sample Size

For most of the protocols, adequate sample size is a must. The sample size is not generally mentioned in the protocol since it depends on a number of factors such as logistics, the scope of the study, available plant material, resources and other facilities. Also each protocol has to be replicated. It should be remembered that the more the sample size and the number of replicates, better and more dependable are the results.

The details of reproductive events, particularly those involving plant–animal interactions, vary between locations and over the flowering period. Therefore, reproductive events cannot be generalized on the basis of the data collected on one population during a part of the flowering period. It is always desirable to study 2–4 populations extending to the entire period of flowering for dependable results. If the study populations happen to be located in disturbed and undisturbed areas or at different latitudes or altitudes, comparative data would provide valuable information on the effects of such variables on reproductive events.

1.4.4 Stage of Flower Buds

In several protocols, especially those on stigma receptivity, controlled pollinations and breeding system, flower buds have to be handled as the starting material for emasculation and bagging. The flower buds to be selected should be before anthesis and anther dehiscence. In many of the species, this applies to flower buds 1 day or <1 day before anthesis. However, in some species anthers dehisce before anthesis. In such species the buds have to be selected before anther dehiscence for emasculation and bagging.

The word anthesis has been used by some authors to indicate various events during the life of the flowers, while others have restricted the term to the opening of the flower. The term anthesis is used in the manual to indicate just the opening of the flower.

1.4.5 Temporal Details of Observations and Fixations

In almost all protocols, observations and/or fixations have to be done over a period of time. Most of these protocols involve developmental events such as the phenoevents, duration of pollen viability, onset and duration of stigma receptivity, pollen germination and pollen tube growth, fruit development, seed germination and seedling establishment. Temporal details of observations to cover all these events are highly variable between species and the aspect of study; it may be in minutes, hours, days, weeks and even months. Thus, no realistic time frame can be given in the protocols. The investigator has to fix frequency of observations and fixations where needed based on preliminary studies and/or available literature.

1.4.6 Recording of Data

The need to maintain meticulous records of all the data cannot be overemphasized. For a beginner, many of the observations appear simple, straightforward and routine and not worthy of record. He/she would soon realize that in research no observation/result is minor or unimportant. Therefore, the results of various treatments have to be recorded in as much detail as possible. This will greatly facilitate writing of the paper or dissertation. Often such details are necessary for revision of the manuscript based on referees' comments.

Similarly the maintenance of proper labels (written with permanent ink) on study plants particularly of perennial species, the biological material used in various treatments, slides, collected/fixed materials and seed samples is very important. This information would be useful even after the specific research programme is completed as the same investigator or any other investigator may use some of those plants/materials fixed/collected to study the same or any other aspect of reproductive ecology. Similarly, the records of phenological events and sexuality particularly of the perennial plants or populations are very useful for long-term studies. Such records are invaluable in studies on the effects of climate change.

1.4.7 Photography

The documentation of observations and results with suitable photographs/diagrams/line drawings is an important part of research on reproductive biology. Photographs and other relevant illustrations of plants, flowers, floral parts, seeds and fruits, pollinators and predators need to be prepared. They can also be supplemented with movies particularly on pollinators, predators and seed dispersers. Several types of digital SLR cameras, flash attachments and video cameras are available in the market. Many of the digital cameras and mobile phones also have options to record movies for a limited time. They may be effectively used to document various aspects of reproductive ecology being studied.

1.4.8 Statistical Analyses

Statistical analyses of the data are essential component of any studies on reproductive ecology. The type of analyses to be applied essentially depends on the objectives of the investigation. The researcher has to be familiar with some basic statistical analyses by using statistical software such as SPSS which are readily available in most of the institutions. A skilled investigator would first choose the statistical test suitable for analysis and then collects the data accordingly. However, this does not bar the mostly used approach, where the data is collected first and then analysed statistically. As a prerequisite, it is essential to determine whether the data is normally distributed. Depending upon the outcome, a suitable parametric or non-parametric test may be applied for the analysis of collected data. If the investigator is not familiar with the type of statistics to be applied, consultation with an expert is desirable before starting the work. For analyses of any biological data, Sokal and Rohlf (1995) is the most useful source of information.

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Study Plants and Sites

2

Studies on reproductive ecology are generally aimed at collecting empirical data on a focal species or a community or testing some concepts/hypotheses, formulated by the investigator based on available information or prevalent in the literature. The most important prerequisite for initiating studies on reproductive ecology on any species is to become familiar with the published literature on the biology of the selected species. This will also help the investigator in framing relevant questions with better focus and refining the objectives of the study. If studies are being undertaken to test a prevailing hypothesis or a concept, a thorough knowledge on the concept/hypothesis to be tested would enable the investigator in selecting suitable system(s) and in effective planning of such studies.

Familiarity with the distribution of the species and its habitat through the use of local *Floras*, *Herbaria* and other published sources would greatly help the investigator in selecting study sites and populations. Considerable care has to be taken in selecting the study sites. A broad survey of the localities where the species naturally occurs would help the investigator to get an idea of the habitat, the extent of disturbances prevailing in the habitat and the density and distribution of the populations. Selection of study site(s) largely depends on the type of enquiry planned, the details of the available populations and also the logistics of the localities. Once the sites of the study are selected, the latitude, longitude and elevation of the selected localities have to be recorded using a GPS system. Apart from recording the topographic details of the study site (such as the contour, whether dry or wet/marshy, along the river or road), a record of the other species in the community particularly those that co-flower with the study species is also necessary. It would be useful to collect the records of maximum and minimum temperatures and rainfall of the previous 3–5 years and during the years of the investigation from the meteorological office of the area. It is also useful to prepare a map of the area indicating the study sites and also the populations used. For perennial species especially trees, it may be necessary to indicate the location of individual trees on an enlarged map.

Correct identification of the selected and other species in the community with the help of Floras, Herbaria or in consultation with experts is one of the most important aspects of any investigation. Any wrong identification of the species would create enormous problems in the literature. It is essential to prepare voucher specimens in the form of herbarium sheets for each population of the species to be studied. Care should be taken not to plunder or denude the population by making too many collections for herbarium sheets or fixation, when the species is critically endangered and the population size is too small. In many protected areas, the collection of plant materials and animals is banned. The investigator should be aware of such restrictions in the study area, and permission has to be obtained from the authorities for the collection of the materials.

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The investigator should be able to describe in detail the habit and habitat of the species and morphological details of the plant and flowers using technical terms (see Lawrence 1964; Radford 1986 or any other book for basic terminologies).

2.1 Labelling of Plants and Flowers

It would be better to label all the plants of the species in the study population by giving serial numbers. As it may not be possible to use all the plants present in a population for detailed studies, a suitable number of plants (depending on the type of study and the resources available) have to be selected either randomly or by demarcating line/belt transects or quadrats. In some studies, particularly on pollination ecology and seed dispersal and recruitment, it may be necessary to make a map of the study site marking individuals of the focal species (and other major species of the area, if needed) using their serial numbers. The type of label and the method of labelling depend on the habit of the plant, the duration of the planned studies and the extent of disturbances the labels are likely to be exposed to public or animals. We have found that soft aluminium tags which can be engraved by writing with a ball pen (by pressing hard while writing) are convenient and long lasting. A metal wire can be inserted through the punched hole at one side of the tag and used to tie the label to the stems of the plants or branches. Aluminium tags of different sizes are available in the market. When the plants are herbs, a wooden stick or a metal rod of suitable size can be pegged to the ground close to the plant, and the labels can be tied to one end of the stick/rod. The stick carrying the label can also be used to support branches or the whole plant if they are delicate. For labelling trees, the labels should be of sufficient size to be recognized easily from a distance and should be tied or nailed to the main trunk or one of the main branches. Alternatively, the trees can also be labelled by using galvanized iron plates of suitable size, labelling them with paint and fixing them to the trunk with nails at breast height. In tree species, it is desirable to measure the height, diameter of the trunk at breast height (BDH) and the spread of the canopy of each tree selected for investigation.

For labelling the flowers, paper tags are adequate for species in which the reproductive events occur for a few weeks. Such paper tags, if not readily available in the market, can be made using a stiff paper sheet. The paper sheet can be cut into about 3 cm^2 or 3×2 cm rectangles and a thread of 8–10 cm long inserted through a hole made at one side of the label for tying. All labelling on the tags should be made with a permanent marker or waterproof ink. If the studies are of short duration lasting only for a few weeks, pencil or ballpoint pen may be used for labelling. If individual flowers cannot be tagged because of their small size or compact arrangement, it is convenient to mark individual flowers by putting a dot on a sepal with a permanent marker; a different colour for each treatment/day may be used.

If phenological events extend for longer duration or occur during the wet season, the labels have to withstand water. Some investigators (Dafni et al. 1997) have recommended the use of electric insulation tape cut to suitable size, labelling them with a marker and twisting it around the pedicel of the flower or peduncle of the inflorescence using the sticky surface. Aluminium tags of smaller sizes are more convenient to use for long-term labelling of individual flowers, inflorescences and branches. If the flowers are small, commercially available aluminium tags can be cut into smaller size, and a hole is punched at one side of the label to insert a wire to tie the label.

2.2 Isolation of Flowers, Inflorescences and Plants

Experimental studies on reproductive ecology, particularly pollination ecology and breeding system, require isolation of individual flowers with suitable devices. Controlled pollinations in which pollination is carried out manually with the pollen of the known type are important

components of any studies on pollination ecology. In these studies, adequate precautions have to be taken to make sure that only the pollen of the desired type land on the stigma by preventing contamination from any unwanted pollen.

In herbaceous species, it is often convenient to isolate the whole plant by covering it with a cage made of pollen/insect-proof cloth with proper supporting frame so that the fabric will not disturb the flowers or delicate parts of the plant. If necessary, the cloth used for isolation may be made of two layers, if the pollen grains of the species are too small or its pollinators are too small. The cage should come in contact with the soil all around the plant so that insects/pollen grains cannot pass through the space between the cage and the soil. Wherever there is a gap, additional soil can be put to cover the gap. Further provision can be made to lift only a part of the cloth to carry out any treatments to the flower after isolation.

Bagging of individual flowers or inflorescences or flowering branches is more convenient; for larger shrubs and trees, this is the only effective method. When the flowers are large, isolation of individual flowers can be conveniently carried out through the use of pollination bags, routinely used by plant breeders for crop species. Pollination bags of different sizes made with a variety of materials such as fabric, wax paper and brown paper are available in the market. The size of the bag to be used depends on the size of the flower to be bagged. The bags have to be tied carefully to the pedicel of the flower with a thread. We have found that folding the bag carefully at the base without damaging the flowers and stapling with a stapler is more convenient than tying. In studies on manual pollination, it is necessary to open the bag to carry out pollination when the stigma becomes receptive and rebag the flower after pollination. In such studies, it would be more convenient to fold the base of the bag carefully into pleats (without leaving much space for insect to pass through) and put a paper clip. The clip can be easily removed without damaging the flower for pollination. After pollination, the bag can be tied or stapled to monitor fruit development. In herbaceous plants, the flowers are often delicate and cannot support the bag. In such species, the bags may need the support of a neighbouring branch or a short stick/cane pegged to the ground.

Bagged flowers have to be labelled properly before or after bagging depending on the convenience. As pointed out earlier, the type of label used to identify bagged flowers depends on the duration of the experiment. For short-term experiments, paper tags are light and convenient for labelling. When the studies are conducted during the rainy season and in species in which the time between the flower and mature fruit lasts for several months, aluminium tags are more convenient. The tags can be tied/hung around the pedicel of the flower.

When the flowers are small or borne in compact inflorescences/clusters, it is not feasible to isolate and bag the individual flowers. In such species, the whole inflorescence can be used for bagging. As the inflorescence contains older flowers, flower buds of different stages and even young fruits, it is necessary to remove fruits, opened flowers and young buds from the inflorescence retaining only those flowers to be treated (generally older buds just before anther dehiscence and anthesis). In some species, it may be more convenient to bag the branch/branchlet bearing a number of flowers of suitable stage. Here also, older flowers, young buds and developing fruits in the branch/branchlet need to be removed before bagging to retain only the buds of the desired stage needed for treatment. For bagging the branchlet/branch, it is desirable to use bags made of a fabric so that it does not affect aeration. Also, larger paper bags needed to bag inflorescences, and branches may get torn during handling. The nature of the cage or the bag should be such that it should cause no change or minimum change in the temperature, light and humidity conditions inside the bag when compared to ambient conditions. Any major change in these parameters may affect floral events and thus induce artefacts in floral responses. Plastic bags are not suitable as they result in increased humidity and temperature inside the bag and markedly affect the microclimate of the flower. Even for paper bags, many investigators recommend making some small holes by piercing them with a

needle to permit some aeration. However, they should be small enough to prevent the passage of any pollen or insects. Paper bags need to be discarded after one use. Cloth bags can be reused, but they have to be decontaminated of their pollen load by keeping them in an oven at about 100 °C for 1–2 h and allowing them to cool before reuse.

When isolations are carried out to exclude some type of pollinators but not all pollinators, the mesh size of the fabric used for bags has to be selected. To check for anemophily, for example, the bags should exclude insects while allowing access to airborne pollen. The use of bags or cages made up of mosquito net or bridal veil with 1–2 mm^2 mesh serves the purpose. When pollen carried in the air as well as insects has to be excluded, a paper bag or a bag made up of fine fabric of single or double layers would be suitable.

In some species, thrips or ants may enter the flower and forage the nectar and or pollen and even bring about pollination; they are difficult to exclude through conventional bagging. To prevent thrips from entering the flowers, some investigators have used an effective insecticide on flowers/inflorescences/flower branches (Baker and Cruden 1991; Sharma et al. 2009). The insecticide used should not affect the plant or the flowers and may have to be sprayed every other day or once in a week depending on the situation, and the concentration of the insecticide has to be standardized.

Entry of ants into the flowers of herbaceous species can be prevented by applying insecticide to the soil around the focal plant. However, in larger shrubs and tree species, preventing ants is more difficult since they move on the plant all the time and can enter through little spaces left when the bags are tied. We have found the application of strong nondrying glue (such as Stickem) or sticky ribbons around the proximal and distal parts of the stem (about 2 cm strip) bearing the flower/inflorescence/flowering branch and then tying the bag firmly around the pedicel of the flower/inflorescence to be effective in preventing ants reaching the flowers. If the flowers are borne on terminal branches, the stem proximal to the flowering part is smeared with the nondrying glue, and the flowering branch is bagged. Ants cannot move across the glue or sticky band as they get stuck to the glue.

Bags made of chicken wire or nylon bird netting (with about 1 cm mesh) can be used to exclude birds but allow entry of insects. Many investigators have grown plants under insect-proof netting or insect-free glass houses for pollination studies. This approach is easier for controlled pollinations, and more importantly it does not alter the microclimate of the flower unlike the use of pollination bags. However, this method of isolation cannot be used to study reproductive ecology of the species in their natural habitat. Also, this method is not suitable even for wind pollinated species as the prevailing conditions under the net house/glasshouse may permit some pollen flow in ambient air.

A few investigators have used, particularly for small flowers, dialysis tubing for isolation (Schemske et al. 1978; Bierzychudek 1987). Dialysis tube is cut to the desired length, and the flower is carefully inserted into the tube. The tube is then tied at both ends with a thread or stapled. Dry dialysis tube cannot be opened easily; it has to be soaked in water for a few minutes before separating the fold and allowing it to dry before using it to cover the flower.

Some researchers have used many other devices such as dried grass straw (Cruzen 1990) or paper/plastic drinking straw available in different sizes (Thomson et al. 1989), to isolate only the stigma/pistil, instead of bagging the whole flower to conduct pollination studies. This method causes least disturbance to the flower and, if carefully done, does not require emasculation. The straws of a suitable width are selected, cut into the desired length and inserted to enclose the pistil. The open end of the straw is blocked with a small amount of cotton. This will allow gaseous exchange but not pollen/insect.

2.3 Field Kit

Field studies regularly require a range of instruments and other items. Special efforts have to be made to make sure that all the items are collected

and taken to the field each time. We have found it very convenient to fabricate a field kit from a fold-out plastic box (*see* Appendix A.6) making provision to keep all the items needed for field studies (Fig. A.2). By carrying the kit, we avoid searching for all the items needed each time we go to the field; it also helps to keep the items together in the field without losing any of them. Another gadget we found very useful is OptiVISOR (Fig. A.2). It is a binocular headband magnifier available in different magnifications (1.5–3.5×) and allows the visor to be tilted out of the way when not needed. It reduces eye strain and leaves both hands free to do field operations.

These days almost all the items, including books, required for studies on reproductive ecology can be ordered online. One can find suitable items needed and their cost in the Internet and make selection. This makes procurement of required items very convenient and fast.

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Phenology

3

Phenology refers to the responses of living organisms to seasonal and climatic changes. In flowering plants various reproductive events, termed phenoevents, such as the onset of flowering and fruiting and seed dispersal, follow a seasonal pattern. There is great variation amongst the species in timing of these events, their duration, intensity and frequency.

Studies on the phenology of populations/communities are important to understand long-term adaptations of the species to prevailing environmental conditions for reproductive success. Studies on the phenology are also important to understand the relationships between plants and animals. Annual variations in phenoevents may occur due to variability in the environmental conditions particularly the rainfall, day length and temperature. The role of environmental variations on various phenoevents from year to year can be analysed by comparing phenological records of plant populations over the years. Also, data on the phenology of populations along the latitudes, longitudes and elevations give general trends of plants' responses to these variations.

Accurate and regular recordings of phenoevents of species along with the prevailing climatic parameters are invaluable for long-term monitoring of the phenology in response to a change in the climate. Phenology is one of the most sensitive reproductive events to climate change, and shifts in various phenoevents over the years help in predicting the degree of such shifts in the coming years. Wherever good records are available, changes in the timing of phenoevents and migration of species to higher elevations and latitude have been consistently observed (Lovejoy 2010). Probably the most accurate long-term records available on the phenology are the flowering time of cherry blossom (*Prunus jamasakura*) for over 1,200 years in Japan. Cherry blossom festival has been an important feature of Japanese culture from time immemorial. Because of its cultural significance and popularity, the records of festival dates (coinciding with the peak of flowering, lasting for 2–4 days) are available since the ninth century. These records show that from 1971 to 2000, cherry blossom plants flowered on an average of 7 days earlier when compared to the average of all previous records (Primack et al. 2009). This shift in early flowering of cherry species has been projected to be around 30 days by 2100 (Allen et al. 2013). Information on flowering phenology, the timing, duration and frequency of various phenoevents particularly of the forest tree species is also important for an effective management of forest resources (Bawa and Ng 1990).

Phenology of flowering plants can be studied at different levels: the flower, individual plant, population and community. The studies should be extended for several years. At the level of the flower, the major phenoevents cover the timings of flower opening (anthesis), anther dehiscence, stigma receptivity, pollination and the longevity of the flower. Phenology of individual plants does not serve much purpose as it does not indicate the

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responses of the population. At the population level, phenological events cover the whole population, and at the community level, they cover all the species growing in the community.

3.1 Population Phenology

The following are the major phenoevents to be recorded in the plant and amongst plants in the population:

Initiation of leaf primordial or leaf flushing (in deciduous species)
Initiation of inflorescences and/or floral buds
Initiation, duration, intensity of flowering
Floral phenology (anthesis and post-anthesis changes until senescence)
Initiation and duration of fruit setting
Fruit ripening
Details of seed dispersal
Initiation and completion of leaf fall (in deciduous species)

Phenological studies are carried out through regular field visits. The frequency of observations and the number of plants to be selected for recording the phenoevents depend on the objectives of the study and temporal progression of various phenoevents in the selected populations. Some of the major phenoevents such as the initiation of flowering and fruiting may be recorded at weekly or fortnightly intervals. However, for floral phenology, it is necessary to take observations at shorter intervals (hourly/daily). For tall tree species, direct observation of the phenoevents on the canopy may not be feasible; a binoculars may be used to observe the details of phenoevents. Often there is a progression of flowering from the base of the canopy to the tip or the other way. This can be easily determined by observing inflorescences and branches at different levels of the canopy by using a pair of binoculars during each visit. As the timing of floral events and their progression are dependent on the prevailing environmental conditions such as temperature, humidity, day length, precipitation and light conditions, it is necessary to record them during the studies and correlate any deviations between years to the variables.

A particular phenoevent in the population is considered to have initiated when about 10 % of the individuals in the population are in the phase. The event is considered to be at its peak when >50 % of the individuals are in the same phase. The event is considered to be at the end when less than 10 % of individuals have remained in the phase. The phenoevent is considered to have been completed when no individuals are in the phase (Dafni 1992).

3.2 Floral Phenology

Floral phenology includes all the events that occur from the opening of the flower until the flower remains functional (able to donate pollen and/or receive pollen). The period for which the flowers remain functional (longevity/life span) is generally indicated by the freshness of the flower. After its functional period, the floral parts lose their turgidity and freshness; the anthers dry up or abscise and the stigma shows signs of senescence. Floral longevity is highly variable between species. Floral phenology has to be recorded frequently in time slots of 1–4 h depending on the species for several days.

In many species, the longevity of the flower is confined to just 1 day or < 1 day. For example, in *Malvastrum coromandelianum*, the flowers remain fresh only for about 2 h (Shivanna 2014). In several others, flower longevity extends for more than 1 day, often several days. In some species of Myrtaceae, the flowers remain fresh for up to 19 days (*see* Lughadha and Proenca 1996). In most of the orchids, the longevity of flowers lasts for many weeks. In *Phalaenopsis*, for example, the flowers remain fresh for about 3 months (Arditti 1979; Zhang and O'Neill 1997). In several species, the longevity of the flower depends on pollination; pollinated flowers start senescing generally within a day after pollination, whereas unpollinated flowers continue to remain fresh for longer periods. In *Petunia*, flowers start senescing soon after pollination, whereas unpollinated flowers remain

fresh up to 7 days (Shivanna and Rangaswamy 1969). To find out if pollination has any role in floral senescence, senescing flowers have to be excised and observed under the microscope for the presence of pollen on the stigma and correlated with pollination. Alternatively, one set of flowers can be manually pollinated at intervals and checked with reference to unpollinated flowers. In a number of species such as *Lantana camara*, pollination results in a change in the colour of the petals, although the flowers remain fresh (Mathur and Mohan Ram 1978). This has implications in attracting floral visitors and directing them to unpollinated flowers. Such details need to be observed carefully in the focal species and recorded.

3.3 Community Phenology

Phenological events in a community give information of plant population dynamics and food resource availability to sustain animals that depend on nectar, pollen, fruits and/or seeds. Marked changes in the phenoevents may disrupt precisely timed relationship between plants and animals which may affect the reproductive success of the species in terms of pollination efficiency, seed set, seed dispersal and seedling recruitment.

For community ecology, the phenoevents described above for population phenology need to be recorded for all the species growing in the community. Therefore, community phenology has to be recorded throughout the year to cover all the species. Studies on community phenology are necessary to not only understand the availability of rewards for floral visitors and fruit/seed dispersers but also a possible competition among plants for pollinators and amongst animals for available resources. It would be rewarding if phenological studies on plant species in the community are combined with the identification of floral visitors and fruit foragers and, if possible, their density; such data will give better information on plant–animal interactions in the community.

3.4 Protocols

3.4.1 Phenology of Populations

The phenology of sufficient number of individuals in the population has to be recorded. Depending on the number of plants available and their density, either all plants of the population (if the number is limited) or a random sampling of plants (if the number of individuals in the population is large, particularly in herbaceous species) may be included for the studies. Random sampling may be made by making a line transect or a quadrat or a circle of suitable size and labelling the enclosed plants for recording the phenoevents. If necessary, these enclosures can be subdivided into smaller segments, and some of them used for studies. If the species is a perennial, it is desirable to label all the plants by giving a permanent number to each plant and record major phenoevents such as the onset and intensity of flowering and fruiting of all the plants. This does not take much time and can be recorded easily each year. In such species the labels used should last for several years. Studies on major phenoevents of all available plants are particularly useful in tree species, in which all plants in a population may not flower every year; in some plants flowering occurs in alternate years and in others it may be irregular. In such species, flowering events have to be recorded for each tree for each year accurately. In dioecious species, it is important to record sexual fidelity of individual plants over the years. It is, therefore, necessary to carry out phenological studies on tagged individuals for 2–3 years to get meaningful results. Details of floral phenology, which requires more time and efforts, may be limited to some selected trees.

3.4.1.1 Special Requirements

Suitable tags/labels to identify individual plant/flowers/inflorescences (see Chap. 2 for details).

3.4.1.2 Procedure

1. Label all the plants to be used for recording the phenoevents. For prolonged observations particularly of trees in a forest, use aluminium

or any other permanent labels which can last for years.
2. Record the date of initiation and duration of flushing of new leaves in each of the selected plants (in deciduous species).
3. Record the date on which the flower buds become clearly visible.
4. Record the date of initiation of flower opening on each plant. As the flowering progresses, record the intensity of flowering (profuse/moderate/poor) and the duration of flowering in selected plants. Based on the records, determine the initiation, peak and end of the flowering in the population.
5. Record the initiation of fruit development (number of days taken from anthesis until the fruits are morphologically recognizable) and its progression until fruits maturity.
6. Record the date when ripened fruits become visible (number of days from the day of anthesis to fruit ripening) and its duration (until all the fruits have ripened). This may take a few days or weeks or even months depending on the species.

3.4.1.3 Modification

Many investigators have recommended quantitative phenology which involves counting the number of flowers that open on each day on each plant during the entire flowering period (see Kearns and Inouye 1993). As this involves considerable work, the number of plants used would be less than those used for qualitative phenology and are selected randomly. Counting of flowers of the entire plant during each observation may not be difficult in herbs and small shrubs. In large shrubs and trees, however, it may not be feasible to count the total number of flowers during each observation. In such species, it would be convenient to tag specific number of inflorescences/branches selected randomly at different parts of the plant/canopy and record phenoevents on these inflorescences/branches. Count the number of flowers that open on each inflorescence/branch on each day until they cease to produce new flowers. If necessary, the data can be extrapolated to the whole plant by counting the total number of branches and suitably multiplying the number of flowers recorded from the counted branches. When the longevity of the flower extends for more than a day, older flowers have to be marked in some way to restrict the counting to freshly opened flowers during each observation. The quantitative data on flower opening can be represented in the form of a graph over the flowering period. Quantitative phenology would be particularly useful in studies of community ecology as it would highlight the quantitative variation in resource availability of different species.

3.4.2 Phenology of Flowers

Recording the details of floral phenology is very important particularly in pollination studies. Studies on floral phenology in species in which longevity of flowers is extended for more than a day are more elaborate when compared to those in which flowers remain fresh just for a day. This is because on any given day, each plant/inflorescence bears the flowers opened on different days; they have to be distinguished by suitably labelling/marking flowers, with different colours or by any other means, opened on each day.

3.4.2.1 Special Requirements

Suitable tags or labels to mark individual flowers.

3.4.2.2 Procedure

1. Tag a good number of older flower buds that would open during the next 1 or 2 days. The buds for tagging have to be selected randomly from several plants (at least 10 plants are recommended). Generally 20 flower buds from each plant in 2 replicates of 10 each are recommended ($10 \times 2 = 20$). When the flower buds/flowers are too small to tag, they can be marked with a permanent marker by putting a dot(s) on the sepal/pedicel in different patterns/colours to keep track of the phenoevents in the labelled flower buds. The flower number can be written on the tag tied to the inflorescence/branch bearing such flowers. It is better to identify each plant and each flower with a label (e.g. label A/1 refers to plant A, flower 1) to record the phenoevents.

2. *Temporal details of flower development*: Observe tagged buds every day and note, for each bud, morphological changes such as the size and colour, extent of emergence of the corolla beyond the sepals and the colour of extended corolla. These details will help in the selection of buds of the desired stage for various treatments.
3. *Temporal details of flower anthesis* (*opening of flower*): Record the date and time of initiation and completion of anthesis (opening) of each tagged flower. In most of the species, this is confined to a few hours. Also the time of anthesis of different flowers in a population may be completed within a few hours or spread over several hours or even the whole day and/or night. If the flowers are borne on an inflorescence, record the type of inflorescence and the sequence of opening of flowers in an inflorescence (acropetal/basipetal/centripetal).
4. *Anther dehiscence*: Record the time and duration of anther dehiscence (in terms of h/days before or after anthesis). In some species all the anthers of a flower dehisce simultaneously (synchronous). In others, particularly in species in which anthers are numerous or at different levels, anther dehiscence may be nonsynchronous. In several species, anthers may dehisce before anthesis. In such species flower buds have to be opened and observed for anther dehiscence. To fix the period of anther dehiscence (h/days before anthesis), the flower bud of comparable stage may be tagged and monitored until anthesis.
5. *Flower longevity*: This is generally expressed from the time/day of anthesis. For studying the longevity of the flower in species in which the longevity lasts for many days, it is better to label each flower on the day of opening (by suitable marking on the flower/on the tag) so that the longevity of each flower can be recorded easily. Keep track of each flower for signs of senescence and record the longevity in terms of the number of days after anthesis. Also check if the longevity depends on pollination or is independent of pollination. Eventually, a labelled flower either abscises or develops into a fruit. In unisexual flowers, all the male flowers abscise, and amongst female or bisexual flowers, some of them develop into fruits and the remaining flowers abscise.

Floral phenology, in a broad sense, also covers the time and duration of nectar secretion, stigma receptivity, pollen viability and pollination events. As these aspects require more elaborate studies, they are generally studied separately, and the protocols are described elsewhere in the manual.

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Floral Morphology and Sexuality

4

Flowering plants show great variation in their size, longevity and morphology. They may be herbs or shrubs or trees or climbers or creepers or epiphytes. Their life span is also variable; they may be annuals or biennials or perennials. Annuals complete their life cycle in 1 year; they produce seeds towards the end of their life cycle and die. Biennials devote the first year to vegetative growth building up the reserve of resources and enter reproductive phase in the second year; after seed production they die. Perennials live for many years. When once they reach a required age (depending on the species), perennials enter reproductive phase repeatedly. In some perennial species, particularly the tree species, flowering may not occur every year; they may flower in alternate years or at intervals of several years. Also the extent of flowering and fruiting may not be the same in each flowering season; some years they produce large seed crops alternating with years of lean or no seed production. In some such species, all the individuals of a population tend to synchronize their flowering event resulting in a bumper crop (masting) in years of large seed production (Kelly 1994; Fenner and Thompson 2005; Sakai et al. 2005).

Plants are classified into two categories based on the number of times they flower in their life span. Monocarpic species produce flowers only once in their life span, while polycarpic species produce flowers repeatedly for an indefinite period. Annuals and biennials are typically monocarpic and perennials are polycarpic. However, even amongst the perennials, a few such as some species of bamboo (*Bambusa bambos*, *Dendrocalamus strictus* and *Phyllostachys bambusoides*) and *Strobilanthes* are monocarpic. They produce flowers gregariously once in their life in a synchronized manner and die. The period of their vegetative growth before flowering varies between a few years to hundreds of years. For example, *Strobilanthes kunthiana* flowers after 12 years, while one of the bamboos, *Phyllostachys bambusoides*, flowers after 120 years. Monocarpic perennial species accumulate reserves over a long period and allocate maximum possible resources to seeds since they flower only once and die. Polycarpic perennials generally allocate lesser resources to seeds each year and thus avoid exhaustion to safeguard future reproduction.

4.1 Morphology of Flower

In angiosperms the flower is the unit of sexual reproduction. Therefore, studies on reproductive ecology should start with an understanding of the structural and functional aspects of the flower. Flowers may be solitary, produced in the axils of leaves or produced in groups called inflorescences. In spite of their enormous diversity in size, shape, colour and morphological features, flowers are built on a basic pattern of four whorls – the calyx (made up of sepals), corolla (made up of petals), androecium (made up of stamens) and gynoecium (made up of carpels). In

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some species calyx and corolla are not differentiated; there is only one whorl termed perianth (made up of sepals). Floral whorls develop from the swollen tip (thalamus) of the floral axis (pedicel). There are innumerable variations in the details of these whorls. The calyx and corolla, although do not play a direct role in the production of fruits and seeds, the end products of sexual reproduction, facilitate reproductive events. The calyx, made up of generally greenish sepals, is traditionally considered as an organ involved in the protection of the flower bud. In some species sepals are conspicuous and serve as organs of advertisement to the pollinators. In some others, they are persistent (remain fresh and green until fruit maturity) and supply photosynthates to the developing fruit. The corolla is made up of coloured petals. The ornamentation of the flower in most of the species is because of the petals which serve as visual attractants to pollinators. In several species, petals develop nectar guides or the guide marks, which direct the pollinators to the nectar. In many of the animal pollinated species, there is a nectary or several nectaries at the base of the ovary between the petals and the ovary. In some species, the corolla changes colour after pollination and enables pollinators to avoid visits to pollinated flowers. In a few species such as *Euphorbia*, and the members of Araceae, the bracts become conspicuous and perform the function of advertisement. When the lobes of calyx and/or corolla are free, such condition is referred to as polysepalous/polypetalous, and when they are fused, the condition is referred to as gamosepalous/gamopetalous. In many species, they are fused at the base but free towards the tip.

The two inner whorls, the androecium and the gynoecium, are the units involved in reproduction. The androecium is made up of stamens. The number of stamens is variable. All the stamens may be of the same size or show differences in their dimensions. Each stamen has an elongated slender stalk termed the filament and a terminal thicker, four-lobed anther. Each anther contains four microsporangia (pollen sacs) in which the male gametophytes, the pollen grains, develop. When pollen grains are mature, the anther dehisces and releases pollen grains. The gynoecium is made up of carpels. In some species, particularly in basal angiosperms, the carpels are free. In most of the species, however, the carpels are fused, and the fused structure is generally referred to as the pistil. The pistil has a swollen basal ovary, elongated style and terminal stigma. The ovary contains ovules which house the female gametophyte, the embryo sac.

Flowers may be actinomorphic (radially symmetrical) or zygomorphic (bilaterally symmetrical). An actinomorphic flower can be divided into two equal halves by any vertical section passing through the centre. A zygomorphic flower can be divided into two equal halves only by one vertical section passing through the centre.

The following are the basic features of floral morphology to be studied:

Arrangement of flowers: Flowers may arise as solitary units in the axils of leaves or in clusters as inflorescences. When flowers are borne on inflorescences, the type of inflorescence and the average number of flowers per inflorescence have to be recorded. It is important to make sure that the number of flowers per inflorescence includes all the flowers produced throughout the life of the inflorescence. This may require counting of open flowers on each day over a period of the inflorescence's active period. This will also provide data for calculating the average number of flowers that open each day per inflorescence.

Morphological features: Apart from describing the overall shape of the flower, the length and width of the flower and morphological details of each floral whorl (number, shape, size, colour, arrangement and any other feature) have to be described in as much detail as possible. For stamens, the length of the filament and the anther need to be recorded. For the pistil, apart from the size of the stigma, style and ovary, the number of ovules has to be determined. For scoring the quantitative details, the number of samples used should be adequate, and the flowers should be collected randomly not only from the same plant but also from other plants in the population so that the data can be used for statistical analyses. Studies on floral morphology in species with

smaller flowers may require the use of hand lens or a stereomicroscope. A millimetre scale can be used to measure flowers and floral parts in most of the flowers to the nearest mm. To measure the length and width of very small flowers/floral organs, an ocular micrometre may be used under low-magnification stereomicroscope; the length and width may be measured in ocular units which can be converted into mm/μm by calibrating the stereomicroscope. A digital caliper is most convenient to measure the length and width of flowers/floral organs and also the diameter of the ovary, fruit or any other three-dimensional structure. The investigator should become familiar with the morphological terms used to describe the flower. These terminologies are adequately explained in many taxonomic books. Lawrence (1951) and Radford (1986) are good sources for such definitions and illustrated descriptions.

4.2 Sexuality of Flowers, Plants and Populations

Flowers of a large number of species are bisexual bearing both the stamens and the carpels; such flowers are referred to as perfect flowers (Fig. 4.1a). In bisexual flowers, the anthers and the stigma may be spatially separated; such condition is called herkogamy. In several species, pollen shedding and stigma receptivity may be synchronous or temporally separated (dichogamy). In a majority of species, stigma receptivity and pollen shedding occur simultaneously or within a short time on the day of anthesis. In several species the stigma becomes receptive before the pollen grains are shed; this condition is referred to as protogyny. In yet others, pollen grains are shed before stigma receptivity; this condition is referred to as protandry. The time gap between stigma receptivity and pollen

Fig. 4.1 (**a**) Bisexual flower: *Passiflora* spp. (**b**) Male flowers in *Schleichera oleosa* with numerous stamens and a sterile pistillode (*inset*) at the base (*After* Gautam et al. 2009). (**c**) Voodoo lily, *Sauromatum guttatum* in bloom. (**d**) *Boxed area* of the spathe in **c** opened and magnified to show the distribution of male (*mf*) and female flowers (*ff*) at different levels in the same inflorescence (monoecy). The inflorescence emanates putrid smell and attracts the pollinator flies

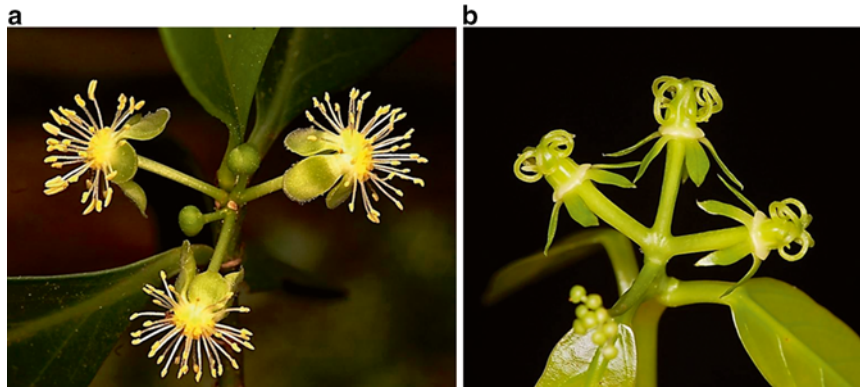

Fig. 4.2 Unisexual flowers: *Blachia denudata*. (**a**) Inflorescence with male and (**b**) female flowers (*Courtesy* Dr M.M. Sardesai)

shedding may vary between a few hours to several days.

There are a number of species in which each flower produces only the stamens or the carpels (imperfect or unisexual flowers) (Figs. 4.1b and 4.2a, b). Thus, at the level of the flower, there are only three sexual forms – bisexual, male and female. Morphologically, two types of unisexual flowers are distinguished; one type exhibits rudiments of opposite sex (staminodes in females and pistillode in males), while in the other type, no rudiments of the opposite sex can be seen. However, there is great variability in the expression of sexuality depending on the distribution of male (staminate), female (pistillate) and bisexual flowers within the plant and between plants in the population/species.

The following are the major types of sexuality in plants and populations:

Hermaphrodite: Individual plants bear only bisexual flowers.

Monoecious: Individual plants bear male and female flowers.

Andromonoecious: Individual plants bear male and bisexual flowers.

Gynomonoecious: Individual plants bear female and bisexual flowers.

Polygamomonoecious: Individual plants bear male, female and bisexual flowers.

Dioecious: Individual plants bear either male or female flowers.

Androdioecious: Individual plants bear either male or bisexual flowers.

Gynodioecious: Individual plants bear either female or bisexual flowers.

Polygamodioecious: Individual plants bear male or female or bisexual flowers.

As sexuality plays a major role in determining pollen flow and the breeding system of the species, it is important to study sexuality at the level of the flower, plant and population in detail. Species with bisexual flowers are most common and make up 72 % of the species, while dioecious species make up only 4 %. Gynodioecy is present in about 7 % of the species, but androdioecy is very rare and is reported only in a limited number of species (de Jong and Klinkhamer 2005).

Heteromorphic species produce two (dimorphic) or three (trimorphic) types of flowers. However, individual plants bear one of the two or three floral morphs present in the species. Thus, each plant can be assigned to a particular floral morph. The floral morphs in heteromorphic species differ in relative position of the stamens and the stigma. In dimorphic species, the two types of flowers are referred to as the thrum (short-styled) and pin (long-styled) morphs. In trimorphic species, the anthers are generally located at two levels, and the third level is occupied by the stigma. The three floral forms are referred to as the short-styled (anthers at long- and mid-levels), mid-styled (anthers at long and short levels) and long-styled (anthers at mid- and short levels) forms (see Chap. 9). Species of *Linum* and *Primula* are the well-known examples of dimorphic species,

while species of *Oxalis* and *Lythrum* are familiar examples of trimorphic species.

4.3 Cryptic Sexuality

In most of the species, sexuality of the flower can be easily determined by careful observation of the stamens and the pistil. In bisexual flowers, the stamens are well developed and dehisce normally, and the pistil shows well differentiated ovary, style and stigma. Both stamens and pistil appear healthy. However, in some species although the flowers are morphologically bisexual with well-developed stamens and pistil, functionally they are unisexual. This condition is termed as cryptic/functional sexuality. In cryptic dioecy, the flowers are morphologically bisexual in all the plants, but the pistil is not functional in male plants and the stamens are not functional in female plants (Dunthorn 2004; Kawagoe and Suzuki 2004). In *Nothapodytes nimmoniana* (Sharma et al. 2010), for example, the plants show typical dimorphic condition with pin and thrum flowers, but pin flowers are functionally female and thrum flowers are functionally male (Fig. 4.3a–d). Many *Floras* have described sexuality of such species wrongly as bisexual. Similarly, in cryptic monoecy (e.g. *Sterculia urens* – Sunnichan et al. 2004, *see* also Vary et al. 2011), the sexuality is morphologically andromonoecious with male and bisexual flowers produced on each plant, but in bisexual flowers, the anthers are not functional. Thus, morphologically bisexual flowers are functionally female, and the sexuality of the species is referred to as cryptic monoecy.

In species showing cryptic sexuality, morphological studies do not reveal the sexuality of the species; functional aspects of the stamens and the pistil have to be studied to determine sexuality. Many a times the plants may switch over to a different sex, thereby exhibiting sex lability. For example, in *Schleichera oleosa*, the male phase in some trees is frequently followed by monoecious phase in the same flowering season. Such trees are known as inconstant males (Gautam et al. 2009). In some plants such as oil palm, there may be alternate male and female phases (Tandon et al. 2001). Thus, regular monitoring of the sexuality of the plant during the flowering season is important to characterize the sexuality of a species (*see* also Sharma et al. 2010).

4.4 Reproductive Allocation

Reproductive allocation (RA) is the proportion of biomass or other resources allocated to reproductive structures relative to the total resources of a plant at a given point of time (see Bazzaz et al. 2000; Fenner and Thompson 2005; Groom and Lamont 2011). It is a measure of how the resources of the plant are partitioned between reproductive and vegetative parts. RA is highly variable between species; it is generally higher in annuals when compared to perennials. Outcrossing species allocate more resources to male flowers than to female flowers (Cruden and Lyon 1985). RA varies even within the species depending on their growth conditions. Under adverse conditions of growth, scarce resources may be preferentially diverted to reproduction as an adaptive response. This is particularly true in weed species (Baker 1974).

Reproductive effort refers to the net investment of resources in reproduction diverted from vegetative activity. However, many investigators do not distinguish between RA, RE and also reproductive cost and use them interchangeably. Most of the investigators assess RA on the basis of the percentage of dry weight of flowers and inflorescences when compared to vegetative structures (mostly above-ground parts: stem and leaves) using the formula:

$$\text{Reproductive Allocation} = \frac{\text{Dry weight of flowers or inflorescences} \times 100}{\text{Total or above ground dry weight of the plant}}$$

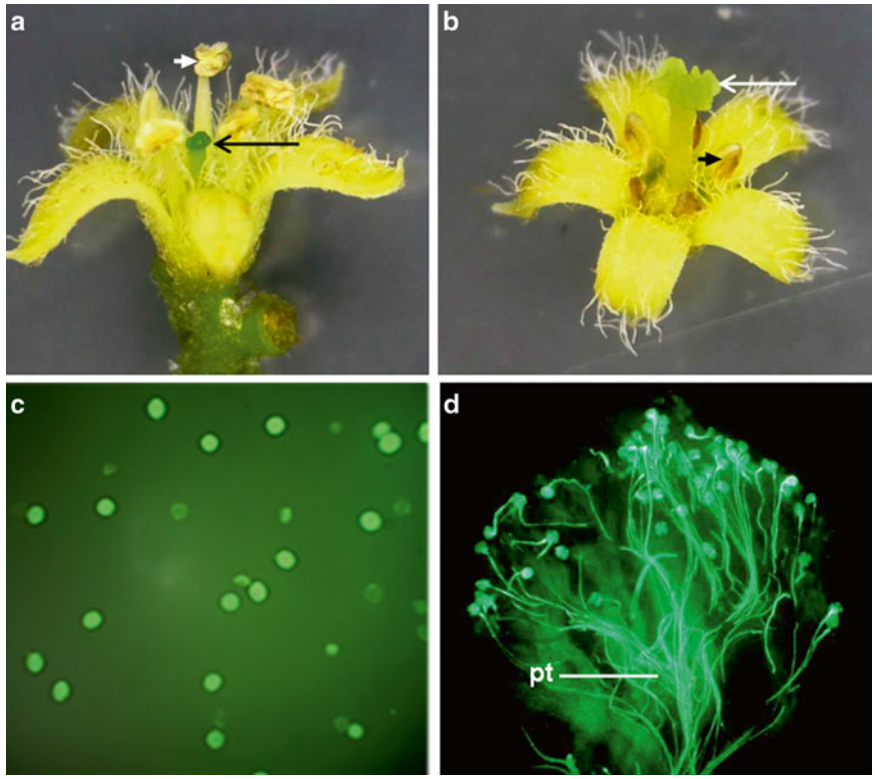

Fig. 4.3 Cryptic dioecy: *Nothapodytes nimmoniana*. The flowers are typically heteromorphic with thrum (**a**) and pin (**b**) morphs. But the thrum morph is functionally male with fertile pollen and pin is female with functional stigma. The anthers of male flower dehisce (*arrow head*), their pollen grains are viable as shown through fluorescein diacetate test (**c**), and they germinate and produce pollen tubes (pt) on the stigma of pin flowers as seen through aniline blue fluorescence (**d**). Anthers of pin morph (*arrow head*) do not dehisce and do not produce pollen. The stigma of thrum flowers (*arrow*) does not support pollen germination (*After* Sharma et al. 2010)

In shrubs and tree species, flowers or inflorescences are collected from unit length of twigs/branches/shoots (Armstrong and Irvine 1989). Dry weight of flowers and the stem and leaves of the selected twigs are taken for estimating RA.

4.5 Protocols

4.5.1 Morphology of Flower

This protocol explains the procedures to record important morphological features of the flower. The sample size should have sufficient number of flowers (at least 20) randomly selected from different plants. When different populations are used for the study, the details should be collected and compared from all the populations.

4.5.1.1 Special Requirements

Measuring scale
Hand lens or stereomicroscope
Digital caliper
Tabulated sheets for recording measurements

4.5.1.2 Procedure

1. Measure the size (length and width/diameter) of the flower and record its colour. The colour of the flower is largely determined by the colour of the petals. If the petals have nectar guides, record their colour, shape and orientation. Also record the fragrance/odour of flowers, if any, by smelling opened flower.
2. Record the number of units in each floral whorl (sepals, petals, stamens and carpels) and measure the size of individual units in each whorl (length and width). If the sepals

and/or petals are fused to form a calyx cup or corolla tube, measure the length and width of the cup/tube.
3. Measure the length of the anthers and of the filaments. When there are two types or levels of stamens, record the measurements for each type/level separately.
4. Determine the number of pollen grains produced per anther and per flower. Follow the procedure given in Protocols 5.5.1 and 5.5.2. When the anthers are of different types, determine pollen production in each type separately.
5. Record the number of carpels in each flower and whether they are fused or free.
6. Measure the length of each carpel (if separate) or the pistil from the base of the ovary to the tip of the stigma. Measure the length and diameter of the ovary, the length of the style and the size of the stigma (diameter/length).
7. Record the relative position of the anthers and the stigma. If the stigma is exerted beyond the corolla, measure the length for which the stigma is exerted.
8. Count the number of ovules in each carpel by dissecting the ovary preferably under a stereomicroscope. Also record the type of placentation (marginal, parietal or axile). If the number of ovules in each flower is large (100 s), follow Protocol 6.3.2 to count the number of ovules.
9. Observe the base of the ovary with a hand lens or a stereomicroscope and record the presence/absence of nectary/nectaries and the nectar. If present, record the shape and size of the nectary/nectaries. In many species, the nectar is not protected, and it accumulates between the ovary and the stamens. In others, the nectar accumulates in the tube of various lengths formed by the fused basal parts of the petals and/or stamens. If the nectar is present in the flower, determine the amount of nectar and its total sugar concentration (Protocol 7.7.1).
10. Study the floral features of each population. Compare the data from different populations, and if there are any marked differences, test their significance statistically and record.

4.5.2 Sexuality of Flowers, Plants and Populations

The determination of the sexuality of the species is important, as it affects pollen flow and to some extent reproductive success of the species. In monoecious and dioecious species, the rewards available in male and female flowers may vary, and it may affect the frequency of floral visitors.

4.5.2.1 Special Requirements

Hand lens or a stereomicroscope
Tabulated sheets for recording the sex of flowers/plants/populations

4.5.2.2 Procedure

1. Select a suitable number of plants in a population and number them with suitable labels.
2. Collect an adequate number of flowers (say about 20) randomly from each plant separately.
3. Score each flower from each plant for its sex (bisexual/male/female) by observing the presence of healthy stamens and pistil. In smaller flowers, a hand lens or a stereomicroscope may be needed to record the sex of the flower. When the flowers are borne in inflorescences, record the sex of all the flowers in randomly selected inflorescences; if the flowers show difference in sexuality, record the position and number of flowers of each sex in the inflorescence.
4. If the flowers are not bisexual, assign the sexuality at the level of the flower/plant/population. Estimate the amount of nectar present in male and female flowers (and bisexual flowers when present) (see Protocol 7.7.1 for details).
5. If the species is monoecious, determine the floral sex ratio (number of male to female flowers) on each plant.
6. If the species is dioecious, determine the sexuality of each plant, label the sex of each plant, and record the number of plants belonging to each sex type (male/female) in the population. For tree species, the sexuality of each tree has to be assessed based on sufficient number of randomly selected flowers.

7. If the species is andromonoecious or gynomonoecious, determine the ratio of male or female flowers to bisexual flowers in each plant.
8. If the species is polygamous, determine the ratio of flowers of each sex.

4.5.2.3 Modification

In several monoecious and dioecious species, the sexuality of a plant may change as the flowering period progresses. Some of the monoecious plants may develop some bisexual flowers; in dioecious plants, some flowers of the other sex or bisexual flowers may develop during the later part of the flowering. In such species, determine the sexuality of the plant during the early, peak and late flowering. This is done by recording the sexuality of plant at suitable intervals (weekly or of 15 days) depending on the duration of the flowering period. Also in perennial species, the sexuality of each plant needs to be monitored for 2/3 years to check its sexual fidelity.

4.5.3 Identification of Cryptic/Functional Sexuality

In these species, sexuality of the flower and of plant is not apparent as they show apparently healthy anthers and the pistil. One has to study their functional aspect with reference to pollen viability, stigma receptivity and fruit and seed set to determine the sex of the flower/plant.

4.5.3.1 Special Requirements

Acetocarmine stain

Requirements needed for controlled pollination (*see* Protocol 9.6.1)

Fluorescence microscope (*see* Protocol 8.5.1)

4.5.3.2 Procedure

1. When flowers of all the plants are apparently bisexual, observe different plants in the population for fruit development. Absence of fruit set in some and their normal development in others is an indication of functional dioecy in the species. The plants with normal fruit set are likely to be females, and those with no fruit set are likely to be males. Further confirmation has to be done by studying the structural and functional aspects of the stamens and the pistil.
2. Make careful observation of anthers of flowers of different plants, and see if they dehisce normally to expose the pollen grains. If anthers dehisce normally in flowers of some plants and not in others, it substantiates the presence of cryptic dioecy. Anthers in female plants generally do not dehisce. This is because pollen grains do not develop in those anthers or even if they develop, pollen grains abort. In some species sterile anthers may show dehiscence, but pollen grains are sterile.
3. To confirm the absence of pollen or presence of only the sterile pollen in such anthers, make acetocarmine squash preparations of undehisced anthers (see Protocol 5.5.3 for details). If pollen grains have not developed in anthers, no pollen grains are visible in squashes; they may often show degenerated meiocytes or microspores. If the pollen grains have developed, the preparations show aborted/sterile pollen grains. Aborted pollen grains appear shrivelled or empty. Score such flowers as male sterile.

   Acetocarmine preparations of pollen grains from fertile anthers (from male plants) show fertile turgid pollen filled with cytoplasmic contents. If facilities are available, the viability of pollen grains may be tested through fluorescein diacetate test (see Protocol 5.5.11). A detailed histochemical study of pollen grains from fertile and sterile anthers may be carried out by fixing flowers/anthers at different stages of development and cutting wax-embedded or resin-embedded sections of suitable thickness (usually 3 or 4 μm). Structural abnormalities in sterile anther may be recorded to validate floral sexuality.
4. Determine the structural and functional aspect of the pistil in potentially male and female plants. Dissect the ovary and check the presence of ovules. If they are present, compare their size and number in both types of flowers. In male flowers, ovules may be shrivelled or may not show any differences between potentially male and female flowers. If the ovules are shrivelled, it indicates that the pistil is not fertile.

5. When there are no differences in the size and number of ovules between potential male and female flowers and the style and stigma show normal morphological features, the only way is to study the functional aspect of the pistil through controlled pollinations.

   Carry out controlled pollinations on the two types of flowers (male and female) using pollen grains from potential male flowers (and female flowers if they produce pollen grains) (for details of controlled pollinations, see Protocol 9.6.1). After 24 h, study pollen germination and pollen tube growth in the pistils of both sets of flowers using aniline blue fluorescence (see Protocol 8.5.1). Pollen grains from functionally male flowers show normal pollen germination on the stigma of female flower but not on the stigmas of the male flowers indicating that the stigma of male flowers is not functional. As expected, pollen grains of female flowers (when they are produced) do not show germination on the stigma of either female or male flowers.
6. Record and label male and female plants in the population and determine their proportion.

4.5.3.3 Modifications

If a fluorescence microscope is not available, pollinated stigmas can be stained with acetocarmine or cotton blue in lactophenol to record pollen germination. If the stigmas are thick, longitudinal freehand sections may be cut before staining. Although this method may not be effective in tracing pollen tubes in the pistil, pollen germination and pollen tube entry into the stigma can be easily observed.

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Pollen Biology

5

Pollen grains represent male gametophytes and are the male partners in sexual reproduction. Following anther dehiscence, pollen grains are exposed into the atmosphere. At the time of shedding, pollen grains contain either 2 cells (a large vegetative cell enclosing the generative cell) or 3 cells (vegetative cell enclosing the two male gametes formed by the division of the generative cell) (Fig. 5.1a, b). The cytology of pollen at the time of shedding (2 or 3 cells) has important correlations with a number of physiological features of the pollen such as viability, storage and in vitro germination. In general, 2-celled pollen show longer viability, store well and germinate on a simple medium when compared to 3-celled pollen. Also the species with 2-celled pollen show gametophytic type of self-incompatibility, whereas those with 3-celled pollen show sporophytic type of self-incompatibility. Mature pollen grains generally contain reserve nutrients in the form of starch (Fig.5.2a) or lipids. The phase of pollen, from their shedding until they land on the stigma, is termed free dispersed phase; it plays a crucial role in plant reproduction as it facilitates gene flow because of their dispersal and transport to other conspecific plants/populations through various pollinating agents.

Pollen grains resemble seeds in many respects (see Chap. 10); both are desiccated units (their moisture content is generally <20 % when shed), dispersal units and units of gene flow. Similar to the refractory seeds of some species, pollen grains of several species are refractory and are shed with higher moisture content (>30 %); they cannot withstand desiccation. Pollen grains are ubiquitous and present in the air, water and soil. Pollen grains of some anemophilous species travel for long distances in air currents. They have been collected in Arctic and Antarctic regions, over 2,000 km away from the nearest seed plants (Linskens 1995). The amount of pollen produced in some tree species which are wind pollinated is enormous.

Pollen grain wall is not just a passive protective layer but a dynamic system performing various vital functions. It is one of the most complex wall systems found in plants. There are three domains of pollen wall – the surface layer (pollenkitt), the exine made up of sporopollenin and the pectocellulosic intine. Sporopollenin is one of the most resistant organic materials known. It can withstand high temperatures and extreme acidity and alkalinity. All the three domains of the pollen wall contain extracellular components particularly a range of proteins including enzymes. Pollen grain surface, especially in species pollinated by insects, contains, apart from other components, lipids and carotenoids which give a range of colour to pollen of different species. The extracellular components present in the three domains of pollen wall play an important role in pollen germination, pollen tube growth and incompatibility responses (see Shivanna 2003 for details).

Detailed studies on pollen biology are important in understanding reproductive strategies and

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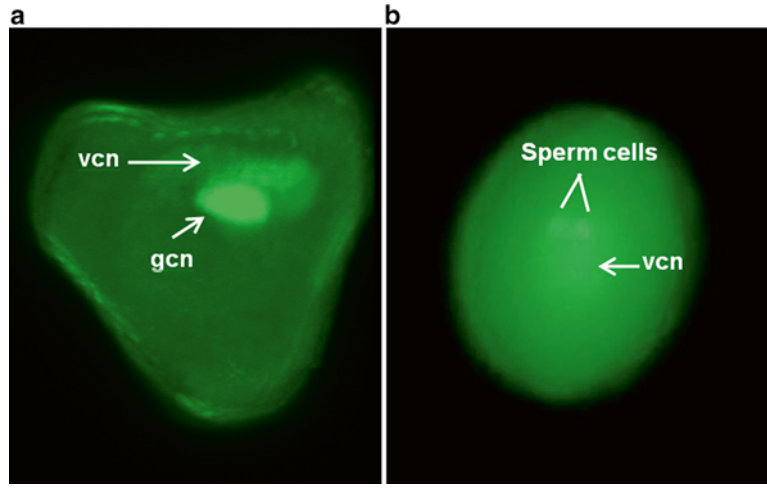

Fig. 5.1 Pollen cytology. (**a**) Fluorescence micrograph of Feulgen stained pollen grain of oil palm to show intensely stained generative nucleus (*gcn*) and moderately stained vegetative nucleus (*vcn*) located close to each other (**b**) Two intensely stained sperm cells (male gametes) in *Dalzelia zeylanica*; vegetative nucleus (*vcn*) is diffuse and thus very lightly stained

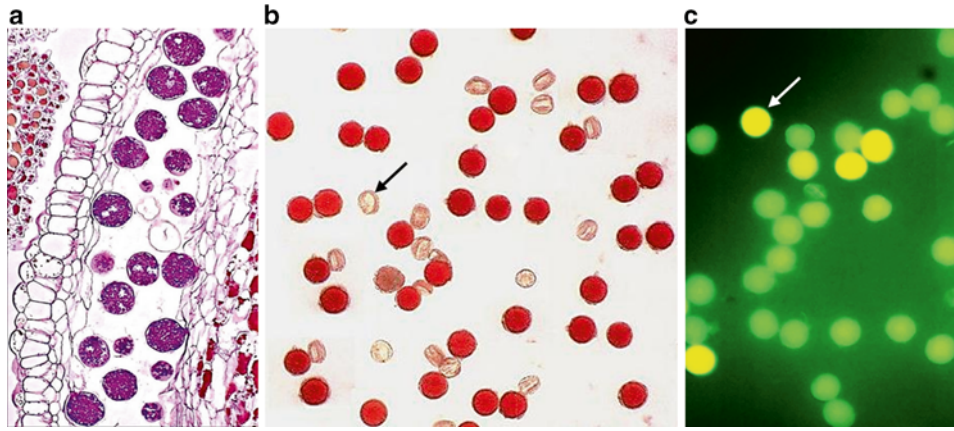

Fig. 5.2 Pollen biology. (**a**) Section of a mature anther to show pollen grains filled with starch grains following PAS staining. (**b**) Acetocarmine test for pollen fertility. Shrivelled pollen without intense colour (*arrows*) are sterile pollen. (**c**) Fluorescein diacetate test for pollen viability. Brightly stained pollen grains (*arrow*) are viable

reproductive success of plant species. Some of the features to be studied under pollen biology are briefly elaborated below.

5.1 Pollen Production

The estimation of pollen production is important to understand many aspects of pollen biology. The amount of pollen produced in each anther/flower varies greatly. In *Cannabis sativa*, for example, each anther produces >70,000 pollen grains, while some of the cleistogamous flowers in species such as *Commelina* produce <100 pollen grains per anther. Thus the resource allocated to the formation of pollen is highly variable between species. Pollen production also gives an indication of the type of pollination. Wind- and water-pollinated species generally produce more pollen when compared to animal-pollinated species. The calculation

of pollen-to-ovule ratio indicates, to some extent, the possible breeding system of the species (Cruden 1977). The ratio is much higher in cross-pollinated species when compared to self-pollinated species. Pollen grains constitute an important reward for pollinators. Pollen counts are also needed to estimate pollen load on pollinators and the amount of pollen removed by pollinators.

5.2 Pollen Fertility and Viability

Plants of hybrid origin often show various degrees of pollen sterility. Even adverse environmental conditions such as high or low temperature and water stress induce pollen sterility to different degrees (see Shivanna 2003). Staining pollen grains with acetocarmine is commonly used to score pollen fertility (Fig. 5.2b) (Protocol 5.5.6).

Pollen viability refers to the ability of the pollen to complete postpollination events on a compatible, receptive stigma and effect fertilization. Even the pollen samples, which are viable at the time of shedding, lose viability over a period of time; this may vary from less than an hour to several months. Prevailing environmental conditions, particularly temperature and humidity, affect the duration of pollen viability. For successful fertilization and seed set, pollen grains have to be not only fertile but also viable at the time of pollination. Thus, pollen viability is critical for successful fertilization and fruit and seed set (see Shivanna 2003). It is, therefore, important to know the extent of viability of pollen at the time of natural pollination and in pollen samples used for experimental studies. Also, it may be necessary to test the viability of the pollen sample collected from the floral visitor.

A number of methods are available to test pollen viability. The most authentic method to test pollen viability is to use pollen sample to carry out controlled pollinations (see Protocol 9.6.1 for details) and assess the extent of seed set. Alternatively, instead of waiting for seed set, one can study pollen germination and pollen tube growth in manually pollinated pistils to get information on pollen viability. However, manual pollinations and in vivo studies on pollen germination are laborious and time consuming. It also restricts the test period to flowering season of the species. Further, this test tends to be qualitative; even a small proportion of viable pollen can yield full seed set particularly in species with lower number of ovules. Thus, fruit and seed set test cannot be used as a routine test for pollen viability. Many alternative methods (see Protocols 5.5.9, 5.5.10 and 5.5.11) have been used to assess pollen viability (Fig. 5.2c).

5.3 Pollen Vigour

Pollen vigour refers to the speed of germination and the rate of pollen tube growth (Shivanna et al. 1991b; Shivanna 2003). It differs from viability since viable pollen samples may show differences in the vigour. Loss of vigour becomes apparent even before the loss of viability. Loss of vigour is well established in the biology of seeds. Until recently pollen vigour was not taken into consideration in assessing the quality of pollen. Several investigations have shown that aging and many environmental stresses, particularly temperature, humidity and desiccation, affect pollen vigour, and it may affect the ability of such pollen to compete with fresh pollen in effecting fertilization (Shivanna and Cresti 1989; Shivanna et al. 1991a, b; Rao et al. 1992).

When the pollen sample landing on the stigma contains a mixture of fresh and aged pollen (coming from older flowers when their longevity lasts for several days) or self- and cross-pollen, pollen may show variation not only in viability but also in vigour. In such a situation, pollen grains which are more vigorous would disproportionately sire more number of seeds than those with reduced vigour. In some studies concerned with pollination ecology and seed biology, it may be necessary to assess the vigour of the pollen in addition to viability.

5.4 Pollen Morphology

Pollen morphology is important in studies related to taxonomy, phylogeny, aeropalynology and paleobotany. Pollen morphology also plays a key

role in several other areas such as archeology and criminology and in testing the purity of honey (Bryant 1990; Shivanna 2003; Warny 2013). As morphological features are specific to each species/genus and also pollen mixtures of each geographic area are different, pollen grains can be used as a tool for identifying the scene of the crime and also in locating the origin of illegally imported drugs, medicines, art objects, food and other products. For effective use of pollen in any of these areas, pollen identification is a basic necessity. The role of pollen morphology in reproductive ecology is limited to describing pollen grains of the focal species and in identifying conspecific and heterospecific pollen load on the floral visitors and on the stigma.

Only a brief description is given here so that the researcher would be familiar with basic aspects of pollen morphology in order to describe and identify the pollen grains of the focal species and also to identify the pollen present on floral visitors. For more details reader may refer to several excellent books available in this field (Erdtman 1969; Nair 1970; Moore and Webb 1978; Faegri and Iversen 1989).

Pollen morphology is generally described in relation to its polarity. Polarity of the pollen grain is established with reference to the arrangement of the microspores in the tetrads. The pore closer to the centre of the tetrad is the proximal pole and the one away from the centre is the distal pole. The hypothetical axis connecting the two poles is the polar axis. The distal half and the proximal half of the pollen is described in relation to an hypothetical line along the equator of the pollen at right angles to the polar axis.

Pollen morphology is largely based on their size, shape, aperture and exine ornamentation (Fig. 5.3a–c). The size and shape of pollen grains vary markedly. Smallest pollen grains (ca 5 µm) are recorded in some members of Boraginaceae, and larger pollen grains are generally seen in members of Cucurbitaceae, Malvaceae and Nyctaginaceae. Perhaps the largest pollen measuring up to 350 µm is recorded in *Cymbopetalum odoratissimum* (Annonaceae) (Walker 1971). In a majority of the species, pollen size ranges between 15 and 50 µm. Filiform pollen grains measuring up to 5 mm are found in marine angiosperms such as *Amphibolis* and *Zostera* (Ducker et al. 1978).

The shape of pollen grains is generally described as seen either in the polar view (from the top) or equatorial view (from the side) (see Moore and Webb 1978 for more details). Pollen grains may be spherical, elliptic, triangular, quadrangular, rectangular or rhombic. The shape may vary, to some extent, depending on whether they are desiccated or hydrated. Desiccated pollen grains are frequently subprismatic because of the shrinkage of the exine, while hydrated pollen tends to be spherical or ellipsoidal.

Apertures are distinct regions in the pollen wall through which the pollen tube emerges. The exine is generally thin or even absent in the region of the aperture. Pollen grains are classified on the basis of the shape, number and position of the aperture. They may be in the form of circular pores or elongated boatlike furrows (Fig. 5.3a, b). Pollen grains which bear pores are termed porate and those with furrows as colpate. Colporate pollen grains show a circular pore at the centre of a furrow. In some species, the colpae fuse to form rings or spiral (Fig. 5.3c). The number of apertures is described with a suitable Latin prefixes, mono-, bi-, tri-, tetra-, penta-, hexa- and poly- along with the type of aperture, for example, mono-porate/-colpate/-colporate, bi-porate/-colpate/-colporate and poly-porate/-colpate/-colporate.

Variations in pollen morphology are largely related to the ornamentation of the exine surface (Fig. 5.3b, c). Outer part of the exine, called the sexine, is sculptured, and the inner part, called the nexine, is homogeneous. The sexine is made up of radially directed rods, the baculae/columella; they may stand free or fused with neighbouring ones. When they are free, the pollen grains are called non-tectate. When they are fused to form a roof, such pollen grains are called tectate.

In non-tectate pollen, the head region of the baculae may be club shaped (clavate), pointed (echinate), short and globular (gemmate), or swollen (pilate). When the baculae are fused with neighbouring ones, they form depressed areas (lumina) and elevated wall-like structures (muri);

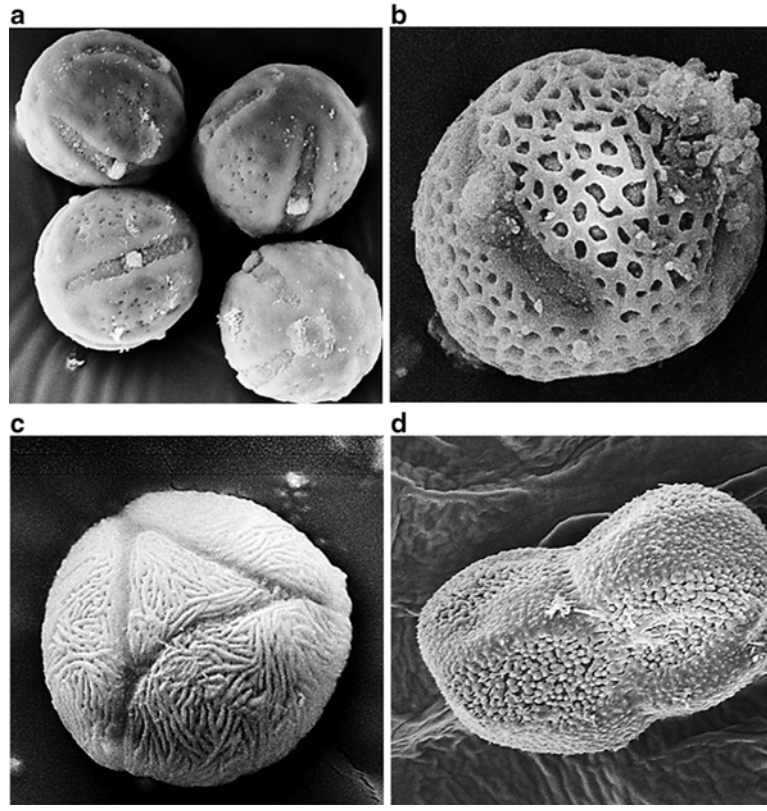

Fig. 5.3 Scanning electron micrographs of pollen grains to show exine ornamentation. (**a**) Colporate pollen grains (*Azadirachta indica*). (**b**) Colporate pollen with reticulate exine ornamentation (*Aegle marmelos*). (**c**) Syncolpate (colpae fused to form rings) pollen with striate exine ornamentation (*Schleichera oleosa*). (**d**) A compound unit of two pollen grains (dyad) in *Zeylanidium olivaceum*

the arrangement of muri and lumina produces different patterns. When the muri form a network, the pollen surface is described as reticulate (Fig. 5.3b), and when they run parallel to each other, as striate (Fig. 5.3c); an intermediate pattern is called rugulate. Instead of rod-like baculae, the sexine may often comprise hemispherical warts (verrucate), tiny flakes (scabrate) or small granules (granulate).

In tectate pollen grains, the baculae are generally referred to as columella and the roof is called the tectum. Pollen grains with partial tectum are described as semi-tectate. Tectate and semi-tectate pollen may bear additional projections on their surface which may be referred to as clavate, echinate, gemmate, pilate, reticulate, regulate, scabrate/granulate, striate or verrucate as in non-tectate grains. To study tectate or non-tectate nature of the pollen, one has to study the sectional view of the pollen wall under compound or electron microscope.

In most of the flowering plants, pollen grains are shed as solitary units (monads). However, there are several families that characteristically release pollen grains in groups (compound pollen). The compound pollen may contain units of two (dyad) (Fig. 5.3d), four (tetrad) or in multiples of four (polyads). In some species such as members of Asclepiadaceae and Orchidaceae, all microspores of a sporangium/anther remain together and are dispersed as massulae or pollinaria. Compound pollen units enhance pollination efficiency; the deposition of a single compound pollen unit may often be sufficient to achieve pollination of all the ovules present in the ovary.

As pointed out earlier, in studies on pollination biology, it is necessary to identify whether pollen grains present on the floral visitor and/or the stigma are conspecific or a mixture of conspecific/heterospecific pollen. A standard method for this is to prepare reference slides of pollen grains (see Protocol 5.5.5) from the focal species and other co-flowering species that are likely to be visited by the floral visitor. Identify pollen preparations collected from the visitor by comparing them with reference slides. Based on the size, shape, details of apertures and exine ornamentation, it is easy to identify the pollen sample (conspecific/heterospecific) carried by the visitor.

In some species lipoidal material on the surface of the pollen may mask its exine ornamentation. The treatment of pollen grains with organic solvents such as hexane, acetone or chloroform would remove the lipoidal material and reveal the surface ornamentation more clearly. The most commonly used technique to study pollen morphology, particularly exine ornamentation, is acetolysis (see Protocol 5.5.4). Acetolysis degrades all other contents of the pollen except the exine, thus revealing the details of apertures and ornamentation more clearly.

5.5 Protocols

5.5.1 Estimation of Pollen Production in Anthers with Limited Number of Pollen Grains

Pollen biologists have been using a number of methods to determine pollen production per anther and per flower. One of the methods used has been through the use of haemocytometre. In recent years, the use of haemocytometre is not very prevalent. One of the reasons is that the amount of pollen suspension used to count pollen is very limited when compared to the total volume. One can also use electronic particle counter, when available, to count pollen grains.

One of the two methods described here counts all the pollen grains produced in an anther; this is ideal when the anthers are small and produce only a limited number of pollen grains. The other method involves the preparation of a pollen suspension in a known volume and counting pollen grains from a sample of the suspension.

5.5.1.1 Special Requirements

Acetocarmine or safranin stain
Permanent black ink pen with a fine tip

5.5.1.2 Procedure

1. Take a microscope slide, and with the help of a permanent ink pen, draw thin parallel lines about 1 mm apart at right angles to the long axis of the slide. The distance between lines should be such that the two neighbouring lines should be visible under the low magnification of the microscope (10×). The width of the lined area on the slide should be wider than the cover glass you would be placing so that the cover glass will not extend beyond the lined area. Alternately you can gently draw permanent lines on the slide with a glass-etching pen. Such a slide can be used routinely for pollen counting.
2. Take a mature but undehisced anther (about to dehisce) and place it on the other side of the slide (without parallel lines) in a small drop of water/stain (acetocarmine or safranin are suitable) placed at the centre of the lined part of the slide.
3. With the help of needles and/or forceps, crush and squeeze the anther to release all the pollen grains. Remove the debris. Gently lower a cover glass of suitable size in such a way that the liquid does not flow/spread outside the cover slip.
4. Place the slide under a microscope to focus left top corner of the cover glass. Make sure that the lines and the space between the two adjacent lines are covered and in focus under the lower-magnification (10×) objective. Count the pollen grains present between the two lines from the top of the cover glass to the bottom of the cover glass by moving the slide (preferably with mechanical stage) along the parallel lines. Include pollen grains present on

the left line while counting. Repeat the counting sequentially covering each parallel line from left to the right of the cover glass so that the whole mounted area is covered for counting. If the mounting medium has spread slightly beyond the boundary of the cover glass, count all the pollen grains present in the mounting medium all around the cover glass. The total number of pollen grains counted from one anther gives the total number of pollen grains produced in an anther.

5. Clean the slide without rubbing the lower surface (marked with parallel lines) and repeat the counting for a number of anthers randomly selected from different flowers and plants. Calculate the average number of pollen grains/anther.
6. Multiply this number with the average number of anthers in a flower to determine the average number of pollen produced in a flower. Present the mean number with SE (standard error)/SD (standard deviation).

5.5.1.3 Modification

When anthers are of different lengths or sizes in a flower, pollen number in each type has to be counted and computed separately.

5.5.2 Estimation of Pollen Production in Anthers with Large Number of Pollen Grains

The procedure described under Protocol 5.5.1 is not suitable for anthers that contain a larger number of pollen grains. As all the pollen grains of an anther cannot be counted, suitable sampling method has to be followed.

5.5.2.1 Special Requirements

A glass/plastic vial of suitable size to prepare pollen suspension

A glass rod to crush the anther

A micropipette (about 100 µl capacity)

Sucrose/glycerin solution of suitable density (2–5 %) or a drop of detergent (such as liquid soap) to keep pollen grains in suspension

Pasteur pipette or disposable syringe (without needle) of about 2 ml capacity

5.5.2.2 Procedure

1. Suspend one mature but undehisced anther in a suitable amount (1–5 ml depending on the size of the anther and number of pollen grains) of sucrose/glycerin solution or water containing a drop of detergent solution taken in a 5–10 ml vial. Crush the anther with a glass rod to release all the pollen grains from the anther. Mix the suspension. Pollen release and mixing can be enhanced by drawing of the suspension from the vial into a Pasteur pipette or disposable syringe (without needle) and expelling it back to the vial with force repeatedly. Remove visible debris with a pair of fine forceps.
2. While gently shaking the suspension, take (using a micropipette) a suitable volume of the suspension (20–50 µl depending on the concentration of pollen in the suspension so that pollen grains spread sparsely making it convenient to count) and place it on a slide marked with 1 mm wide parallel lines as described in Protocol 5.5.1. The optimization of the amount of liquid used to make pollen suspension and the amount of suspension taken would require some trial runs.
3. Lower a cover glass and count the number of pollen grains as described in the Protocol 5.5.1.
4. Repeat steps 2 and 3 at least for 10 samples of the suspension.
5. Estimate the total number of pollen grains in the suspension by using the following formula:

$$\frac{\text{Total volume of pollen suspension} \times \text{No. of pollen grains in the sampled suspension}}{\text{Volume of suspension used for counting}}$$

6. Repeat pollen counts (using the above procedure) for the required number of anthers selected randomly from different flowers of the same plant as well as other plants.
7. Calculate the average number of pollen per anther.
8. Multiply this number with the number of anthers in a flower to determine the average number of pollen per flower.
9. Present the mean number with SE/SD.

When anthers are of different lengths or sizes in a flower, pollen number in each type has to be calculated and computed separately.

5.5.2.3 Modifications

1. Pollen suspension, instead of placing on the slide as a drop, is spread on the slide in the form of a narrow band (the width of which can be covered by low magnification of the microscope). The pollen grains present can be counted by moving the slide from one end of the band to the other.

   If the pollen grains are sufficiently large to be clearly seen under a stereomicroscope, the band can be slightly broader and pollen grains can be counted under a stereomicroscope.
2. Kearns and Inouye (1993) have described another simple method. In this one, pollen grains are suspended in a known volume of ethanol and a sample of the suspension is placed on a microscope slide. Addition of a few drops of ethanol onto the slide would enable better spread of pollen. Allow ethanol to evaporate and count the pollen grains under a microscope. Drawing parallel lines with a marker as described in Protocol 5.5.1 would make it convenient to count.

5.5.3 Estimation of Pollen Fertility

5.5.3.1 Special Requirements

Acetocarmine stain

5.5.3.2 Procedure

1. Collect pollen from freshly dehisced anthers and place them in a drop of acetocarmine taken on a clean microscope slide.
2. Mix pollen grains with the help of a needle.
3. Lower a cover glass. Warm the slide gently over a spirit lamp/burner and apply gentle pressure over a piece of blotting paper placed on the cover glass to remove excess stain but do not rupture the pollen. Leave the slide for about 5 min for pollen to take up stain. As fertile pollen grains are filled with contents, they take up deep and uniform stain (Fig. 5.2b). Sterile pollen grains are shrivelled/incompletely filled or empty; they do not take up deep, uniform stain.
4. Score pollen grains for sterility/fertility under a microscope using bright illumination. Under each microscope field, count the number of pollen grains with uniform deep colour as fertile and those which are or unstained or partially stained as sterile. Record data using at least ten microscopic fields. Repeat this with a sufficient number of anthers collected from different flowers and plants. Calculate per cent fertility.

5.5.4 Acetolysis of Pollen Grains

This method is used to study exine ornamentation in detail. Acetolysis removes all non-sporopollenin components of the pollen (the contents, intine and components present in and on the pollen wall) leaving only the exine intact.

5.5.4.1 Special Requirements

Acetolysis mixture: acetic anhydride and concentrated sulphuric acid (9:1). Add the acid slowly to acetic anhydride in the fume hood; as it results in exothermic reaction, the container gets hot.

Low-speed centrifuge.

Glacial acetic acid.

5.5.4.2 Procedure

1. Collect sufficient amount of pollen grains (2–4 mg) and suspend them in about 10 ml of acetolysis mixture taken in a test tube.
2. Heat the suspension to boiling in a water bath for 1–2 min. Remove the tube and allow it to cool.

3. Transfer pollen suspension into a centrifuge tube. Remove acetolysis mixture by low-speed centrifugation (about 2,000 rpm).
4. Add about 5 ml of glacial acetic acid to the centrifuge tube. Mix pollen pellet and remove acetic acid by centrifugation.
5. Add about 5 ml of water to centrifuge tube and mix pollen pellet in water.
6. Mount pollen grains on a slide in water or 10 % glycerol and observe under the microscope. They can also be mounted in glycerin jelly as semi-permanent preparations for later observations and also as reference slides (see Protocol 5.5.5).

5.5.4.3 Modification

If acetolysed pollen grains have turned dark, bleach the pollen in chlorine. For this, add 2 ml of glacial acetic acid +2–3 drops of saturated sodium chlorate solution +1–3 drops of concentrated hydrochloric acid. As chlorine is released, gently stir the mixture for about a min. Remove bleaching mixture by centrifugation and suspend pollen grains in water. If necessary, acetolysed pollen grains can be transferred to 70 % ethanol and stored for later studies.

5.5.5 Mounting of Pollen Grains in Glycerin Jelly

For any morphological study such as size, shape, apertures and exine ornamentation, pollen grains have to be mounted in a suitable medium. Although temporary preparations can be made in acetocarmine or safranin, the structure of the pollen grains may not be clear. Glycerin jelly, containing some stain such as basic fuchsin, has been used routinely as a mounting medium by pollen morphologists. Glycerin jelly becomes solid at room temperature, and it has to be melted for mounting. Both fresh and acetolysed pollen can be mounted in glycerin jelly.

5.5.5.1 Special Requirements

Glycerin jelly (see Appendix A.3 for details of preparation)
Spirit lamp/hot plate
Paraffin wax

5.5.5.2 Procedure

1. Take a small amount of pollen sample (to be mounted) on the middle of a clean microscope slide.
2. Scoop a small piece of glycerin jelly by using forceps or a spatula and place the jelly in contact with the pollen sample so that pollen grains adhere to the jelly.
3. Warm the slide gently on a spirit lamp/hot plate until the jelly melts. Do not allow it to boil.
4. Keep the slide under a stereomicroscope and spread the pollen in the melted jelly by gently stirring with a needle. Rewarm the jelly if it solidifies during stirring.
5. Lower a cover glass carefully over the jelly. Allow it to spread into a circle of 4–5 mm and rewarm the slide, if necessary.
6. While the slide is still warm, place the slide inverted (cover slip facing down) with support on both the sides with some solid material so that the cover glass remains hanging without touching anything. This will allow pollen grains to settle near the cover glass so that pollen grains can be observed under higher magnification of the microscope.
7. Observe the preparation or store for later observation.

Glycerin jelly slides remain in good condition for many weeks or even months. They should be stored under dust-free atmosphere.

5.5.5.3 Modification

The slides can be made permanent by sealing the slide with paraffin wax. After step 6, place a small amount of paraffin close to the edge of the cover glass on one side and heat the slide until the wax melts. The wax is drawn under the cover glass and surrounds the glycerin jelly mount. Scrape off the excess wax with a blade or clean the slide with xylene and store in a slide box.

5.5.6 Acetocarmine Squash Preparations to Study Pollen Cytology

The number of cells in the pollen (2 or 3 celled) at the time of shedding is important as it is

correlated with a number of physiological responses of the pollen. Pollen cytology can be studied through acetocarmine/Feulgen squash preparation or the use of DNA fluorochromes.

5.5.6.1 Special Requirement

Acetocarmine stain

5.5.6.2 Procedure

1. Transfer a small amount of pollen to a drop of acetocarmine taken on a slide.
2. Mix pollen suspension with a needle and lower a cover glass. Warm the slide over a flame but do not boil. Cool the slide. Take out excess stain with a piece of blotting paper.
3. Keep the slide on a flat surface, spread two or three layers of blotting paper pieces on the cover glass and apply pressure with the thumb avoiding any lateral movement of the cover glass. The aim is to rupture the pollen wall and spread the contents evenly. It is desirable to maintain the identity of the contents of individual pollen so that the vegetative nucleus and the generative nucleus/sperm nuclei can be assigned to individual pollen.
4. If the slide is to be observed some time later after the preparation, seal the edges of the cover glass with nail polish or paraffin wax and maintain the slide in a slide box free from dust.
5. Observe the preparation under a high-power/oil immersion objective of the microscope and look for the vegetative nucleus and the generative nucleus/the two sperm nuclei. Generally, the vegetative nucleus takes up faint stain because of its dispersed chromatin, while the generative nucleus/sperm nuclei take deeper stain as they have condensed chromatin. Observe nuclear details in 15–20 pollen grains and record whether the pollen grains are two/three celled.

5.5.7 Feulgen Squash Technique to Study Pollen Cytology

The basis of this technique is hydrolysis of the purine deoxyribose linkages of DNA with warm HCl to open up aldehyde groups on the deoxyribose sugar. The fuchsin reacts with the open aldehyde and produces an insoluble DNA-specific coloured complex.

5.5.7.1 Special Requirements

Feulgen stain: Dissolve basic fuchsin (0.5 g) in 100 ml of boiling distilled water, cool to ca 50 °C and filter. Add 10 ml of N-hydrochloric acid and 0.5 g potassium metabisulphite. Shake thoroughly, close the lid tightly and store in dark until use.

Table-top centrifuge.

5.5.7.2 Procedure

For sequential changes of pollen (steps 2–7), use low-speed centrifugation of about 2,000 rpm:

1. Fix mature anthers before they dehisce in acetic alcohol or in Carnoy's fluid (see Appendix A.1) taken in a vial for 2–6 h.
2. Transfer anthers to 70 % ethanol and release pollen by crushing with a glass rod.
3. Hydrolyze pollen in 10 % HCl for about 6 min in a water bath at 60 °C.
4. Rinse 2–3 times in distilled water.
5. Transfer pollen to Feulgen stain for 10–15 min.
6. Remove unbound stain with distilled water.
7. Transfer pollen to 40 % acetic acid.
8. Take a small drop of pollen suspension on a slide and squash pollen sample (follow the procedure of acetocarmine squash, see Protocol 5.5.6).
9. Observe under the microscope. The vegetative and generative/sperm nuclei stain purplish red. The cytoplasm stains green.

5.5.8 DNA Fluorochromes to Study Pollen Cytology

The use of DNA fluorochromes is the most convenient method to study pollen cytology, if a fluorescence microscope is available (Hough et al. 1985). The method is simple, rapid and consistent and can be used to study details of nuclei not only of the mature pollen but also during pollen development, pollen germination and pollen tube growth.

5.5.8.1 Special Requirements

Several DNA fluorochromes are available commercially. We have routinely used DAPI (4′,6-diamidino-2-phenylindole) or ethidium bromide (3,8-diamino-5-ethyl-6-phenyl phenanthridinium bromide).

DAPI: stock solution – 1 mg/ml dissolved in water. Working solution: 5–50 µg/ml (10 µl of stock solution made up to 2 ml with distilled water = 5 µg/ml working solution).

Ethidium bromide: stock solution – 0.1 % in distilled water stored in a fume hood at room temperature. Working solution – 100 µg/ml in distilled water (stock solution diluted 10 times in distilled water).

5.5.8.2 Procedure

1. Take fixed or fresh pollen sample on a microscope slide and add a few drops of working solution of DAPI/ethidium bromide.
2. Lower a cover glass and apply gentle pressure. Sometimes, the reserve material in the pollen may mask the nuclei. For such pollen apply more pressure on the cover glass to rupture the pollen.
3. Observe the preparation under a fluorescence microscope. For DAPI, use UV filter combination; the nuclei show blue fluorescence. For ethidium bromide, use blue filter combination; the nuclei show yellow/orange fluorescence.
4. For later observation, seal the preparation in paraffin/nail polish and store in dark in a refrigerator.

5.5.8.3 Modifications

1. This protocol can be used for cultured pollen grains to study the movement of vegetative nucleus and generative cell into the pollen tube and its division (2-celled pollen) and the movement of sperm cells in the pollen tube (3-celled pollen). Follow the above procedure for fixed or fresh in vitro germinated pollen tubes after allowing pollen tubes to grow for different lengths.
2. DNA fluorochromes have been used as vital stains (Hough et al. 1985). They can be incorporated into the germination medium, and the details of pollen nuclei can be followed during pollen germination and pollen tube growth by observing pollen suspension taken after different time intervals.
3. For details of other DNA fluorochromes such as Hoechst 33258 and mithramycin, see Shivanna and Rangaswamy (1992).

5.5.9 Tetrazolium Test for Pollen Viability

One of the simple methods used in earlier studies to assess pollen viability has been the tetrazolium test (TTC test). TTC test is based on the demonstration of the activity of dehydrogenases (an important group of enzymes in the respiratory cycle) in the pollen cytoplasm. When pollen grains are mounted in a solution of colourless soluble tetrazolium salt, dehydrogenases of the pollen, if active, reduce colourless soluble tetrazolium salt to a reddish insoluble substance called formazan. Formazan accumulates in the pollen cytoplasm and gives reddish colour to pollen. 2,3,5-triphenyl tetrazolium chloride and nitroblue tetrazolium are the most commonly used tetrazolium salts.

5.5.9.1 Special Requirements

2,3,5-triphenyltetrazolium chloride (0.2–0.5 % solution prepared in a suitable concentration of sucrose solution to prevent bursting of pollen). TTC solution needs to be stored in a brown bottle as it undergoes photo-oxidation. It can be stored under refrigeration for a couple of weeks.

Simple humidity chamber (see Appendix A.5).

5.5.9.2 Procedure

1. Mount pollen grains in TTC solution and lower a cover glass.
2. Incubate the slide in a humidity chamber in dark (such as table draw) at laboratory temperature for 30–60 min.
3. After the incubation period, observe the slide under the microscope and score pollen grains that have turned red as viable. Oxygen inhibits reduction of TTC; it is better to score pollen grains from the central part of the cover glass.

Pollen grains located along the margin of the cover glass may show variable degree of red colouration due to higher oxygen availability.

4. Calculate per cent viable pollen in the sample.

In our experience the results of tetrazolium test are not consistent. It tends to give false-positive results in many pollen samples. Even the heat-treated pollen samples, which fail to respond to germination test, have been reported to give high levels of positive values in TTC test (Heslop-Harrison et al. 1984). Another limitation of this test is that pollen grains often show a gradation in colour development (from very light to deep red) with the result fixing a cut-off point for colour intensity becomes difficult for scoring. In recent years, TTC test is being used very rarely.

5.5.10 In Vitro Germination Test for Pollen Viability

In vitro germination test is based on the ability of the pollen grains to germinate in vitro in a suitable nutrient medium. This is a rapid and reasonably simple test and, in an optimal germination medium, shows correlation with fruit and seed set. In vitro germination test has been a routine test used for pollen viability. This test requires some preliminary studies to standardize the medium that permits optimal germination and the period of incubation. This test cannot be used for those species in which pollen grains cannot be germinated in vitro.

There are a number of methods such as hanging drop cultures, sitting drop cultures, suspension cultures and surface cultures available for in vitro germination. Here, only the sitting-drop culture which is one of the simplest, most convenient and effective methods for in vitro pollen germination is described. For details of other methods, refer to Shivanna and Rangaswamy (1992).

5.5.10.1 Special Requirements

Suitable nutrient medium (see Appendix A.4) for composition of various media

Humidity chamber (see Appendix A.5) for preparing simple humidity chamber

Acetocarmine stain

5.5.10.2 Prehydration of Pollen

Pollen of many species particularly 3-celled pollen require prehydration/controlled hydration/vapour-phase hydration to give consistent results. This is carried out by exposing pollen grains to high humidity for about 30 min. One of the easier methods of prehydration is to spread the pollen sample on a dry slide and keep the slide in a humidity chamber for 30 min.

The plasma membrane in desiccated pollen is not in a functional state. When they are cultured directly in the liquid medium, they leak soluble metabolites into the surrounding medium. This imbibitional leakage seems to be the major limitation for achieving optimal germination. Prehydration of pollen by exposing them to high humidity permits gradual hydration which is suitable for the restoration of membrane integrity (see Shivanna 2003). As prehydration does not have any negative effects, we routinely expose pollen sample to prehydration before culturing.

5.5.10.3 Procedure

1. Collect pollen sample to be tested for viability and spread them as a thin layer on a dry microscope slide.
2. Keep the slide in a humidity chamber for prehydration for 30–60 min.
3. Collect suitable amount of prehydrated pollen with a clean needle or forceps from the slide maintained in the humidity chamber and disperse them in a drop (about 20–30 µl) of standardized germination medium taken on a microscope slide. As the pollen grains tend to clump and settle at the periphery of the drop, try to make homogeneous distribution of pollen in the drop with the help of a clean and dry needle. Pollen density is also important; too dense or too dilute suspension would not yield optimal results. A good sitting drop culture should appear neither turbid nor transparent. Take minimum time to prepare sitting drop culture; too much of preparation time results in evaporation of the medium and

Table 5.1 Arbitrary scores for calculating pollen germination and pollen tube growth in pollen cultures raised to test pollen viability*

Microscope field no.	No. pollen grains in the field	No. pollen grains germinated	Length of individual pollen tubes (ocular units) in each field									
			Pollen tube no.									
			1	2	3	4	5	6	7	8	9	10
1	18	15	8	12	9	10	12	8	9	16	13	8
2	20	16	10	13	12	8	10	12	17	10	12	10
3	15	12	12	8	12	9			12	8	15	9
4	10	7	8	10	8	12	8	10	13	7	8	12
5	14	9	15	15	12	10	11	15	10	12	9	13
6	12	8	9	7	10	7	15	16	11	13	10	12
7	16	10	12	9	11	15	13	13	12	13	12	10
8	20	16	13	11	14		10	9	8	14	10	15
9	15	13	8	12	9		8	12	14	9	7	10
10	12	11	14	15	11		12		13	10	11	8
Total	152	111	Total no. of pollen tubes measured = 94									
			Total length of all 94 pollen tubes measured = 1,056 ocular units									

Per cent pollen germination: 111/152 × 100 = 73.02 %
Mean length of pollen tube: 1,056/94 = 11.23 ocular units
For converting ocular units to micrometre, the microscope using the same objective (say 10x) has to be calibrated using a stage micrometre. One mm of the stage micrometre is divided into 100 units of 10 μm each. Suppose, if five ocular units cover six stage micrometre units which is equivalent to 60 μm, then 1 ocular unit is equivalent to 60/5 = 12 μm.
Mean pollen tube length: 11.23 ocular units × 12 μm = 134.76 μm
*This data from one culture is taken as one replicate. A suitable number of replicates have to be raised and scored for each time interval for each sample

consequent changes in the concentration of its constituents. Transfer pollen cultures into a humidity chamber.

4. Maintain the pollen cultures in the humidity chamber for optimal period on the basis of your preliminary studies (1–4 h depending on the species). For each sample, an enough number of replicates have to be cultured (4–6 sitting drop cultures).
5. At the end of the incubation period, add a small drop of a fixative (FAA/acetic alcohol) or a drop of acetocarmine, remix pollen suspension as uniformly as possible and lower a cover glass.
6. Score the cultures to calculate per cent germination. Pollen grain is considered germinated when the length of its tube is more than the diameter of the pollen grain. For each culture, it would be desirable to score pollen from 6 to 10 microscope fields, selected randomly. Enter the scores of each microscope field as shown in the sample Table 5.1. To eliminate the probability of scoring the same sample of pollen grains more than once, it would be desirable to move the preparation under the microscope in consecutive rows and score 2–3 arbitrary fields in each row. From each microscope field, count the total number of pollen grains and the number of pollen grains germinated.
7. Calculate per cent pollen germination as shown in sample Table 5.1.

5.5.11 Fluorescein Diacetate Test for Pollen Viability

Fluorescein diacetate (FDA) test is also referred to as fluorochromatic reaction (FCR) test. The test was introduced by Heslop-Harrison and Heslop-Harrison (1970). Since then it has become one of the standard tests to assess pollen viability. It is simple and can be performed within a few minutes. The only limitation is that it requires a fluorescence microscope. In this test, pollen grains are mounted in FDA solution. The nonpolar

and nonfluorescent FDA readily enters pollen cytoplasm as the plasma membrane does not act as a barrier. Cytoplasmic esterases, if active, hydrolyze FDA and release fluorescein, which is polar and fluorescent. Unlike FDA, fluorescein passes through the intact plasma membrane sparingly and therefore accumulates in the cytoplasm of viable grains and gives a bright green or yellowish green fluorescence under the fluorescence microscope (Fig. 5.2c). If the plasma membrane of the pollen is not intact, fluorescein comes out readily through the membrane into the mounting medium to give a dull fluorescence of the mounting medium; such pollen grains do not show bright fluorescence. Similarly if pollen grains lack active esterases, they do not produce fluorescein and thus do not fluoresce. FDA test, therefore, assesses two properties of the pollen grain: the integrity of the plasma membrane and the activity of esterases. The FDA test has been found satisfactory for pollen of a range of species and correlates with in vitro germination test (Shivanna and Heslop-Harrison 1981; Heslop-Harrison et al. 1984; Shivanna et al. 1991a, b). We have been using FDA test routinely to assess the pollen viability (Kuriakose et al. 2009; Sharma et al. 2010).

5.5.11.1 Special Requirements

Fluorescein diacetate (FDA): stock solution – since FDA does not dissolve in water, stock solution has to be prepared in acetone (2 mg/ml). Stock solution can be stored in a refrigerator for months.

Sucrose solution of suitable concentration to prevent bursting of pollen grains. 8–10 % works well for most of the pollen systems.

Humidity chamber (Appendix A.5)

5.5.11.2 Procedure

1. Prepare working solution of FDA by taking 2–5 ml of sucrose solution/germination medium in a glass vial and adding drops of FDA stock solution with a pipette until the solution shows persistent turbidity. The working solution should be used within 30 min of its preparation; otherwise most of the FDA would precipitate.
2. Take a drop of working solution on a microscope slide and suspend sufficient amount of pollen grains in the drop and mix the suspension with a needle and lower a cover glass.
3. Incubate the preparation in a humidity chamber to prevent evaporation of the solution for 3–5 min. The incubation time may vary depending on the species. Pollen of some species show bright fluorescence soon after making the preparation.
4. After the incubation period, observe the preparation under the fluorescence microscope using blue filter combination.
5. Score pollen grains that fluoresce brightly as viable by following the procedure described under Protocol 5.5.10. Scoring has to be done within a few minutes of preparation. If they are left for longer period, the fluorescence of pollen becomes dull as the fluorescein continues to move from pollen cytoplasm into the medium although at a slower rate and the background also starts showing dull fluorescence.

5.5.11.3 Modification

Pollen grains of many species may require prehydration for 30–60 min to provide better conditions for restoration of membrane integrity to enhance pollen response to the FDA test. In such species, it would be better to prehydrate pollen samples (see Protocol 5.5.10 for details) before testing for viability.

5.5.12 In Vitro Germination Test for Pollen Vigour

One of the simple methods to assess the vigour of pollen samples is to use pollen samples for controlled pollinations. One set of pistils is pollinated with fresh pollen which are expected to show maximum viability and vigour and another set with pollen sample to be tested for vigour. Some pistils from each set are fixed at intervals and used to study pollen germination and pollen tube growth using aniline blue test (for details see Protocol 8.5.1) and record the extent of tube growth in terms of mm/cm

(depending on the length of the pistil) in a unit time. Less vigorous pollen show slower growth through the pistil. For example, pistils pollinated with fresh pollen may show pollen tubes in the middle part of the style at a given time, whereas those pollinated with test pollen may show pollen tubes in the upper part of the style. This procedure again requires too much of time and also a fluorescence microscope for clear documentation of pollen tube growth in the pistil.

In vitro germination assay can also be used to assess pollen vigour. In the protocol to assess pollen viability, per cent pollen germination is scored at one time without consideration of the time factor; the cultures are scored after maintaining them for a much longer period than that required for germination. To assess pollen vigour, however, germination and tube length in fresh pollen samples and test pollen samples are scored at regular intervals after culture and the responses are compared. The results on pollen germination and tube length are presented in the form of a graph. Pollen grains with reduced vigour take longer time when compared to control (fresh pollen) to attain maximum germinability (Shivanna and Cresti 1989). Similarly, pollen tube length remains significantly shorter at any given time in pollen samples with reduced vigour.

5.5.12.1 Special Requirements

As in Protocol 5.5.10 on in vitro pollen germination test

5.5.12.2 Procedure

1. Raise sitting drop cultures of fresh pollen and test pollen as described in Protocol 5.5.10. As the same culture cannot be used for scoring at regular intervals, raise more number of cultures for each pollen sample depending on the scoring time intervals. Scoring intervals depend on the time taken for germination and the rate of pollen tube growth. Record the time of raising each culture.
2. Incubate the cultures in the humidity chamber.
3. After each time interval (for many species, 30-min intervals are satisfactory, and others may require 1-h intervals), take two cultures from each pollen sample, add a drop of fixative or acetocarmine, mix the pollen suspension and lower a cover glass. Keep the slide under humidity chamber to prevent drying if they are not scored immediately.
4. Repeat the procedure at selected time intervals.
5. Score all the cultures (2 from fresh pollen and 2 from test pollen, at each selected time interval) for pollen germination (following the procedure given under Protocol 5.5.10 and Table 5.1). Measure pollen tube length from each field used for scoring germination as follows:
   (a) Adjust the intensity of microscope illumination with the help of diaphragm so that the boundary of each grain and tube are clearly visible. From each microscope field used for germination score, measure the length of 10 pollen tubes selected randomly (in ocular units) by using an ocular micrometre. Omit curved pollen tubes which cannot be measured accurately. If the number of pollen tubes in each microscope field is <10, measure all the pollen tubes in the field. If the number of tubes is >10, measure 10 randomly selected pollen tubes.
   (b) Enter both germination and tube length data in a tabular form (see the sample Table 5.1). Repeat the scoring for all the cultures. Calculate mean pollen germination and pollen tube length for each pollen sample at each time interval and present the same with SE/SD. If necessary, ocular units can be converted into micrometres by calibrating the microscope with the use of stage micrometre (see Table 5.1 for details).
6. Present the results for germination and tube length in the form of a graph over time for each reading.

5.5.12.3 Modification

The semi-vivo method can also be used to test pollen vigour. The details are described in Protocol 8.5.3.

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Pistil Biology

6

The pistil represents the female partner in sexual reproduction of flowering plants. It consists of the stigma, the style and the ovary. The stigma is the recipient of pollen and shows wide variation in its morphology (Heslop-Harrison and Shivanna 1977). The stigma is grouped into two broad types, dry and wet, depending on the presence or absence of secretary fluid (stigmatic exudates) on its surface at the time of pollination. Each type has been classified further (Table 6.1). The receptive surface of the stigma invariably contains extracellular heterogeneous components – proteins (both enzymic and non-enzymic), lipids, carbohydrates, amino acids and phenols (Mattsson et al. 1974; Heslop-Harrison and Shivanna 1977; Shivanna 2003). These are present as a part of the exudate in wet types or in the form of a dry extracellular lining outside the cuticle in dry types.

The style is basically of two types – solid (closed) and hollow (open). In solid style, a core of transmitting tissue (TT) is present along the whole length of the style. In hollow style, a canal, termed stylar canal, connects the stigma with the ovarian cavity. In solid style, the TT is made up of elongated cells connected end to end through plasmodesmata. There are no plasmodesmatal connections on their longitudinal walls. In transections, the cells of the TT are circular with massive intercellular spaces filled with extracellular matrix (ECM) secreted by the cells of the TT. In hollow style, the stylar canal is lined with one or two layers of glandular cells, the canal cells. The secretion from the canal cells may fill the canal (as in *Lilium*) or form a lining around the inner tangential wall of the canal cells (see Shivanna 2003 for details).

The ovary houses the ovule(s). The ovules are the seat of female gametophyte, the embryo sac. The embryo sac is derived from the megaspore mother cell which undergoes reduction division to give rise to four megaspores. Three of the megaspores degenerate and the remaining one divides through mitotic divisions and gives rise to the embryo sac. There is great variation in the details of development and cell numbers of the mature embryo sac (Shivanna 2003). The most common type of embryo sac present in >80 % of the flowering plants is termed '*Polygonum* type'. It consists of the egg apparatus at the micropylar pole, made up of the egg cell and two synergids, three antipodal cells at the chalazal pole of the embryo sac and a large central cell with two haploid nuclei, the polar nuclei, located just below the egg apparatus. The polar nuclei eventually fuse to form the secondary nucleus.

Following successful pollination, pollen grains land on the stigma where they germinate. The resulting pollen tubes grow through the tissues of the stigma and style. In solid styles pollen tubes grow through the intercellular spaces of the transmitting tissue and in hollow styles on the surface of the canal cells. Pollen tubes enter the ovary and eventually the embryo sac located inside the ovule through one of the synergids. They discharge male gametes in the synergid; one of the male gametes fuses with the egg to

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Table 6.1 Morphological diversity of the stigma

Dry stigma (without apparent fluid secretion)
Group I: Plumose – receptive surface dispersed on multiseriate branches
Group II: Receptive surface localized to stigma
A. Stigma-surface non-papillate
B. Stigma-surface distinctively papillate
1. Papillae unicellular
2. Papillae multicellular
(a) Papillae uniseriate
(b) Papillae multiseriate
Wet stigma (fluid secretion present on the stigmatic surface)
Group III: Receptive surface papillate
Group IV: Receptive surface non-papillate
Group V: Receptive surface covered with copious exudates in which detached secretory cells of the stigma are suspended

Based on Heslop-Harrison and Shivanna (1977) and Shivanna (2003)

give rise to the zygote, and the other fuses with the secondary nucleus located in the central cell to give rise to the primary endosperm cell. The zygote develops into the embryo, and the primary endosperm cell gives rise to the endosperm, a nutritive tissue which nourishes the embryo. Although fertilization takes place inside the embryo sac, situated deep inside the ovary, the stigma and style play a very important role in fertilization. It is, therefore, necessary to study the structural and functional details of stigma and style to understand pollen–pistil interaction and fertilization.

6.1 Stigma Receptivity

Stigma receptivity refers to the ability of the stigma to promote pollen germination and pollen tube growth following compatible pollination. Receptivity of the stigma is crucial for pollination success; unless the stigma is receptive at the time of pollination, pollen grains cannot germinate and consequently result in the failure of fertilization. In dichogamous species, stigma may become receptive a few hours or days before anther dehiscence (protogyny) or a few hours or days after anther dehiscence (protandry).

In several species, stigma receptivity can be identified on the basis of morphological changes that occur in the stigma. In some species with lobed stigma, the lobes are closed in the non-receptive stage but open out when the stigma becomes receptive. However, in most of the species, stigma receptivity cannot be recognized externally. The identification of stigma receptivity in such systems requires controlled pollinations followed by subsequent studies on pollen germination and pollen tube growth.

Many investigators have assessed stigma receptivity on the basis of the presence of some enzymes particularly esterases (Fig. 6.1a–c) and peroxidases (Fig. 6.1d) on the surface of the stigma (Dafni et al. 2005). However, the result may not be categorical. For example, in *Crinum*, although younger buds do show the presence of surface esterases and support pollen germination, pollen tubes fail to enter the stigma (Shivanna and Sastri 1981). Our experience has been that although stigma-surface enzymes are always present at the time of stigma receptivity, their presence need not necessarily indicate stigma receptivity (non-receptive buds often show surface enzymes). It is better to confirm stigma receptivity on the basis of pollen germination in manually pollinated pistils (see Protocol 6.3.5).

6.2 Ovule Receptivity

Apart from stigma receptivity, ovule receptivity is also important for successful fertilization. In several orchids, although the stigma is receptive and permits pollen germination, the ovules are not even differentiated at the time of pollination (Arditti 1979; O'Neill 1997). Pollination stimulates ovule differentiation, and the time lag between pollination and fertilization varies from a few weeks to months. Pollen tubes cease their growth in the ovary until ovule differentiation. They regain growth after ovules are fully differentiated, enter the ovules and effect fertilization. In *Narcissus* also (Sage et al. 1999), cross-pollination induces differentiation of additional ovules. Thus, the status of ovules is important for successful fertilization.

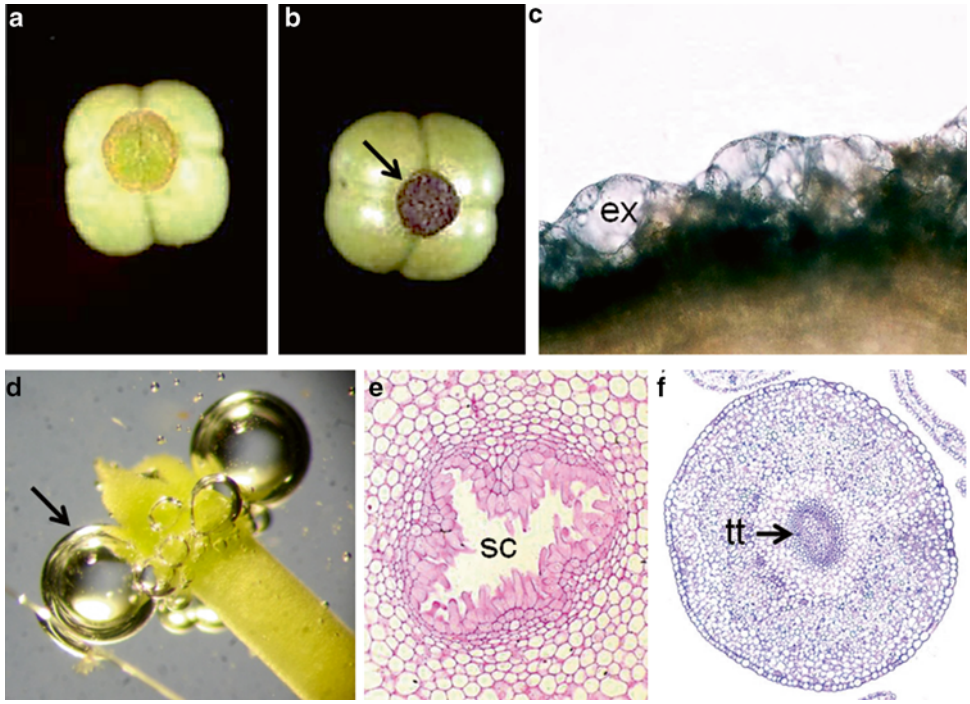

Fig. 6.1 (**a**, **b**) Localization of esterases on the stigma surface of *Crateva adansonii* (*After* Mangla and Tandon 2011). (**a**) Control stigma incubated without the substrate; there is no colouration of the stigma. (**b**) The stigmatic surface in the presence of the substrate showing intense colour (*arrow*). (**c**) Whole mount of the wet stigma of *Aegle marmelos* after incubating in the presence of the substrate. The stigmatic exudate (*ex*) has taken colour due to the activity of esterases. (**d**) A receptive stigma of *Azadirachta indica* incubated in peroxidase mixture. The bubbles (*arrow*) emanating from the stigma indicate peroxidase activity. (**e**) Transverse section of the style in *Elaeis guineensis* showing the hollow style with the stylar canal (*sc*) bordered by canal cells. (**f**) Transverse section of the style of *Brassica juncea*. The style is solid with a core of transmitting tissue at the centre (*tt*)

In many multiovulate species, not all the ovules develop into seeds. Resource optimization, parent–offspring conflict, sibling rivalry and maternal regulation of genetic quality of offspring by selectively aborting genetically inferior progeny have been implicated as causative factors for the abortion of ovules and even developing seeds. One of the important morphogenetic events associated with fertilization is the change in the direction of pollen tube growth towards the ovule from the placenta, which involves curvature of the pollen tubes by about 90°. This change in the direction has been shown to be controlled by the embryo sac particularly synergids in a few species (Hulskamp et al. 1995; Higashiyama 2010). Although we still do not know the components responsible for this guidance, receptive ovules of several species have been shown to secrete an exudate in the micropylar region which seems to be necessary for pollen tube entry (*see* Shivanna 2003; Kawashima and Berger 2011). Micropylar exudate responds to polysaccharides and proteins. Recently Sengupta and Tandon (2010) have assessed ovule receptivity in 24 multiovulate species on the basis of the presence of micropylar secretion by staining with toluidine blue O'. In all the species studied, the number of receptive ovules was lower than the total number of ovules in the ovary, indicating that not all the ovules were receptive. Most of the ovules in younger buds were not receptive. More interestingly, there was a positive linear correlation between per cent receptive ovules and per cent seed set indicating the development of lower number of seeds when compared to ovules is likely to be due to lack of fertilization of non-receptive ovules.

6.3 Protocols

6.3.1 Morphology and Anatomy of the Stigma and Style

6.3.1.1 Special Requirements

Acetocarmine or safranin

6.3.1.2 Procedure

1. Collect freshly opened flowers and bring them to the laboratory; describe the morphology of the pistil. Excise the pistil and observe the stigma under a stereomicroscope and describe its morphology. Record whether the stigma is of the wet or dry type based on the presence or absence of the exudate. In some species the exudate is very thin and creates difficulty in categorizing it as dry or wet. In such species a general criteria to distinguish wet from dry stigma is to consider whether pollen grains after pollination come in contact with the exudate or on the dry surface of the stigma. If pollen grains come in contact with the exudate, it is considered as wet type, and if they come in contact with dry surface, it is considered as dry type. Also record whether the stigma is papillate or non-papillate. When the stigma is thick, freehand longitudinal sections of the stigma may reveal the structure more clearly.
2. If the stigma is papillate, mount a part of the stigma along with some papillae under the microscope to observe cellular nature of the papillae. Record whether the papillae are unicellular or multicellular, and if multicellular, whether they are uniseriate or multiseriate.
3. Cut freehand transactions of the style in the middle part of the style; stain the sections with safranin or acetocarmine and make temporary mounts. Observe the sections under a microscope, and record the nature of the style (solid or hollow) (Fig. 6.1e, f). If solid, identify the transmitting tissue, which may be one or several depending on the number of carpels. Even in species with many carpels, the transmitting tissue of different carpels may be fused to form one bundle at the centre. If it is hollow style, try to locate canal cells around the stylar canal (Fig. 6.1e). If the sections are thin enough, some of them can be stained with Coomassie blue to localize extracellular matrix in the inner surface (facing the stylar canal) of the canal cells. In some legumes, the style just below the stigma is solid and becomes hollow further down. To rule out such possibilities, it would be better to cut sections just below the stigma and middle part of the style.
4. Cut longitudinal and transverse sections of the ovary and observe the type of placentation (marginal/free central/basal).
5. Count the number of ovules in each ovary by careful dissection. Use sufficient number of ovaries to determine the average number of ovules in an ovary. If the number of ovules is too large to count through dissections, follow the procedure given in the next protocol (Protocol 6.3.2).

6.3.2 Estimation of Ovule Number in Species with Large Number of Ovules

The number of ovules in each flower is highly variable between species; it ranges from one to several hundreds. In species with a limited number of ovules, their number can be easily determined by careful dissection of the ovary and counting the number of ovules. In species with a large number of small ovules such as *Nicotiana*, *Lilium* and *Passiflora*, all the ovules cannot be counted through dissection. Similarly in orchids, the number of ovules is in thousands. This protocol is for such species with a large number of ovules.

6.3.2.1 Special Requirements

Watch glass or any wide-mouth vial of about 5 ml capacity

A few drops of any detergent (soap solution would do)

Graduated Pasture pipette

6.3.2.2 Procedure

1. Excise the ovary from a freshly opened flower and slit it longitudinally into 4–8 segments (depending on the size of the ovary and the

number of ovules) with a clean blade. As far as possible, avoid cutting the ovules by confining the cut to the ovary wall and pulling out each segment to expose the ovules.
2. Take 2–4 ml of water (depending on the number of ovules) containing a drop of any detergent in a watch glass or any wide-mouth glass or plastic vial convenient to scrape ovules. We use a well of a titre plate routinely used in immunological studies. Take one segment of the ovary with a pair of forceps, and carefully scrape all the ovules into the water using a stereomicroscope; discard the wall portion of the segment. Repeat this with all the ovary segments. Now the ovule suspension contains all the ovules of the ovary.
3. Stick a piece of graph paper below a clean slide.
4. Take 0.5 ml of the suspension while gently shaking the vial in a graduated Pasture pipette and put it on the slide (prepared in step 3) in the form of a narrow band of 2–4 cm.
5. Count the number of ovules under a stereomicroscope (with epi-illumination) from one side of the band to the other along the graph paper squares.
6. If the volume of ovule suspension is small (say 2 ml), the entire suspension can be used in 4 replicates of 0.5 ml for counting the total number of ovules. If the volume is larger, 4–6 replicates of 0.5 ml sample can be used, and the total number of pollen in the suspension can be calibrated as follows (similar to pollen counts, see Protocol 5.5.2):

$$\frac{\text{Total No. ovules in the suspension used for counting} \times \text{Total volume of ovule suspension}}{\text{Volume of suspension used for counting}}$$

7. Repeat the procedure for 10–15 ovaries and calculate average number of ovules/ovary and present it with SD/SE.

6.3.3 Cytochemical Localization of Stigma-Surface Esterases

Irrespective of the morphological variations of the stigma, the receptive surface invariably contains extracellular matrix (ECM) on the surface. The ECM is heterogeneous and is made up of lipids, proteins and glycoproteins, a variety of carbohydrates, amino acids and phenols. ECM plays an important role in pollen–pistil interaction. A number of enzymes such as esterases, acid phosphatases and peroxidases have also been localized in the ECM. The receptive surface of the stigma can be easily recognized by localizing one of these surface enzymes. Cytochemical localization of non-specific esterases is a convenient method. This method essentially involves incubating the stigma in the presence of a substrate, α-naphthyl acetate. Stigma-surface esterases hydrolyze the substrate to produce colourless α-naphthol and acetate. α-Naphthol forms a reddish insoluble complex with the coupling reagent, fast blue B (Mattsson et al. 1974; Ghosh and Shivanna 1984). The development of reddish colour on the stigma indicates the presence of esterases. Incubation solution without the substrate (α-naphthyl acetate) acts as the control in which the reddish colouration would be absent.

6.3.3.1 Special Requirements

α-Naphthyl acetate
Phosphate buffer (0.15 M, pH 6.8): see Appendix A.2 for details of the preparation
Acetone
Fast blue B
Pasture pipettes
Electronic balance
Sucrose

Reaction mixture: prepare two solutions[a] A and B as given below

Reagents	Solution A (with substrate)	Solution B (control, i.e. without substrate)
α-Naphthyl acetate[b]	5 mg	0 mg
Phosphate buffer 0.15 M	10 ml	10 ml
Sucrose (as osmoticum)	ca 1 g	ca 1 g
Fast blue B	25 mg	25 mg

[a]The solutions should be prepared shortly before use and should be used within 15–30 min of preparation
[b]As α-naphthyl acetate is not soluble in phosphate buffer, it has to be dissolved (5 mg) in a few drops of acetone in about 15 ml vial. Then add 10 ml of phosphate buffer, sucrose and fast blue B; shake gently

6.3.3.2 Procedure

1. Collect freshly opened, unpollinated flowers and bring them to the laboratory. If anthers dehisce before anthesis, flower buds need to be emasculated and bagged before anther dehiscence to prevent pollination.
2. Carefully excise the pistil from 6 to 10 flowers, and place them on a dry slide kept in the humidity chamber (see Appendix A.5). Take care not to injure the stigma while handling the flowers; handle them at their pedicel with a pair of forceps. If the stigma is injured, cytoplasmic esterases of the cells of the injured part will also produce colour and obstruct localization of surface esterases.
3. Take a few drops or ml of solution A and B on separate microscopic slides (for smaller stigmas)/cavity slides/watch glasses (for larger stigmas). Avoid contamination of solution A and B scrupulously through glass ware or tools used to handle the stigma.
4. Dip the stigma of half of the number of pistils in solution A and the other half in solution B; make sure that the stigma is completely dipped inside the solution. If necessary add some more solution. Try to keep the remaining part of the pistil away from the solutions. Avoid bringing the cut end of the style/pedicel in contact with the solution as the cellular esterases at the cut end would respond and give deep colour.
5. Incubate the preparations in both the solutions for 10–20 min at laboratory temperature or at 25–30 °C. The incubation period varies for different systems. The development of reddish colour on stigmas kept in solution A and no colour in those kept in solution B is a good indication of adequate incubation.
6. After incubation period, remove the pistils from each solution separately and rinse thoroughly in phosphate buffer taken in a watch glasses/Petri plate. It would be better to remove and rinse the pistils from the control solution (B) before the substrate solution (A) to prevent the substrate contaminating the control solution through instruments. If the same set of forceps is used for both, wash them thoroughly before using them for the other solution.
7. Make whole mount preparation of the stigmas in 10 % glycerin. If the stigma is thick as in the members of Solanaceae, slice it longitudinally or cut freehand longitudinal sections and mount.
8. Observe the preparations under the microscope. The location of esterases depends on the type of the stigma. In dry type of stigma, esterases are visible in the pellicle layer as a thin lining along the margin of the papillae (in papillate stigma) or on the stigma surface (non-papillate stigma). In wet stigmas, esterases appear in the exudate.

6.3.3.3 Modifications

1. Other enzymes such as acid phosphatases may also be studied. For phosphatases use α-naphthyl phosphate (1 mg/ml) as the substrate and fast garnet GBC (1 mg/ml) as the coupling reagent in 0.1 M acetate buffer (pH 4.0) and two drops of 10 % $MgCl_2$.
2. By studying stigma-surface enzymes at different stages of flower buds, developmental details on the accumulation of surface enzymes on the stigma can be analysed.
3. Localization of surface enzymes can also be combined with stigma receptivity to study the relationship between the appearance of the stigma-surface enzymes and stigma receptivity. Select flower buds of different developmental stages and opened flowers. Arrange buds of

different stages and opened flowers into two groups. Use one group of each stage for localization of surface enzymes and the other group to carry out controlled cross-pollination and studies on pollen germination (see Protocols 6.3.4 and 6.3.5). Compare the presence or absence of stigma-surface enzymes of each group and their ability to support pollen germination and pollen tube entry into the stigma.

6.3.4 Demonstration of Peroxidase Activity on the Stigma Surface

Several investigators have assessed stigma receptivity by localizing the activity of peroxidases on stigmatic surface (Galen and Plowright 1987). The addition of hydrogen peroxide, which is the substrate for the enzyme, results in the release of nascent oxygen as bubbles from the stigmatic surface. The rate at which the bubbles are released indicates the intensity of reaction.

6.3.4.1 Special Requirements

Hydrogen peroxide (H_2O_2)
Benzidine
Ethanol

Prepare solutions A and B as follows:

Solution A (with substrate): 1 % benzidine prepared in 60 % ethanol:H_2O_2:distilled water (4:11:22 v/v)
Solution B (without substrate, control): 1 % benzidine prepared in 60 % ethanol:distilled water (4:33 v/v)

6.3.4.2 Procedure

1. Follow the steps 1–3 as explained in Protocol 6.3.3.
2. Immerse the stigmas of half of the pistils in the solution A and the other half in solution B.
3. Keep observing both sets of pistils soon after incubation as the stigmas in solution A start releasing O_2 bubbles within 2–3 min. No bubbles are released in solution B.
4. Count the number of bubbles released per minute. The values should be averaged from a set of five replicates of each developmental stage.
5. Floral stages with positive reaction are considered as receptive, and the stage with greatest mean number of bubbles is the one with maximum receptivity.

6.3.4.3 Modifications

Modifications 2 and 3 as described under Protocol 6.3.3.

6.3.5 Assessment of Stigma Receptivity Based on Pollen Germination

The most authentic method to assess stigma receptivity is on the basis of its ability to support pollen germination and pollen tube entry into the stigma following controlled compatible pollinations.

6.3.5.1 Special Requirements

Pollination bags and tags

6.3.5.2 Procedure

1. Bag the flower buds of different stages including those 1 day before anthesis and anther dehiscence. If the flowers are bisexual, emasculate (before anther dehiscence) the oldest stage of flower buds (which would open the next day) before bagging to prevent self-pollination. Continue this step for a few days so that on the day of pollination, there are flower buds at different stages of development, freshly opened flowers and, if the longevity of flowers extends beyond 1 day, unpollinated flowers at intervals of 1 day after anthesis until initiation of senescence. See step 2 for guidance.
2. Group bagged buds and opened flowers into different sets on the basis of days to anthesis or length of flower buds and days after anthesis.

   The following are the suggested stages if the longevity of the flower extends for 2 days:
   (a) One day before anthesis
   (b) Soon after anthesis
   (c) 6 h after anthesis
   (d) 12 h after anthesis
   (e) 24 h after anthesis
   (f) 48 h after anthesis

However, in some species, the flower may remain fresh for many days. For example, in members of Solanaceae and Liliaceae, the stigma remains receptive for 7–9 days if it remains unpollinated. In such species, emasculated buds have to be collected at 1-day interval until the flowers remain fresh. The required number for each stage has to be selected on the basis of the availability of the flowers and the scope of the study. Preferably each set should have 6–10 buds/flowers.

3. Carry out controlled cross-pollinations (to make sure that lack of pollen germination is not due to self-incompatibility in self-incompatible species) on each stigma with adequate amount of fresh pollen. See Protocol 10.6.1 for details of controlled pollinations. Bag pollinated flower buds and flowers.
4. Excise each set of pistils 4–6 h after pollination and fix them in a fixative (make sure that all sets of pollinated pistils are given the same time gap after pollination) (see Appendix A.1 for fixatives). If fixed pistils have to be studied later for pollen germination, they can be transferred to 70 % ethanol and kept for weeks. Note that the pollinated pistils are not maintained for longer periods (24–48 h); this would permit pollen germination even in pistils which were not receptive at the time of pollination but become receptive later (6–24 h) after pollination.
5. Process each set of fixed pollinated pistils separately to study pollen germination and pollen tube growth through aniline blue fluorescence (see Protocol 8.5.1 for details).
6. Score each stigma of each set for pollen germination and pollen tube entry into the stigma. Calculate per cent stigmas of each set that support pollen germination. If possible, count the number of germinated pollen on each stigma; estimate average number of pollen grains germinated per stigma.

6.3.5.3 Modification

If a fluorescence microscope is not available, stain the stigmas with acetocarmine or cotton blue in lactophenol. Gently tease stigma with a pair of needles, lower a cover glass, apply gentle pressure on the cover glass and observe under a light microscope. Although this method is not suitable to trace pollen tubes through the style, pollen germination on the stigma and their entry into the stigma can be seen clearly. If the stigma is thick, cut freehand longitudinal sections, mount them in a drop of the stain and observe for pollen germination and pollen tube entry into the stigma.

6.3.6 Pollination of Excised Flowers/Pistils for Stigma Receptivity

The protocol described above (Protocol 6.3.5) is ideal to study stigma receptivity. However, it is laborious and inconvenient particularly in tree species. Alternatively, stigma receptivity can be studied by pollinating excised pistils/flowers/inflorescences maintained in the laboratory under suitable conditions.

6.3.6.1 Special Requirements

Beakers
Petri plates
Agar plates, if necessary (see Appendix A.3 for preparation)

6.3.6.2 Procedure

1. Excise flower buds of different stages and opened flowers (emasculated and bagged in the bud stage to prevent pollination) with their pedicel intact. Immediately after excision, keep them in a beaker containing water with their cut ends in dipped in water. If the flower buds and flowers are small and delicate, excise the inflorescences/small branches bearing flowers and keep in the beaker with their cut ends in water.
2. Group flower buds into different sets (on the basis of their size or days to anthesis) and label each set of buds and also opened flowers. Each group has to be handled separately in subsequent steps.
3. If the flowers are large, keep each set of flower buds and opened flowers separately. If the flowers are extra large as in *Lilium*, the pistils can be excised along with their pedicels and maintained in beakers.

If the flowers are smaller, implant each stage of excised flower buds and flowers in agar plates through their pedicel. Make sure that the stigmas of excised flowers and pistils do not become wet. Use at least 6 pistils/flowers for each set.

4. Carry out cross-pollination on each stigma with adequate amount of fresh pollen. Leave them for 4–6 h.
5. Follow the Protocol of 6.3.5 for subsequent steps of fixing the pistils and processing them to study pollen germination and pollen tube growth and for scoring.

6.3.6.3 Modification

When the longevity of the flowers extends for several days, flowers to be used for post-anthesis stages have to be emasculated before anther dehiscence and bagged on the plant itself. They are excised at suitable periods (starting from flowers on the day of anthesis and continued at regular intervals of 12–24 h, depending on the longevity of the flowers, until the day of senescence) and implanted in beakers or agar plates for pollination followed by subsequent steps.

6.3.7 Assessment of Ovule Receptivity

In this protocol, ovule receptivity is assessed on the basis of the presence of micropylar exudate in the ovules by staining fresh ovules with toluidine blue O' which stains insoluble polysaccharides blue to intense magenta at pH 4.4 (Sengupta and Tandon 2010). As micropylar exudate contains insoluble polysaccharides, it responds positively to this stain.

6.3.7.1 Special Requirements

Toluidine blue O' (TBO) (see Appendix A.1.1 for preparation)

6.3.7.2 Procedure

1. Collect fresh flowers on the day of anthesis.
2. Count the number of ovules and calculate average number of ovules per flower.
3. Carefully dissect all the ovules form each flower in a drop of phosphate buffer (see Appendix A.2) taken on a slide.
4. Remove the buffer with a piece of dry filter paper and immediately add a drop of toluidine blue without allowing the ovules to dry.
5. Gently lower a cover glass and immediately observe each ovule for the presence of bluish-magenta spot near the micropyle. Score such ovules as receptive and those which do not show the colour as non-receptive (Fig. 6.2a, b). Do not get confused with the colour development at the cut end of the funicular part of the ovule; it invariably develops colour because of the injury caused by breaking of the funiculus during ovule separation from the ovary. Count the number of receptive and of non-receptive ovules.
6. Repeat the scoring for a number of flowers (about ten ovaries), and calculate the average number of receptive ovules per ovary.
7. Collect mature or nearly mature fruits (using the same sample size as the flowers used) and count the number of seeds developing in each fruit. Calculate the average number of seeds per fruit.
8. Compare the average number of receptive ovules per flower and average number of seeds per fruit. Interpret the results on the basis of the relationship between the number of receptive ovules in opened flowers and the number of seeds in mature fruit.

6.3.8 Clearing of Ovules to Study Embryological Details

Ovule clearing method is relatively an easier and much quicker method to study various embryological details when compared to studies using wax and resin sections (Fig. 6.3). The developmental details of megasporogenesis, megagametogenesis, early embryogenesis and also the possible occurrence and type of apomixis can be studied in whole mounts of cleared ovules. One of the major requirements for using this method is the availability of a good photomicroscope with Differential Interference Contrast (DIC)

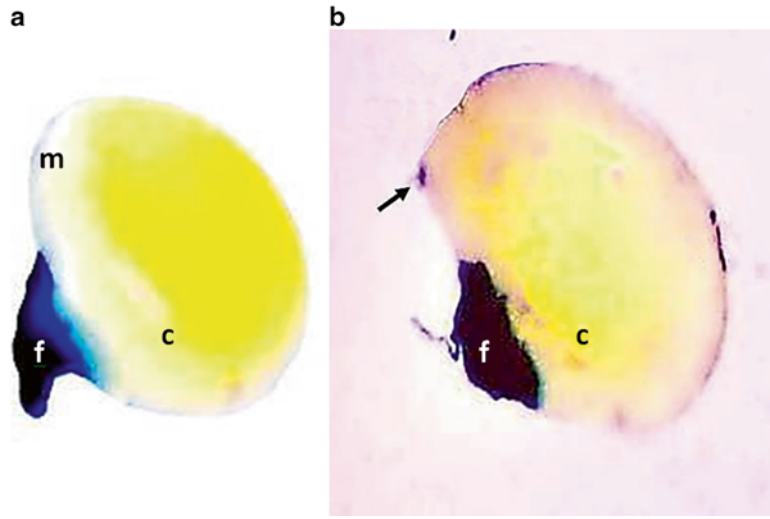

Fig. 6.2 Test for ovule receptivity in *Pisum sativum* (*After* Sengupta and Tandon 2010). Whole mount of ovules from opened flower mounted in control buffer (**a**) and in toluidine blue O' dye (**b**). The micropylar exudate stains magenta (*arrow*) in **b** indicating its polysaccharidic nature (*c* chalaza, *f* funiculus, *m* micropyle)

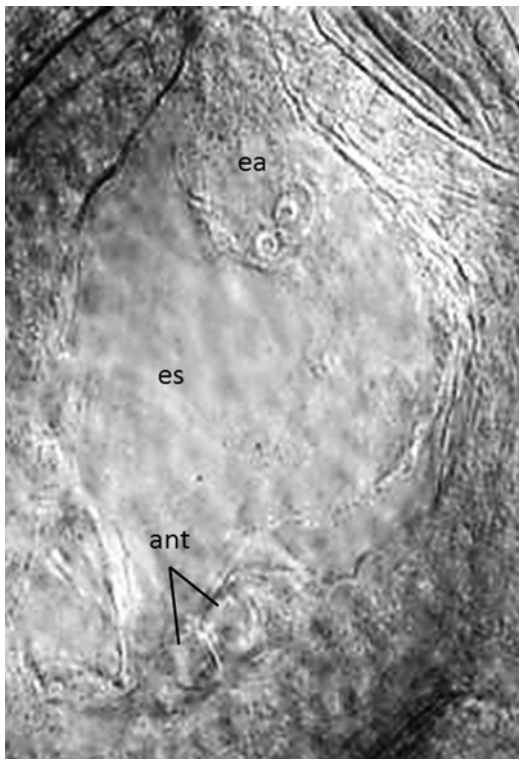

Fig. 6.3 A cleared ovule of *Cenchrus ciliaris* with internal details of embryo sac (*es*) as observed using DIC microscope; the egg apparatus (*ea*) and the antipodal cells (*ant*) are noticeable (*Courtesy* Dr Vishnu Bhat)

attachment. Standardization of suitable clearing method poses problems when the ovules are thick: the duration of fixation and temperature range for clearing has to be standardized. With the application of suitable stains (DAPI, Feulgen, etc.), even the cytological features in an organized tissue can be studied.

There are a number of clearing methods available (Herr 1971; Young et al. 1979; Braselton et al. 1996; Siddiqi et al. 2000; Dreni et al. 2011). Amongst them, methyl salicylate method, presented here, is one of the most commonly employed methods. For ovules with inclusions (such as silica) in their cells as in members of Podostemaceae, we found Herr's ovule clearing method (given under 'Modifications') to be more effective.

6.3.8.1 Special Requirements

A Differential Interference Contrast (DIC) attachment with Nomarski filters

Methyl salicylate solution

6.3.8.2 Procedure

1. Fix the flowers or the dissected ovaries of the required stages in FAA for about 24 h. Wash the material in 70 % ethanol twice;

and the material can be used directly or stored in 70 % ethanol for later use.
2. Carefully dissect the ovules (from the fixed ovaries).
3. Dehydrate the ovules by passing them through an ascending ethanol series (80, 90 and 100 % ethanol) by giving two or three changes for 30 min in each. Keep the material in 100 % ethanol overnight. (If ovules are very small, use low-speed centrifugation for changes.)
4. Transfer ovules to a sequential series of ethanol and methyl salicylate mixtures for 30 min each, 1:1 and 1:3, and finally to methyl salicylate. The duration in each solution may be increased to 1 h for harder tissues. After standardizing the duration of clearing, the ovules may be stored in fresh methyl salicylate.
5. When the clearing is satisfactory, study the details by mounting the cleared ovules in a drop of methyl salicylate under DIC microscope and record observations.

6.3.8.3 Modifications

1. As the nuclei persist after clearing, cytological details of developing embryo sac can be studied by staining the cleared ovules in Feulgen stain or DNA fluorochrome such as DAPI (see Protocols 5.5.7 and 5.5.8).
2. There are a number of other clearing agents. The procedures of some of them are given below:
   (i) *Herr's clearing solution* (Herr 1971)
   Fix the ovaries in FPA (formalin 5 ml, propionic acid 5 ml, ethanol 90 ml) for 24 h. Transfer fixed ovaries or dissected ovules from fixed ovaries to the clearing fluid consisting of lactic acid (85 %), chloral hydrate, phenol, clove oil and xylene (2:2:2:2:1 by weight) for 24 h. The ovules are then mounted in a drop of the same fluid taken on a slide and observed under the DIC microscope.
   (ii) *Chloral hydrate and lactic acid* (Dreni et al. 2011)
   Pistils fixed in FAA are used. Dissect the ovules from fixed pistils and transfer them to Herr's clearing solution (lactic acid, chloral hydrate, phenol, clove oil and xylene: 2:2:2:2:1 by weight) for 2 days. Transfer them to lactic acid saturated with chloral hydrate for another 2 days. The ovules are then mounted in the same solution and viewed under DIC microscope.

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Pollination Ecology

7

Pollination is one of the most critical events in sexual reproduction of flowering plants. Pollination is the basis of gene flow and genetic recombination. Studies on pollination ecology were prevalent even before Darwin; they became intense after Darwin formulated the concept of co-evolution between flowers and pollinators. The number of publications on pollination perhaps exceeds those in any other area of reproductive ecology. Pollination is simply the transfer of pollen grains from an anther to the stigma. Pollination ecology is the study of pollen transfer through understanding of interactions between plants and pollinators in relation to the prevailing habitat. Except in some apomictic species which do not depend on fusion of the second male gamete with polar nuclei for endosperm development (pseudogamy), effective pollination is a prerequisite for successful seed development. A comprehensive understanding of pollination ecology of a species needs a thorough familiarity with the phenology, floral morphology and sexuality of the species.

The most important feature of pollination that drives all relevant adaptations is that plants are sedentary and they have to make use of external agencies to achieve pollination. Wind, water and animals are the three pollinating agents. Nearly 90 % of the flowering plants are pollinated by animals (biotic pollination/zoophily), and the remaining 10 % of the species use abiotic agents – largely wind (anemophily) – and a small proportion of them use water (hydrophily) (Ollerton et al. 2011).

Depending on the origin of pollen, pollination is categorized into the following three categories:

Autogamy – transfer of pollen grains from the anther to the stigma of the same flower

Geitonogamy – transfer of pollen from the anther to the stigma of another flower of the same plant or of another plant of the same clone (ramet)

Xenogamy – transfer of pollen from the anther to the stigma of a different plant (not of clonal origin, genet)

Autogamy does not require pollinating agents, while geitonogamy and xenogamy require pollinating agents for pollen transfer. Another term, allogamy, has often been used in the literature to indicate transfer of pollen from one flower to the other irrespective of whether it is from the same or a different plant.

In several species, autogamous self-pollination (without external agents) takes place to different degrees. Cleistogamy is one such mechanism in which flowers never open and the stamens and the pistil remain enclosed within the flower bud (Lord 1981; Richards 1986; Kaul and Koul 2009). In cleistogamous flowers, the stigma and anthers are in contact with each other; pollen grains germinate inside the anther, or after coming in contact with the stigma, pollen tubes enter the stigma and grow through the pistil. Most of the cleistogamous species such as *Commelina benghalensis* and species of *Viola* produce both cleistogamous and chasmogamous (flowers that open) flowers. Often the production of cleistogamous flowers depends on the prevailing

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environmental conditions particularly the temperature and light. *Ruellia*, for example, produces chasmogamous flowers during the summer and cleistogamous flowers during the winter under Delhi conditions. Cleistogamous flowers are exclusively autogamous.

Even in several chasmogamous species, autogamous pollination has evolved as a means of reproductive assurance in the absence of pollinators (Kalisz and Vogler 2003; Eckert et al. 2006). Dioecious species and those which are strictly self-incompatible are exclusively xenogamous. Most other species show mixed mating system; both cross- and self-pollinations occur to various degrees depending on the structural features of the flower and the visitation frequency and efficacy of the pollinators.

Some of the major floral adaptations of the three pollination syndromes are summarized below:

7.1 Anemophily

Amongst the abiotic mode of pollinations, anemophily (wind pollination) is more prevalent than hydrophily. Anemophily is common in several families such as Poaceae, Juncaceae and Cyperaceae. Anemophilous species are generally characterized by non-showy flowers. The perianth is much reduced or even absent, and the flowers lack colour, nectar and smell. Wind-pollinated species produce a large amount of pollen grains to compensate for the uncertainties of pollen landing on the stigma. Pollen grains are dry and powdery with a smooth surface. The stamens are large and borne on long filaments and their anthers are well-exposed to the air. The stigma is also large, well-exposed and often feathery to trap airborne pollen. The number of ovules in the ovary is generally reduced, often to just one. Wind pollination is limited in tropical forests (about 5 %), where the movement of wind below the canopy is highly reduced and frequent rains wash the pollen from the air, when compared to temperate forests.

Wind pollination is considered as secondarily derived (Endress 1994; Bronstein et al. 2006; Waser and Ollerton 2006). This is based on the prevalence of predominantly insect pollination in fossil records of primitive angiosperms, lack of wind pollination in extant basal angiosperms and occurrence of wind pollination in specialized families of angiosperms.

7.2 Hydrophily

Hydrophily (water pollination) is rare and is limited to just about 30 genera of 11 families, largely monocotyledons (McConchie 1983). Water acts as a vector in the transportation of pollen in hydrophilous species. A majority of plants growing in water are not hydrophilous; their flowers emerge above the water level and are pollinated by other agents as in terrestrial plants (largely by biotic agents). Marine angiosperms are exclusively hydrophilous.

Basically, there are two types of hydrophily – epihydrophily or ephydrophily and hypohydrophily or hyphydrophily. This distinction is largely based on the movement of pollen; in the former it is two dimensional, and in the latter it is three dimensional. In epihydrophilous species, pollination takes place on the surface of water. In several of them, the reproductive organs are carried just above the water surface (dry epihydrophily), and pollen grains do not come in contact with water during pollination. In others, pollen floats on the surface of water (wet epihydrophily). In hyphydrophilous species, the flowers are submerged in water, and thus pollen grains are dispersed below the water surface and come in contact with submerged stigma. Hypohydrophily is reported in 18 genera of which 17 are monocots and 12 are marine species (Cox 1988, 1993).

Water-pollinated species tend to show unisexual flowers with reduced perianth and absence of colour, nectar and smell. The number of ovules is generally reduced, usually to just one, and the stigma is rigid with a large surface. Adaptations of pollen grains to water medium are found in only those species which come in direct contact with water (wet ephydrophily and hyphydrophily). In such species, the exine of the pollen grains is highly reduced or even absent. Pollen grains are covered with a coating of mucilage to prevent

them from wetting in water. Pollen grains of many such species are filamentous which facilitates their movement in water.

Studies on pollination biology of hydrophilous plants are more difficult when compared to those pollinated by other agents, and the information available is limited (Ducker and Knox 1976; Cox 1988, 1993; Cox et al. 1990). There are not many methods that have been described to study hydrophily. Epihydrophilous species may be studied by direct observation. Exclusion methods used for terrestrial plants need to be modified to suit water medium. Floating rafts or small boats are used to approach flowers in water bodies. For epihydrophilous species, metal or plastic cages suitably modified to prevent the movement of pollen grains on the surface of water can be used in exclusion experiments. Pollination studies on hyphydrophilous plants are more difficult and require diving gears. Growing plants in aquariums is more convenient to study floral biology and pollination biology in hydrophilous species when compared to natural conditions.

7.3 Zoophily

An essential feature of zoophily/biotic pollination is that plants have to develop effective devices to attract suitable animals to visit their flowers in a sustainable way by providing them with some rewards and use them effectively for pollination services. Further, plant species have to use some degree of discretion and restrict the number of animal species visiting the flowers of each species to a reasonable number. If they attract animals indiscriminately, all potential pollinators, present in the habitat, may visit all synchronously flowering plant species; this would bring about extensive heterospecific pollination which seriously reduces the fitness of the plant species. The aim of pollination ecologists is to understand how plants perform these conflicting demands of attraction and restriction of animal species for pollination services. Studies on pollination ecology so far have largely been focused on understanding the details of attraction. Only limited studies have been carried out on the details of restriction of potential pollinators (Shivanna 2014).

As pointed out earlier, the number of species pollinated by animals far exceeds those pollinated by abiotic agents. Zoophilous flowers exhibit an amazing variety in the size, shape, arrangement, colour, scent and sexual system. The evolution of such matchless variety of flowers obviously facilitates the flowers in performing the dual function of attraction and restriction of pollinators. Figure 7.1 presents some of the variations found in flowers and their pollinators. The flowering plants, although last to evolve amongst plant groups (over 100 million years ago in the early and middle cretaceous period), became the most successful group and occupied a pre-eminent position amongst all groups of plants. Evolutionary success of angiosperms has been attributed to the origin of the flower and associated evolution of biotic pollination to bring about cross-pollination (*see* Pellmyr 2002; Shivanna 2003; Willmer 2011). Amongst animals, insects are the major pollinators. According to one estimate, of the 13,500 genera of angiosperms, 500 contain bird-pollinated species, 250 contain bat-pollinated species, and 874 contain wind- or water-pollinated species; the remainder contain mostly insect-pollinated species (Renner and Ricklefs 1995). Hymenoptera (bees, wasps and ants), Lepidoptera (butterflies and moths), Coleoptera (beetles) and Diptera (flies) are the major orders of insects involved in pollination. Birds and bats are the other important pollinators.

Bees show great variation in body size and length of proboscis. Bees (both medium and large sized) are the most important pollinators. Bees are good in recognizing colours and scents and efficient foragers of nectar and pollen. Unlike humans, many bees can perceive light in ultraviolet range but cannot visualize shades of red which appear black to them (Kevan 2005). Butterflies are active during the day and land on the flower before foraging. Beetles are a very diverse group of insects. Beetles tend to move around the flowers chewing floral parts indiscriminately and get covered with pollen.

Thrips (Thysanoptera) are frequently present in flowers and feed on pollen and nectar. In several

Fig. 7.1 Diversity of flowers and pollinators. (**a**) *Cynopterus sphinx* bat pollinating the flower of *Oroxylum indicum*. (**b**) A giant Asian honeybee (*Apis dorsata*) on the flower of *Aegle marmelos*. (**c**) *Parotis marginata* moth on the flower of *Wrightia tomentosa*. (**d**) Graceful awlsnail (*Lamellaxis gracilis*) pollinating the flower of *Volvulopsis nummularium* (*after* Sarma et al. 2007). (**e**) Three-stripped squirrel pollinating the flower of *Butea monosperma* and (**f**) a syrphid fly (*Helophilus fasciatus*) amidst the flowers of *Schleichera oleosa*

species, thrips have been shown to be the major pollinators (Mathur and Mohan Ram 1978; Ananthakrishnan 1993; Garcia-Fayos and Goldarazena 2008). Their limited flights largely promote self-pollination. However, movement of thrips, which are very small insects, is often assisted by wind, and in such situations, their pollen may be carried for longer distances (Ghazoul and Sheil 2010).

Depending on the position of anthers in the flower in relation to the entry of the insects, pollen grains may be deposited on the body of the insect in a diffuse manner, or they may be deposited on the upper (nototrobic) or lower (sternotrobic) surface of the body. The position of the stigma is generally such that it comes in contact with the surface of pollen deposition and the pollination is brought about. Diffuse distribution is generally

associated with primitive flowers and unspecialized insects, while the other two types are commonly found in advanced flowers and specialized insects (Faegri and van der Pijl 1979).

Bird-pollinated (ornithophilous) species have been reported in about 65 families (*see* de Wall et al. 2012). Amongst birds, humming birds (family Trochilidae) are one of the major pollinators and are restricted to the New World; they forage while in flight by hovering near the flower. Sunbirds (Nectariniidae) and sugarbirds (Promeropidae) are common pollinators in Asia and Africa. Sunbirds are capable of hovering, but perch if a perch is available. The other major bird pollinators are honeyeaters (Meliphagidae), restricted to Australia, and honey creepers (Drepanididae) endemic to Hawaii (Kearns and Inouye 1993). In India, as many as 58 bird species belonging to 16 families have been reported to be involved in pollination of 93 species of flowering plants (Subramanya and Radhamani 1993). The information available on bird pollinators is much less than those on insect pollinators. Bird-pollinated flowers are generally bright coloured, most of them being red and scentless. The nectar is the main reward for bird pollinators; bird-pollinated flowers produce copious amount of nectar with low viscosity. The nectar is located in long corolla tubes.

Bats are nocturnal pollinating agents. Bats involved in pollination belong to the order Megachiroptera in the Old World and Microchiroptera in the New World. Bat pollination (Chiropterophily) has been recorded in a large number of tropical and semitropical plant species of over 40 families (Endress 1994; Gibson 2001). Bats can hover, cling or perch while foraging the flowers. Moths (Lepidoptera) are the other major group of animals involved in nocturnal pollination.

Apart from insects, birds and bats, there are other groups of animals particularly nonflying mammals such as marsupials, rodents and primates (Kress 1993; Endress 1994; Ghazoul and Sheil 2010) which are involved in pollination in a limited number of species. Some of these unusual pollinators are cockroaches (Nagamitsu and Inoue 1997 – *Uveria*), mice (Wester et al. 2009 – Pagoda lily), squirrels (Tandon et al. 2003 – *Butea*), snails (Sarma et al. 2007 – *Volvulopsis*) and lizards (Olesen and Valido 2003; Ortega-Olivencia et al. 2012 – *Scrophularia,* Hansen et al. 2007 – *Trochetia*).

Pollination brought about by each group of pollinators has been given a specific terminology (Table 7.1). Variation in floral attractants, rewards and flower morphology determine, to a large extent, the type of animal species that visit the flowers and the specificity of the visiting species. The literature on pollination is full of a large number of terminologies. Some of these terminologies with reference to the flowers and pollinators are presented in Table 7.2.

7.3.1 Floral Attractants and Rewards

Plant species pollinated by animals have to advertise their presence and provide rewards for the visiting animals to sustain their visits. Floral colours, sizes and shapes act as visual attractants, and olfactory attractants are in the form of floral scents. In most of the species, advertisements are provided by floral organs, although in a few, extra floral organs such as bracts may take part in attraction. Most of the species with hidden nectar have contrasting patterns on the corolla termed nectar guides (Fig. 7.2a, b) that guide the visitor to the source of nectar; their size and shape are highly variable. Many investigators have experimentally shown that these nectar guides do play a role in guiding pollinators to the site of nectar (Leonard and Papaj 2011; Hansen et al. 2012).

Several plant species have evolved traits that are beneficial to both the plant species and the pollinator. Postpollination colour change is one such feature that has been recorded in over 214 species of 74 geographically and taxonomically diverse families (Gori 1983; Weiss 1991). In these species the pollinated flowers, instead of senescing, change colour and are retained on the plant. In *Lantana camara*, for example, the flowers are yellow on the day of anthesis and offer pollen and nectar to the pollinators. They turn orange and then red on subsequent days and do not offer rewards (Mathur and Mohan Ram

Table 7.1 Pollinator groups and their respective floral syndrome traits

Pollinator group	Technical term	Floral syndrome characteristics and some examples
Beetles	Cantharophily	Flowers unspecialized, open, generally large, cylindrical or bowl shaped, sex organs exposed, dull coloured, no nectar guides, rewards easily accessible, strong odour, pollen deposited all over the body, often thermogenicity involved in attraction. Members of Annonaceae, Lauraceae, Myristicaceae and Dipterocarpaceae
Flies	Myophily	Flowers small, radial, dull coloured, no nectar guides, decaying odour, sex organs generally hidden, pollen deposited all over the body. Members of Anacardiaceae, Araceae, Aristolochiaceae
Bees	Melittophily	Flowers highly variable in morphology, simple open to complex often zygomorphic with landing sites and nectar guides, brightly coloured, odours mild, nectar hidden but not deep, sexual organs concealed, stamens few, precise pollen placement on the body, offer nectar and pollen. Members of Fabaceae, Bignoniaceae, Melastomaceae and Euphorbiaceae
Butterflies	Psychophily	Flowers tubular, erect, diurnal blooming, mild odour, brightly coloured, nectar ample and deeply hidden in tubes/spurs, pollen deposited on proboscis and head. *Delonix, Caesalpinia, Ixora, Mussaenda*
Moths	Phalaenophily	Flowers horizontal or pendent, nocturnal blooming, pale and heavily scented, nectar deeply hidden in long tubular corolla or spurs, pollen deposited on proboscis and head. Rubiaceae, Apocynaceae, Meliaceae, Mimosoideae
Ants	Myrmecophily	Flowers variable, often small and open, pollen placement diffuse
Birds	Ornithophily	Flowers fairly robust, diurnal blooming, rigid, vividly coloured often red, long tubular corolla, copious nectar with low sugar concentration, odours absent, pollen placed on beak, head or precisely on body, nectar sole reward. *Erythrina, Butea, Spathodea*
Bats	Chiropterophily	Flowers nocturnal blooming, generally pale and large, strong odour at night, large mouthed and stiff, copious nectar, anthers often numerous, pollen placed on the head, nectar sole reward. Members of Bombacaceae, Bignoniaceae and Myrtaceae
Thrips	Thripophily	Small unspecialized flowers, often pale and white, faintly scented. Members of Anacardiaceae, Annonaceae, Dipterocarpaceae, Moraceae

Based on Faegri and van der Pijl (1979), Turner (2001), and Ghazoul and Sheil (2010)

1978). Red flowers are retained on the plant for several days depending on the variety and the weather conditions. Experimental studies have shown that the retention of older flowers on the inflorescences increases plant's attractiveness to pollinators from a distance (Weiss 1991). They discriminate the colour of the flower from a close range and visit yellow flowers significantly more often than orange or red flowers. Thus, the retention of pollinated flowers not only increases the visibility of the flowers to pollinators but also guides them to rewarding flowers.

Olfactory cues are in the form of volatile fragrance compounds emitted by flowers. The chemical composition of floral scents is one of the most extensively investigated areas of floral biology sine long because of their commercial value in perfume industry. The role of floral volatiles in pollinators' attraction is comparatively recent. The fragrance is in the form of complex mixtures of a large number of volatile compounds. Floral fragrance is largely made up of monoterpenoids, sesquiterpenoids, phenylpropanoids and benzenoid compounds (Williams 1983; Knudsen et al. 2006).

The complex mixture of volatiles is characteristic for each species. No two species, even if they are closely related, have been shown to produce identical mixture of volatiles. The specificity of fragrance of a species is established not by individual fragrance compound but a combination of compounds. Insects are able to distinguish

Table 7.2 Various terminologies used to describe the relationship between the flower and animal vector

Terminology	Characteristics
Based on flower	
Allophilic	No morphological adaptations; visited by unspecialized short-tongued visitors
Hemiphilic	Imperfectly adapted; visited by intermediate degree of specialized visitors
Polyphilic	Flowers pollinated by many species
Oligophilic	Flowers pollinated by several related species
Euphilic/monophilic	Flowers highly specialized and pollinated by one or a few closely related species
Based on vector	
Dystrophic	Unadapted, no relation to flower organization, often destructive but may bring about pollination
Allotropic	Poorly adapted for utilization of floral resources
Polylectic	Pollinators foraging on several species of plants
Oligolectic	Pollinators restricting foraging to a single genus, tribe, subfamily or family
Eulectic/monolectic	Pollinators foraging on a single species

Based on Faegri and van der Pijl (1979)

complex mixtures of floral volatiles from different species and respond accordingly (Dudareva and Pichersky 2000; Riffell 2011). Maximum emission of fragrance generally coincides with the activities of their pollinators, and the emission often follows endogenous rhythm (Dudareva and Pichersky 2000). Floral scent often changes after pollination and thus enables the pollinators to avoid pollinated flowers (Schiestl et al. 1997).

Pollen grains also emit odours that differ from those of other floral parts and are characteristic of the species (Dobson 1988; Dobson and Bergstrom 1996, 2000). Insects are able to discriminate pollen odours of different species. Flowers of many species emit unpleasant odours, due to the presence of amine-containing compounds which serve as chemical attractants to some insects particularly beetles and flies.

Plant–pollinator interactions are largely mutualistic; they result in reciprocal benefits to both the partners. It is a form of "biological barter" and involves exchange of resources of the plant such as pollen and nectar with the services of the pollinator (Ollerton 2006). Pollen and nectar form the most important rewards for the pollinators. A few species pollinators collect oils, and some others collect nest materials such as resins and waxes (Armbruster 2012). Nectar is largely made up of sugars secreted by the nectary. The major sugars present in the nectar are sucrose, glucose and fructose. Apart from sugars, nectar also contains small amounts of amino acids and traces of lipids, phenolics, alkaloids and proteins (Baker 1977; Nicolson and Thornburg 2007; Heil 2011). The amount of nectar produced per flower (common range 0.1–500 µl) and the concentration of sugars (common range 5–45 %) present in the nectar are highly variable. Nectar of several species has been reported to contain bacteria (Freidman et al. 2012) and a few contain yeasts (Herrera et al. 2009). The role of bacteria and yeasts in nectar is not well understood. In some species, the nectar is coloured (Hansen et al. 2007) and in several, it is scented (Raguso 2004).

Pollen grains are highly nutritious; apart from carbohydrates (ca 25 %), proteins (ca 25 %), amino acids (ca 10%) and lipids (ca 5 %), they are rich in vitamins and minerals (Schmidt and Buchmann 1992; Roulston and Cane 2000). Pollen grains are needed for the larvae and young ones of insects. Bees gather pollen in special parts on their body, the pollen baskets/corbiculum. Pollen baskets in honeybees and bumblebees are on the hind legs, but on leaf-cutting bees, they are under the abdomen.

Many Neotropical orchids produce fragrant compounds largely terpenoids and aromatics to attract as well as reward male euglossine bees. Euglossine bees collect fragrant compounds and store them in their modified hind legs (Dressler 1982). The bees are thought to use these compounds to produce sex pheromones that are released to attract females (Tan 2006).

In some species, the flowers reward the larvae of the pollinators with young seeds (nursery

Fig. 7.2 Nectar guides on the lower petals of *Adhatoda vasica* (**a**) and *Digitalis* sp. (**b**). (**c**) A generalized open flower of *Syzygium* sp. (*arrow* points to the stigma). (**d**) Specialized flower of *Impatiens* sp.

pollination). The pollination of *Ficus* species by fig wasps and of *Yucca* by yucca moth (*Tegeticula*) represents highly specialized obligate nursery pollination systems; each plant species is pollinated by a specific wasp/moth species (Machado et al. 2005). Neither the plant nor the pollinator is able to reproduce without the other. Most of the *Ficus* species are monoecious and produce male and female flowers in specialized inflorescences termed syconia. The female wasps enter the receptive syconium through the terminal ostiole. They lay their eggs in a proportion of female flowers. The larvae of the wasps feed on the gall formed in oviposited flowers, and the remaining pollinated flowers develop into seeds. The emergence of adult wasps from the larvae coincides with the maturation of male flowers. The wingless male wasps are short lived; they mate with the females, cut an exit tunnel in the wall of the syconium and die. The females loaded with pollen come out through the exit tunnel and enter another receptive inflorescence (which is in the female phase) through the ostiole to reproduce; they bring about pollination during their movement inside the syconium. Several investigators have shown that volatile compounds emitted by the receptive syconia are responsible for the attraction of their specific pollinators (Khadari

et al. 1995; Proffit et al. 2008, 2009). The syconia emit volatile compounds only during the period of receptivity of female flowers.

7.3.2 Nocturnal Pollination

In a great majority of species, pollination is diurnal and takes place during the daytime. However, in some species, pollination occurs during night-time. In yet others, pollination occurs during the day as well as night (Young 2002). In species with nocturnal pollination, flowers remain open during the night. In several species with diurnal and nocturnal pollination, the life span of flowers lasts for several days. This strategy of nocturnal and diurnal pollination ensures seed set when pollinators are scarce or unpredictable (Dar et al. 2006 and references therein). Beetles, moths, particularly hawkmoths, bats, and a few species of bees and rodents are the common nocturnal pollinators (Muchhala et al. 2009). Hawkmoths have the longest tongue amongst insects. They are confined to tropical areas.

Although visual cues are the predominant attractants for diurnal pollinators, they are not reliable during night-time; acoustics and olfaction are the principal means to locate flowers by nocturnal pollinators. Flowers of nocturnal pollination species are not brightly coloured but emit strong odour. Bat- and rodent-pollinated flowers produce large quantity of nectar. Bats of Microchiroptera (New World) can produce ultrasonic sound to locate flowers. The sound is reflected by the petals of bat-pollinated flowers, and bats have the ability of recognizing this reflected sound (echolocation) (von Helversen and von Helversen 1999). Echolocation ability is not developed in most of the Megachiroptera (Old World); they depend largely on olfactory and visual cues to locate flowers. When compared to diurnal pollination, the information available on nocturnal pollination is limited largely due to the difficulty of observing flowers and pollinators at night. Logistic problems in conducting observations particularly in tropical forests also deter studies on nocturnal pollination.

7.3.3 Pollination by Ants

Studies on plant–pollinator interactions are largely centred on flying insects as they are the most important pollinators around the world. Ants are active 24 h a day either as individual species or as overlapping guilds of species foraging for particular periods during the day or night. Although ants are amongst the most abundant insects on earth and visit flowers frequently, ant pollination has not evolved as a major pollination syndrome. Ant pollination has been documented in less than 20 species (Beattie et al. 1984; Beattie 1985). Several hypotheses have been put forward to explain the reasons for the absence of frequent evolution of ant pollination syndrome (*see* Beattie 1985). There are some evidences to indicate that the secretion of antimicrobial substance on their body surface (as a means of protection against bacteria and fungi in the soil) is harmful to the pollen also. So far, reports of ant pollination have been confined to herbs and small shrubs.

7.3.4 Non-mutualistic Pollination

In a number of species, flower–pollinator interactions are non-mutualistic. The flowers do not provide rewards for their pollinators (rewardless flowers), or the floral visitors exploit floral rewards without affecting pollination (nectar and/or pollen robbers) (Wiens 1978; Dafni 1984; Dettner and Liepert 1994; Renner 2006; Bronstein et al. 2006). Rewardless flowers exploit pollinators by signalling the presence of reward without providing the reward (deception). Non-mutualistic plant–pollinator interactions have evolved in all major groups of flowering plants. Orchids form the major group of non-mutualistic interactions; about one third of orchids (ca 3,000 species) are reported to be deceptive (Renner 2006).

Food deception is one of the widely recorded non-mutualistic pollinations. Non-rewarding species (mimic) coexists with rewarding species (model), and the flowers of the mimic resemble the flowers of the model. Floral visitors draw rewards from the model but do not discriminate strongly against

non-rewarding flowers. Food deception is largely mediated by visual signals; olfactory signals do not seem to play a major role. The fragrance compounds in several food deceptive orchids have been shown to be quite different and week when compared to its model (Galizia et al. 2005; Salzmann et al. 2007).

A number of orchid species, particularly of *Ophrys*, achieve pollination through sexual deception – the shape and colour of their flower mimic the female insect of its pollinator, and the floral scent mimics the pheromones of the female insect. The pheromones attract mate-seeking males to the flowers from a distance. The visual cue serves as a close-range attractant and induce sexually stimulated males to land on the flower; the male tries to copulate (pseudocopulation) with the flower and brings about pollination (Ayasse et al. 2003; Schiestl et al. 2003; Huber et al. 2005; Phillips et al. 2014). Although sexual deception is predominant in orchids, it has been reported in a few other species (Asteraceae, Ellis and Johnson 2010; Iridaceae, Vereecken et al. 2012).

Several species belonging to ten families such as Annonaceae, Araceae and Aristolochiaceae mimic brood sites and attract insects whose larvae feed on dung/carcasses. Their flowers (*see* Wiens 1978; Bernklau 2012) mimic the odours of dung and/or carrion to attract coprophilous beetles and flies that oviposit or feed on carrion or dung. These odours smell like decaying proteins or faeces and are very unpleasant to humans. The odours are composed of sulphide compounds, ammonia, alkyl amines, cadaverine and putrescine. Faecal-like odours are also produced by skatole and indole compounds (Dettner and Liepert 1994).

In some species showing brood site mimicry, the odours are enhanced by the production of heat in the flower/inflorescence (thermogenesis). It has been reported in members of Nymphaeaceae, Annonaceae, Araceae, Arecaceae, Aristolochiaceae and Magnoliaceae (*see* Thien et al. 2009). Flowers of some of these species have developed trapping mechanisms to retain insects in the flower for one to several days. In species of *Aristolochia* (Murugan et al. 2006; Trujillo and Sersic 2006), the flowers are protogynous and emit fragrance during the female phase. The pollinators are attracted to the fragrance and enter the flower through narrow corolla tube. The insects cannot escape because of the presence of downwardly pointed hairs on the inner surface of the corolla tube. When the flower enters the male phase, the insects get coated with the pollen; by this time the downwardly pointed hairs senesce and allow the insects to escape. They are attracted to another flower that is in the female phase; they enter the flower and deposit the pollen on the receptive stigma.

7.3.5 Ambophily

Generally, anemophilous and entomophilous species do not show combination of both the syndromes; however, there are several species in which both the types of pollinations are combined (ambophily). Some examples of ambophily are *Plantago lanceolata* (Stelleman 1978), *Urginia maritime* (Dafni and Dukas 1986), species of *Salix* (Sacchi and Price 1988; Tamura and Kubo 2000; Karrenberg et al. 2002) and *Thymelaea velutina* (de la Bandera and Traveset 2006). In *Ceratonia siliqua*, for example, the flowers are showy and entomophilous; they show a combination of diurnal and nocturnal entomophilous pollination as well as wind pollination (Dafni et al. 2012). Wind pollination in species showing entomophilous syndrome compensates low frequency of insect visits. Similarly insect pollination in species showing anemophilous syndrome reduces uncertainty associated with wind pollination.

7.3.6 Floral Visitors and Pollinators

Flowers of most of the species are visited by a number of animal species. All of them need not be pollinators; some of them may rob pollen and/or nectar without affecting pollination. It is, therefore, necessary to distinguish pollinators from non-pollinating floral visitors. Many investigators have identified pollinators on the basis of their visit to the flowers and/or presence of pollen load on their body. However, careful studies have shown that all the floral visitors and those with

pollen load may not be pollinators (Sinu and Shivanna 2007; Sinu et al. 2011). Thus, presence of pollen load is not an authentic method for identifying pollinators. To confirm a floral visitor as a pollinator, one has to demonstrate the transfer of pollen grains to the stigma and/or seed set after a visit of a potential pollinator to a virgin flower. When there is more than one pollinator of a given plant species, their frequency of visits and pollination efficacy of each species in terms of pollen removal and/or pollen deposition may vary greatly. The pollination efficiency of some of them may be so low that they may not have any perceptible role in overall pollination success of the plant species.

7.3.7 Restriction to Pollinators

Any natural habitat is made up of a number of plant species and diverse animal species, many of which are potential pollinators. However, each plant species attracts only a proportion of potential pollinators to visit its flowers but prevents the visit of several others present in the habitat. As pointed out earlier, for an efficient pollination system, restriction of visits to a reasonably limited number of pollinator species is important to ensure their visits to largely conspecific flowers. Restriction to potential pollinators to the flower may act at different levels – morphology of the flowers, species-specific fragrance and quantitative and/or qualitative features of the nectar and pollen rewards.

7.3.7.1 Morphological Filters

Flowers of many species are generalized type; their rewards (both pollen and nectar) are open and accessible to any visitor (Fig. 7.2c). Flowers of a large number of species show structural diversification, and their rewards are not accessible to all the visitors (Fig. 7.2d). Such flowers restrict the visits to a limited number of animals. One such floral diversification is the change from radial symmetry to bilateral symmetry. Bilateral symmetry enables the flowers to guide the approaching pattern of the visitors to harvest the rewards efficiently. Flowers of such species are visited only by those animal species that are able to locate and harvest the rewards. Another type of elaboration is the evolution of a long corolla tube or a spur in which nectar is located (Richards 1986; Pellmyr 2002). A number of species in Orchidaceae have spurs of various lengths often reaching up to 40 cm. The nectar in such flowers with spurs or corolla tubes is accessible only to structurally suited animals; only those that have the proboscis/beak of suitable length can harvest the reward. Many studies have shown that pollinator species with different tongue lengths tend to specialize on plant species with matching spur/corolla tube lengths (Pleasants 1983).

Pollination Syndromes: The role of morphological traits of flowers that permit some species of pollinators (and prevent some others) was known since long. Traditional concept, elaborated by Darwin, on pollination systems has been that the evolutionary tendency of a species is towards greater refinement making the pollinator and the flower mutually interdependent. This led to the view that the combination of floral traits reflects pollinator type and each pollinator type is the result of selection pressure exerted by different pollinators. This eventually resulted in the formulation of various "pollination syndromes" (Faegri and van der Pijl 1979; Turner 2001). A pollination syndrome is a combination of floral traits associated with the attraction and utilization of a specific functional group of animals for pollination (Table 7.1). Pollination syndrome concept can explain floral diversity and convergence of floral forms across angiosperms pollinated by similar pollinators (Bronstein et al. 2006). A high level of specialization has the advantage for both the pollinator and the plant species as long as both the partners are adequately available. This also has inherent weakness; if one of the partners becomes rare or absent, the other is also bound to fail unless it evolves compensatory mechanisms such as self-compatibility, autogamy, vegetative propagation or apomixis. High specialization also binds both the partners for spreading to new areas.

There has been a considerable discussion in literature in recent years on specialization and

generalization in plant–pollinator interaction (Waser et al. 1996; Waser and Ollerton 2006; Johnson and Steiner 2000). Many recent studies have shown that plant and pollinator assemblages are mostly generalized; a majority of plant species are visited by taxonomically diverse groups of pollinators, and most pollinator species visit several plant species. These studies have questioned the concept that floral traits associated with a pollination syndrome constitute an adaptive response (*see* Mitchell et al. 2009). An advanced level of specialization is seen in a very limited number of species such as some orchids, *Yucca* with yucca moth, and *Ficus* with Ficus wasp, in which pollination of each species is brought about by one specialized insect.

Several investigators have emphasized the utility of pollination syndromes in understanding the mechanisms of floral diversification (Fenster et al. 2004) and have elaborated the evolutionary aspects of mutual interaction between pollinators and the plant species (Mitchell et al. 2009). They argue that costs and benefits of plant–pollinator interactions play an important role in determining whether these interactions are more 'generalized' or 'specialized'. If a plant species has many visitors which provide similar and comparable pollination services, there is little incentive for plants to specialize to attract a particular group of pollinators. On the other hand, if some floral visitors are more effective in the quantity or quality of pollen transfer, selection should favour traits promoting these effective pollinators (Mitchell et al. 2009). Variation amongst floral visitors in pollination efficiency is therefore a requirement for the evolution of specialization (Schemske and Horwitz 1984). These pollination syndromes (permitting some and preventing other visitors) are likely to function in combination with floral fragrance.

7.3.7.2 Fragrance Filters

As pointed out earlier, the composition of fragrance is unique to each plant species and attracts a specific pollinator or a group of pollinators. The fragrance acts as a filter as its attraction is restricted to some species; those animals which are not attracted to the fragrance do not visit the flowers (Williams and Dodson 1972; Omura et al. 2000). In species with obligate specialization, fragrance may be the only filter. A meta-analysis of 18 studies on the response of animals to floral scents by Junker and Bluthgen (2010) has highlighted the dual function of floral scents; obligate floral visitors are attracted to floral scent, while those which are facultative and antagonists are repelled by floral scents.

7.3.7.3 Nectar Filters

Studies on nectar have so far highlighted largely the role of its nutritive components, sugars and amino acids, as rewards for the visitors. The amount of nectar present in the flower and its sugar concentration are, to some extent, correlated with the type of animals visiting the flowers (Baker and Baker 1983). Bee-visited flowers generally have lower amount of nectar with higher sugar concentration, while bat- and bird-visited flowers have higher nectar volume with lower sugar concentration. These features are well recognized and form a component of pollination syndromes.

Several studies have highlighted the role of non-nutritive metabolites such as alkaloids and phenolics in attracting or repelling floral visitors (Stephenson 1981; Adler 2000; Irwin et al. 2004; Adler and Irwin 2005; Raguso 2004). Nectar traits often deter nectar robbers without affecting the visits by pollinators. The floral nectar of *Catalpa speciosa*, for example, contains iridoid glycosides, catapol and catalposide, which adversely affected potential nectar thieves (ants and a skipper butterfly, *Ceratomia catalpae*); the legitimate diurnal bee pollinators were not affected by these glycosides (Irwin et al. 2004). Similarly, some South African species of *Aloe* produce dark brown nectar with a bitter taste because of the presence of phenolic compounds. Bulbuls and white eye, which are effective pollinators, are unaffected by the bitter taste of nectar, while bees and sunbirds which are not the pollinators are deterred by the phenolics in the nectar (Johnson et al. 2006).

Floral nectar of several species is scented. Various components of the fragrance have been shown to have positive or negative effects on

different animals. In *Nicotiana attenuata* (Kessler and Baldwin 2006), benzyl acetone attracted the pollinators (moths and hummingbirds); methyl salicylate repelled ants but attracted moths. Thus, the nectar, at least in several species investigated, has the potential to filter flower visitors, favouring some and deterring others.

7.3.7.4 Pollen Filters

Nutritional quality of pollen is highly variable; some of them lack several essential nutrients and some are poor in proteins (Roulston and Cane 2000; Rasmont et al. 2005), and yet others contain secondary compounds which are repellent or toxic to insects (Pimentel de Carvalho and Message 2004; *see* Hargreaves et al. 2009; Sedivy et al. 2011). Several studies indicate that pollen can act as a filter to select floral visitors. Analyses of pollen loads of several bee species have shown that some species are specialists at the level of plant families or subfamily or even genera, while others are generalists visiting the flowers of up to 15 plant families (Schmidt 1982; Müller and Kuhlmann 2008; Sedivy et al. 2008). Thus, floral visitors show preferences to pollen of some species and avoid visiting the flowers of other species. Such choices in pollen foragers may be physiologically constrained.

A few studies have been conducted on the effects of host and non-host pollen on the development of larvae of bee species (Sedivy et al. 2011). Pollen of *Sinapis arvensis* (Brassicaceae) and *Echium vulgare* (Boraginaceae) failed to support larval development of *Colletes* bee species specialized on pollen of *Campanula* (Praz et al. 2008). Similarly, pollen of Asteraceae and Ranunculaceae permitted larval growth of only those bee species that are specialized to harvest pollen from plants belonging to these families; their pollen failed to support larval growth of other bee species. These studies clearly indicate that palatability of pollen can act as an effective filter to restrict the number of floral visitors. Pollen of non-host species may hamper the digestion of the larvae, and the bees seem to have adapted their metabolism to digest pollen of host species (Leonhardt and Bluthgen 2012).

7.4 Pollination Efficiency

Pollination efficiency has been defined in various ways by pollination ecologists. Often different and confusing terminologies have been used (*see* Dafni et al. 2005). Pollination efficiency can be referred to the efficiency of individual floral visitor to bring about pollination. In this manual pollination efficiency of the visitor is defined as the number of pollen grains deposited on the stigma of a virgin flower after one visit by the pollinator. It can also be assessed on the basis of the number of seeds induced by the pollinator in one visit. Pollination efficiency can also be referred to overall pollination efficiency of all the floral visitors combined. Pollination efficiency under field conditions refers to the per cent of flowers that gets pollinated irrespective of the number of visiting species or number of visits of each species.

7.5 Pollination Limitation

Pollination limitation refers to the reduction in seed production by inadequate deposition of conspecific compatible pollen on the stigma. Pollination limitation has been reported in a large number of species; some of the studies indicate that over 60 % of the species may show pollen limitation under certain conditions (Burd 1994; Wilcock and Neiland 2002; Knight et al. 2005). This is one of the major constraints that often drive the populations to vulnerability.

7.6 Pollen Travel and Gene Flow

The movement of alleles physically through space is referred to as gene flow. Pollen grains and seeds are the agents of gene flow. Often the distance for which pollen travels from its source before it lands on the stigma is used as a measure of gene flow. There are different methods such as staining pollen (before their dispersal) with vital or fluorescent stains and labelling of pollen with radioactive carbon, which can be used to measure

the distance of pollen travel. The use of genetic or molecular markers to identify the progeny sired by the pollen of a marked plant is the most authentic method to study gene flow (for details *see* Kearns and Inouye 1993; Dafni et al. 2005).

7.7 Protocols

7.7.1 Estimation of Nectar Volume and Concentration of Total Sugars in Nectar

Nectar is an important reward for biotic pollinators. It is largely made up of sugars. The amount of nectar and its sugar concentration are highly variable and to some extent reflect the nature of the pollinator. The dynamics of nectar secretion, its location in the flower, the quantity and concentration of sugar are important in understanding pollination ecology.

7.7.1.1 Special Requirements

Isolation bags and tags

Calibrated capillary tubes or micropipettes (1–50 µl depending on the amount of nectar present in the flower of the focal species)

Portable refractometer (with 0–50 % range is sufficient)

7.7.1.2 Procedure

1. Bag flower buds before anthesis (preferably the previous evening of anthesis) to prevent visitors from foraging the nectar before estimation.
2. Soon after anthesis, remove the bags and excise flowers for collecting nectar.
3. Collect nectar by gently inserting calibrated capillary tube of suitable capacity (1, 2, 5, 10, 25 and 50 µl capacity are available), depending on the amount of nectar present, and allow sufficient time for the movement of nectar into the capillary tube through capillary action. When the amount of nectar is abundant as in some bird-/bat-pollinated flowers, micropipettes/microsyringes of various capacities (0.1 ml/1.0 ml) may be used.
4. Estimate the amount of nectar for each flower. As the lumen of the calibrated capillary tubes is uniform, the amount of nectar can be estimated by measuring the length of the tube filled with nectar and calculating its amount on the basis of the total length of the capillary tube up to the calibration point. Generally, 10–20 flowers for each reading are satisfactory.
5. Calculate the average amount of nectar per flower and present with SD or SE.
6. Dispense nectar from capillary tube/micropipette onto the surface of the calibrated portable refractometer. Lower the lid of the refractometer slowly without allowing air bubbles to be trapped. The amount of nectar should be sufficient to cover the entire surface of the refractometer when its lid is lowered. The readings of the refractometer indicate nectar concentration as percentage of sucrose equivalents. Repeat this estimation at least for 6–10 flowers and calculate the average.

7.7.1.3 Modifications

1. Instead of bagging the flower buds the previous evening (step 1), flowers can be collected soon after anthesis before they are visited by any floral visitors.
2. For species in which the longevity of flowers extends for several days and also those that show protandry and protogyny, it would be necessary to study the details of nectar secretion every day until flower senescence, particularly during male and female phases. In such species, more elaborate planning for nectar estimation is needed depending on the objective of the study. Larger number of flower buds is bagged, and specific number of flowers is excised at a given time (with reference to the time of anthesis) for nectar estimation. There should be sufficient number of flowers for each set. The results on the amount of nectar are generally presented in the form of a table/graph/histogram over the period with SD/SE for each value.
3. To check if the nectar is secreted every day, carefully remove the nectar from flowers in the evening and bag them. Next morning open the bags and estimate the amount of nectar secreted and concentration of sugars in the

nectar. This would give the amount of nectar secreted during the night.

4. In some studies, information on the amount of nectar consumed by the visitor and resorption of unused nectar by the flower before senescence may also be required. Comparison of the amount of nectar in bagged flowers and those in which the visitor has foraged nectar would give the amount of nectar foraged by the visitor. Comparison of nectar amount in fresh flowers and those bagged until initiation of senescence would indicate if the nectar is resorbed in non-visited flowers. When there is active resorption, there is hardly any nectar left in senescing flowers. Marginal reduction in the amount of nectar may be due to the evaporation of the nectar; in such cases, the amount of sugars generally shows a marginal increase.
5. In several species, the amount of nectar present in the flowers is very small (<1 μl). In such species it is difficult to collect nectar through capillaries and estimate sugar concentration using a refractometer. McKenna and Thomson (1988) suggested a sensitive method to estimate total carbohydrates in the nectar in such species. Instead of capillaries, small filter paper strips are used as wicks to collect available nectar. The paper strips are dried and stored until used for sugar estimation using calorimetric method (for details of protocol, refer to McKenna and Thomson 1988).

7.7.2 Floral Visitors and Frequency of Their Visits

Studies on floral visitors, their visitation frequency, foraging time and behaviour are important aspects of pollination ecology. These studies are needed not only to document floral visitors but also to determine their role in pollination of the species. The frequency of visits (number of visits per flower per unit time) would indicate their possible involvement in pollination and also, to some extent, serve as an indicator of pollinators' abundance. Foraging time (the visitor spends on the flower) indicates the extent of reward available in the flower. The way the forager harvests the reward from the flower (whether legitimate or illegitimate) and patterns of their movements between flowers of the same and other plants would indicate their efficiency in pollination and also the type of pollen they are likely to deposit on the stigma.

7.7.2.1 Special Requirements

Suitable tags to label flowers/inflorescences. In herbaceous species, pegs and thread may be required to encircle selected patch for observation.

Tabulated field notebook – although there is no standard format for preparing the table to record the details, it should contain, on the top, the name of the plant species, date of the study and the number of focal flowers marked for observation. A sample table is given for guidance (Table 7.3). The investigator may modify the same or prepare the table in his/her own format convenient for recording the details.

7.7.2.2 Preliminary Studies

This exercise requires some preliminary studies. It would be desirable to spend a couple of time slots (15/30 min in an hour) to become familiar with all the visitors to the flower and to understand the details of recording various events in the tabulated sheet:

Selection of the focal flowers: Depending on the number of visiting species, their frequency and the size and density of flowers, the investigator has to decide on the number of flowers/inflorescences to be kept under observation on each day so that all the visitors can be easily recorded during the observation time.

Observation period of the day: Depending on the active period of the visitors (in some species, the visitors may be active the whole day, and in others the activity may be confined to the morning and/or evening hours), the period of record has to be selected.

Suitable time slots: When the studies are carried out by one person, it is not feasible to make observations on the visitors continuously throughout the day. Generally, 15/30 min slot for each h is selected depending on the

Table 7.3 A sample table for recording the frequency of visits of each floral visitor

Plant species			Observation date	
No. flowers in the study patch: 15			Weather conditions	
No. of flowers visited in each bout[a]				
Time slot	Visitor A	Visitor B	Visitor C	Visitor D
06.00–06.30	3, 4, 2, 3, 3, 4, 1	–		
07.00–07.30				
08.00–08.30				
09.00–09.30				
10.00–10.30				
11.00–11.30				
To continue				

Arbitrary data is presented only for one of the visitors (A) for one time slot of 30 min. The record has to be for all the time slots for all the visitors covering the entire period of observation. Prevailing weather conditions include sunny or cloudy or rainy day, temperature and any other condition that may affect the visitors needed to be recorded. The number of flowers in the focal patch is arbitrarily put as 15 for the purpose of calculation

Calculation of the frequency of visits for each time slot (based on arbitrary values recorded in the sample table)

Total number of flowers visited in 30 min: 3 + 4 + 2 + 3 + 3 + 4 + 1 = 20

Total number of flowers visited in 01 h (06–07 h) = 20 × 2 = 40

Total number of flowers in the study plot: 15

Frequency of visits (no. of visits/flower/h) = 40/15 = 2.66

For presentation of the results, the observations have to be replicated for 3 or 4 days using different batch of flowers and the mean values for each time slot have to be presented

[a]When flowers are visited by many visitors simultaneously in high frequency, it may be difficult to keep track of the visits of all the species. In such a situation, only one/two visitors may be recorded during each time slot. Alternatively, two persons may take observations and divide the species to be recorded between them

frequency of visits. In some species depending on the visitation details, it may be necessary to observe continuously during the day. In such situations, it is better to have two investigators recording alternately on the same set of flowers.

Identity of floral visitors: Identity of each animal species that visits the focal flowers needs to be recorded. If you do not know the name of the species, record each unknown species as a morphospecies for later identification. A few individuals of each of the unknown species have to be collected by using either a sweeping net or an aspirator (*see* Protocol 7.7.7), depending on the size of the visitor, and store them in rectified spirit for identification. Give each morphospecies a letter (A, B, C) so that it can be used for regular recording of its visits.

Mode of their landing/entry: The approach may be legitimate (when it comes in contact with the anthers and the stigma) or illegitimate when it robs the pollen or nectar without touching the anthers and/or the stigma. In actinomorphic flowers, the visitors generally land on the top of the flower and move amidst anthers and stigma. In zygomorphic flowers the visitors may land on one part of the flower and then move for foraging the pollen and/or nectar.

Foraging pattern: The visitor may forage only pollen or only nectar or both. Some insects forage exclusively pollen/nectar during some period of the day. These features have to be recorded.

Foraging time: The time spent by the visitor on each flower indicates the reward available in the flower.

Contact with the anthers and the stigma: It is important to observe whether the visitor comes in contact with the stigma and the anthers during their foraging. This is essential for effective pollination.

Behaviour of the visitor after its exit from the flower: When the visitor exits from a flower, it is desirable to note whether it visits other flowers of the same plant and if so the number of flowers of the same plant visited in each bout before moving to the flowers of a neighbouring plant. Sometimes it may move to the flowers of another species. These details would indicate the type of pollen deposited on the stigma.

Since the above features generally do not change for each visitor, some of the above details can be recorded during the preliminary studies and need not be repeated during all the time slots.

7.7.2.3 Procedure

1. Select a small compact flowering patch containing 10–30 fresh flowers from a single plant or a group of plants which can be observed from the observation spot and mark the patch or the flowers with small tags or by any other means. If the number of species visiting the flowers and their frequency are high, it would be better to select a smaller number of flowers so that the details of all the visitors can be conveniently recorded. If the frequency is low, a larger area with larger number of flowers can be selected. The main consideration for selecting the plot is to ensure that the investigator can clearly see any visitor visiting any of the flowers in the selected patch. Record the number of flowers in the selected area. In annuals, the study plot is generally made up of a group of plants which can be kept under observation from the observation spot without difficulty. If necessary, the selected patch may be enclosed with a square/rectangle/circle made with the help of pegs and thread. If it is a large shrub or a tree, the study unit has to be confined to some branches or inflorescences.
2. Select a comfortable place to sit (for insect visitors, 0.5–2 m away from the selected flowers is satisfactory as they are not very sensitive for human presence), and make sure that all the flowers in the selected plot are clearly visible. Birds are particularly sensitive to human presence. They may even avoid visiting the flowers when the humans are present nearby. For observing birds, it is better to select observation spot several metres away from the focal flowers/inflorescences/branches behind other bushes or branches to camouflage the sitting area.
3. Record the visits of all the visitors to selected flowers in the tabulated sheet (*see* Table 7.3) during each time slot. In compact inflorescences where visits to individual flowers are difficult to record, the inflorescence may be taken as a unit; the frequency is expressed as the number of visits/inflorescence/h. When the movements of foragers are very swift, it would be more convenient to record observations by using a video camera/handycam for later analysis. One of the advantages of video recordings is that the temporal record of the foraging time of pollinators/robbers can be ascertained with ease, and the recordings can be observed several times when more than one pollinator is visiting the focal flowers.

   Apart from recording the frequency of each visitor to the focal flowers, observe and record the following in the pre-tabulated sheet:

Details of foraging: The visitor may forage only the nectar or only the pollen or both; they can be recorded by the letters P/N/B, respectively. Many of the visitors may forage pollen as well as nectar in the morning hours, but confine only to nectar in the afternoon and evening hours when pollen is no more available. Also record whether the visitor's body comes in contact with the anthers and/or the stigma during their entry/foraging/exit.

Duration of visit: Depending on the reward available, the duration of visits on each flower may vary from less than a second to several seconds, often minutes. The duration may change depending on the depletion of nectar/pollen in the afternoon and evening. Duration of visits can be recorded with the help of a digital stopwatch of the mobile phone or digital wristwatch. This method, although most accurate, is cumbersome to record the time spent by each visitor on each flower. Alternatively, start the stopwatch when the visitor lands on the first flower in the observation patch, continue the count as it enters other flowers in the observation patch, and stop the watch when the visitor leaves the last flower of the observation patch. Calculate the duration of visits by dividing the total time spent on the selected patch by the number of flowers visited during each bout. Another method some investigators use to measure the duration of visits is to practise and standardize the counting in seconds so that foraging time starting from landing until departure can be counted and recorded. Combine the results of all the foraging trips for each time slot, and calculate the average time spent on each flower.

It is often difficult to record the number of flowers visited by each visitor and the time spent on each flower. We have found it convenient to record only the number of flowers visited by each visitor (frequency) in the observation patch during each bout in a 15/30 min time slot of each h; the remaining time of the h can be spent in recording other details such as the time the visitor spends on each flower, its foraging behaviour and whether the body of the insect comes in contact with the stigma and anther and so on.

As the pollinators' activity is affected by environmental conditions particularly temperature, light and precipitation, these parameters have to be recorded on each day of the study period.

Observations are discontinued for the day after the visitors cease to visit the flowers.

7.7.2.4 Modifications

1. In tall tree species, it would be better to construct a platform so that the investigator can sit comfortably near the selected flowers for longer periods and record the details. In the absence of a suitable platform, the investigator may be able to find a convenient branch to sit for observation. Alternatively, for small trees, a ground patch may be selected, and a good pair of binoculars may be used to observe visitors. However, by this method many of the smaller insects may be missed particularly at higher canopy. While recording observations with the binocular, it would be convenient to have two investigators, one to vocally announce and the other to record the details. Alternately one can use the recorder.
2. In many species, the flowers remain fresh only for a day; in several others, however, they remain fresh for more than a day, often for several days. In such species the observations have to be extended for the same set of flowers every day until the flowers do not receive any more visitors or until the flowers start senescing. Any new flowers that open in each study patch may be removed to confine observations to older flowers.

The above studies have to be replicated at least for 3–5 days for each population of the focal species using different randomly selected patches each day. It may also be necessary to record details of the visitors and visitation frequency during early, mid- and late periods of flowering as the visitors' guild/their frequency may change over the flowering period.

After completion of the studies on each patch of flowers, analyse the data. Calculate visitors' frequency (average number of visits per flower per h or any unit time), duration of foraging (average foraging time per flower in seconds) and foraging pattern (nectar/pollen/both) at different time slots studied during the day. The data on replicates on different days of the same time slot may be combined to determine the mean with ±SD/SE of the means. Represent the frequency of the visits and duration of foraging in the form of suitable graphs/histograms/box plots. Depending on the frequency of visits over time, many investigators combine the data of 2–4 h time slots to plot the graph/histogram (e.g. 06.00–09.00, 09.00–12.00, 12.00–15.00, etc.) instead of plotting for each time slot. Determine the total observation time spent for each species (number of hours spread over the number of days).

7.7.3 Identification of Pollinators Based on Pollen Transfer to the Stigma

As pointed out earlier, all the floral visitors may not be pollinators. Many visitors rob the nectar and/or pollen without affecting pollination. Some animals may visit flowers to feed on smaller insects present on the flowers. Identifying pollinators from non-pollinators is very important in pollination ecology.

7.7.3.1 Special Requirements

Tags and isolation bags

7.7.3.2 Procedure

1. Select a set of focal flowers as described in Protocol 7.7.2 and sit at a convenient place to record floral visitors. Based on your earlier/preliminary studies, make sure to start observations before the visits start so that none of the flowers in the selection plot has received a

visitor until the start of the observation. If this is not possible, the focal flowers have to be bagged one day before anthesis, and the bags are opened just before the start of the observation. This ensures that you start with a set of virgin flowers.
2. Keep track of the visits with reference to each visitor. It is better to focus on one visitor at a time. Chase away other visitor that approach the focal flowers by a stick or a small branch; do not allow them to land on any of the focal flowers.
3. Soon after the selected visitor exits a focal flower, excise the flower, label it with the name of the visitor, and keep it in a container with as little disturbance as possible for later studies. Continue observation until you excise enough number of flowers for each visitor (say 10–15 on each observation period).
4. Repeat these studies until you cover all the visitor species. If this cannot be done in one sitting, the studies can be spread to several sittings to cover all the visitors with enough number of replicates.
5. Take all the excised flowers to the lab without disturbing their stigmas. In bisexual flowers, it is important to make sure that self-pollen grains do not get deposited on the stigma during handling of the flower.
6. In the laboratory, take the stigma/pistil of each flower visited by each visitor individually and observe under a stereomicroscope the presence of pollen grains. If the pollen grains are too small to be seen under a stereomicroscope, mount the stigma in a drop of safranin/acetocarmine and observe under a compound microscope to record the pollen load. If the stigma is thick, tease the stigma, before observation. The presence of conspecific pollen on a substantial proportion of stigmas confirms that the visitor is an effective pollinator. Calculate the percentage of flowers that get pollinated by one visit of the specific visitor. If the conspecific pollen is absent on the stigma of any of the flowers visited by a particular visitor, it is not a pollinator. If such visitors (without pollen load on the stigma) forage pollen and/or nectar during their visit, they can be termed as pollen and/or nectar robbers.

7.7.3.3 Modifications

1. *Pollination efficiency based on pollen load on the stigma:* Pollination efficiency of different pollinators can be compared by calculating the percentage of stigmas pollinated by a single visit of each pollinator to the virgin flower. A further modification is that the number of pollen grains can be counted on each stigma under the microscope. If the number of pollen grains deposited is less than the number of ovules in the ovary, such stigmas are generally considered as insufficiently pollinated. If the pollen grains are more than the number of ovules, they are considered as sufficiently pollinated.
2. *Pollination efficiency of multiple visits by pollinators:* More often, pollinators keep visiting the same flower repeatedly, and in some instances, the same flower may receive as many as 8–10 visits by the same pollinator or different pollinators. In many such species, a single visit to a virgin flower may not be sufficient to deposit sufficient number of pollen grains. The above procedure may be modified to collect flowers visited by once, twice, three times or during the whole day of a specific pollinator.

   The following is the brief procedure when the flowers are visited by a *single pollinator*:

   (a) Give sequential numbers to all the focal flowers kept under observation in the study patch. Each flower may be labelled by an inconspicuous tag (so that it will not distract the visitor), or a rough diagram can be made on the notebook to indicate the position of all flowers under observation and each flower be given a serial number. As the forager visits each flower, note the serial number of the flower visited in the notebook and continue this record for the entire observation period. At the end of the observation period, count the number of visits for each flower. Excise all the flowers at the end of the observation period and categorize the flowers on the basis of the number of visits it has received.

   (b) Take the flowers to the laboratory without disturbing their stigmas. Observe all

flowers of each category (visited 1 to *n* number of times) under the microscope and calculate per cent of flowers sufficiently and deficiently pollinated for each category.

(c) Represent the results in the form of a table/histogram for each visitor. This is reasonably simple when the flowers are visited by a single pollinator.

In species with multiple pollinators, the same flower is likely to be visited by more than one visitor. Such species require more time and efforts to get data on multiple visits of each pollinator to focal flowers. Under each observation period, only the selected pollinator has to be allowed to visit the flower; others need to be chased away by a stick/small branch. This has to be repeated for each pollinator.

3. Efficiency of pollinator based on fruit set: Pollinators can also be confirmed and their efficiency determined based on the fruit set after one visit or multiple visits by a potential pollinator to a virgin flower.

   Follow the same procedure as described in the Protocol 7.7.3. Allow each pollinator species to make one or several visits as explained above. At the end of observation period, label each flower for the specific pollinator visited and the number of visits made. Bag the flowers and allow them to develop into fruits. Determine per cent fruit set and average number of seeds per fruit for each pollinator (for one visit and/or several visits) and compare the same for different pollinators to establish their efficiency.

7.7.4 Pollination Efficiency Under Field Conditions

In the above protocol, pollination efficiency is measured only in flowers visited by a specific pollinator. This does not indicate the pollination efficiency under field conditions. Depending on the density of pollinators, all fresh flowers may or may not be visited by pollinators. Also there may be variation in the number of times the pollinators have visited each flower. Unless there are chances for natural autogamy, the flowers which do not receive visitors will not get pollinated and hence do not set fruits and seeds. This protocol gives the methodology to determine pollination efficiency under field conditions.

7.7.4.1 Special Requirements

None for the main protocol
Tags for modification

7.7.4.2 Procedure

1. Excise the senescing flowers (which cease to attract pollinators) randomly from different plants in the population. The number of flowers to be selected depends on the number of individuals in the population and the area of the study site. The plants need to be selected randomly to cover the entire population. At least ten plants in a population of 50 individuals are recommended. The total number of flowers in such a population should be around 50–100.
2. Study the stigma of excised flowers under the microscope for the presence of pollen and score them as described under Protocol 7.7.3.
3. Determine the percentage of flowers pollinated. In multiovulate species, the percentage of deficiently pollinated and sufficiently pollinated flowers can also be determined by counting the number of pollen grains on the stigma of each flower.

As pollination efficiency is affected by a number of factors, these studies have to be carried out during the early, peak and late flowering season of the population.

7.7.4.3 Modification

Based on Fruit Set: Tag sufficient number of flowers, selected randomly, in the population, and keep them under observation until they abscise or develop into fruits. Count the number of fruits and the number of seeds in each fruit. Calculate per cent flowers that set fruits and mean number of seeds per fruit. Represent pollination efficiency as per cent fruit set and/or mean number of seeds per fruit.

7.7.5 Estimation of Pollination Efficiency Using Spear's Pollination Index

Many investigators have used Spear's pollination index (Spears 1983) to evaluate pollination efficiency of each pollinator. In this method, a pollinator is allowed to make one visit to a virgin flower and is bagged (*see* Protocol 7.7.3). Pollination efficiency (PE) is assessed on the basis of the number of seeds formed per visit. The PE is calculated as follows:

$$(\mathrm{PE}) = \frac{Pi - Z}{U - Z}$$

where *Pi* is the average number of seeds in the fruit that received only one visit by the pollinator *i, Z* is the average number of seeds in the fruit that received no visits by the pollinator *i* and *U* is the average number of seeds in the fruits that received unrestricted visits. The values of the pollination index range from 0 (when there is no contribution by a given pollinator) to 1 (when the production of seeds or fruits by a given pollinator is equal to that of flowers which received unrestricted visits by pollinators).

7.7.5.1 Special Requirements

Isolation bags and tags

7.7.5.2 Procedure

The procedure is basically the same as described under protocol 7.7.3, but requires the following three sets of virgin flowers:

1. Prepare the following three sets of flowers, each with sufficient number:
   (i) Flowers bagged the previous evening of anthesis (no visits).
   (ii) Allow one visit of each pollinator to each flower and bag them as in Protocol 7.7.3. This set has to be repeated for each pollinator.
   (iii) Tag flowers and allow them for unrestricted visits.
2. Monitor all the three sets of flowers to develop into fruits or until their abscission, and record the response of each flower in each set.
3. Harvest the fruits for each set separately. Open each fruit and collect the seeds and count them.
4. Calculate average number of seeds/fruit for each set of flowers.
5. Calculate pollination efficiency using the above formula. The flowers of set (i) give information on seed set as a result of autogamy (*Z*). Flowers of set (ii) give information on seed set after one visit by specific pollinator (*Pi*), and flowers of set (III) provide data on seed set after unrestricted visits by the pollinators (*U*).

Treatment (i) need not be repeated for each pollinator. Seed set data for *Z* and *U* obtained for one pollinator can be used to calculate pollination efficiency of all other pollinators.

7.7.5.3 Modification

This formula can also be used to assess pollination efficiency on the basis of the number of pollen grains deposited on the stigma. Instead of bagging and allowing the three sets of flowers to develop into fruits, excise and label them, and bring them to the laboratory. The flowers of sets (i) and (iii) have to be excised at the end of flower longevity and the flowers of set (ii) have to be excised after one visit of the pollinator.

In the laboratory, count the number of pollen grains on each stigma in each set of flowers instead of scoring them for the presence or absence of pollen. For counting pollen grains on the stigma, place the stigma in a drop of acetocarmine and tease the stigma with a pair of needles to release all the pollen grains into the stain. If the stigma is large, remove the debris, lower a cover glass and count the pollen grains carefully (see Protocol 5.5.1 and 5.5.2 for pollen counts).

7.7.6 Estimation of Pollination Limitation

In most of the plants, particularly in large shrubs and trees, a large proportion of flowers do not produce fruits but abscise resulting in low fruit set. Many causative factors for low fruit-set have been identified (Burd 1994; Larson and Barrett 2000; Wilcock and Neiland 2002; Knight et al. 2005). Pollination limitation is one of the major constraints for low

fruit set. One of the standard methods to estimate pollination limitation is through pollen supplementation experiment. In these studies, the fruit set in flowers allowed to open pollinate is compared to that in flowers subjected to pollen supplementation (manual pollination).

7.7.6.1 Special Requirements

Tags to label flowers

7.7.6.2 Procedure

1. Tag a suitable number of freshly opened flowers. The number of flowers to be tagged depends on the availability of flowers. At least 50–100 flowers would be needed to get meaningful results.
2. Make them into two sets. Leave set I flowers as such. This set would represent open pollination. Carry out supplementary pollination with cross-pollen (just to avoid self-incompatibility as a factor for lack of seed set if the species is self-incompatible) on flowers of set II.
3. Monitor open-pollinated and manually pollinated flowers and record their fruit set.
4. When the fruits mature, harvest them. Collect seeds from each fruit and count them.
5. Calculate per cent fruit set and average number of seeds per fruit in both the sets and tabulate the results for open- and manually pollinated flowers.

If the per cent fruit set and average number of seeds per fruit in manually pollinated flowers is significantly more than those in open-pollinated flowers, pollination is a constraint. If there is no significant difference between the two sets of flowers, pollination is not a constraint.

7.7.7 Trapping of Flower Visitors

Trapping of floral visitors is necessary for their identification, analysis of their pollen loads and in some experiments for marking them. There are many devises available to collect various floral visitors. Kearns and Inouye (1993) and Dafni et al. (2005) have given a comprehensive account on collecting and preserving pollinators. A few standard methods are elaborated here.

7.7.7.1 Special Requirements

Suitable devise to trap insects. Any of the following devises can be used:

1. Sweeping net: The use of insect sweeping nets, available in various sizes, is the most commonly used method to trap pollinating insects. The sweep should be swift enough to force the insect to the bottom of the collection net; the net should be turned immediately so that the bottom of the bag folds over the rim of the net to trap the insect inside.
2. Aspirator: Another method of collecting insects particularly the smaller ones is to use an aspirator. A manually operated aspirator is available with suppliers of laboratory apparatus or can be easily constructed (see Appendix A.7, Fig. A.3). It consists of a plastic or glass vial of suitable size closed with a rubber stopper with two holes; two hoses are inserted through these holes in the stopper, one is for sucking through user's mouth and the other is used as the inlet for the insect. The end of the sucking hose inside the vial is tied with a nylon mesh to prevent insects entering the hose during sucking.
3. Wide-mouthed vial of suitable size: We have been employing routinely a wide-mouthed plastic vial (25–50 ml capacity) to collect smaller insects of many species. After the insect has entered the flower, the vial is held in the mouth of the flower. When the insect exits the flower, it enters the vial. The lid of the vial is closed immediately to prevent its escape. Keeping a piece of tissue or cotton dipped in ethyl acetate inside the bottle before trapping would also help in immobilizing (by keeping for a shorter time) or killing (by keeping for longer time) the insect. Ethyl acetate should not be inhaled during the capture of insects.

7.7.7.2 Procedure

1. Capture the floral visitor by using any of the above devises based on availability, convenience and the size of the insect.
2. Transfer the captured insect to a killing jar.

There are several types of killing jars available in the market (see Kearns and Inouye 1993). The killing jars contain a killing agent,

usually ethyl acetate or crystals of sodium/potassium cyanide. Ethyl acetate is a routinely used killing agent for insects. Alternatively, a piece of tissue dipped in ethyl acetate may be kept at the bottom of the vial before trapping. When an aspirator is used to trap insects, the killing agent should not be put inside the aspirator; trapped insects should be transferred to a killing jar, similar to those trapped in the sweeping net. The time of exposure of insects to the killing fumes varies and depends on the size and type of insect. Small insects such as bees and flies are killed in minutes, while larger insects, especially beetles, may take many hours.

Caution: Killing agents have to be handled carefully. As they are highly toxic, one should be careful not to inhale the fumes.

Insects can also be killed by placing the vials in a freezer or by transferring them to ethanol. They can be stored in ethanol for longer periods.

3. Killed insects can be pinned to insect boxes and stored for identification or long-term record.

It is more difficult to trap other pollinating agents such as birds, bats and rodents. For catching birds and bats, mist nets have been commonly used. If studies are being carried out in protected forests, permission has to be taken from the authorities to use mist nets for trapping of these animals. Careful observation with a pair of binoculars, photographing (with a zoom lens) and/or video recordings of the animals while foraging may help in their identification without the need for trapping. Their pollination efficiency may be determined by examining the stigmas of flowers after their visits (see Protocol 7.7.3). For trapping rodents such as rats, suitable traps available in the market may be used by keeping bait inside the trap.

7.7.8 Estimation of Pollen Load on Floral Visitors

Studies on pollen load on flower visitors are important for a number of reasons. The presence of pollen load of conspecific species indicates the possible role of the floral visitor in pollination of the species. These studies also indicate whether the visits are confined to flowers of conspecific species or extend also to synchronously flowering heterospecific species. Species with generalist type of flowers (flowers in which rewards are available to any visitor) are visited by a variety of insects and all of them may not be pollinators. Even if they are pollinators, their efficacy may vary. For effective pollination, the visitor should carry sufficient pollen on its body parts that come frequently in contact with the receptive stigma.

It is important to note that most of the pollen harvested actively and packaged as pollen baskets becomes a part of the larval food in the bee hives and is not available for pollination. Also their viability may be affected. It is only the pollen that remains on the body parts that frequently come in stigmatic contact constitute the pollen used for pollination. Often a distinction is made between the 'total pollen load' (pollen grains collected from the total wash of the pollinator) from 'functional pollen load' (pollen that has the chance to reach the target stigma due to its location on the animal body). From pollination point of view, it is the functional pollen load that is important. In many instances, the presence of pollen load on the visitors' body may not be sufficient to confirm them as pollinators as their pollen-loaded parts may not come in contact with the stigma. For determining the pollen load, killing of insects in ethanol should be avoided, as it may dislodge pollen grains from the body unless the entire ethanol used for storage is used for pollen counts.

Qualitative analysis of pollen deposition on the foragers may be done with a powerful magnifying glass or under a dissecting microscope. Pollen identification may also be made by scraping some pollen grains from the body onto a slide and observing them under the microscope. They are compared with the reference pollen mounts prepared from the pollen of the focal and co-flowering species for identification. If all the pollen grains on the insect belong to the same species, it indicates the constancy of the visitor during the study period. If the pollen grains are of more than one species, it shows that the visitor forages flowers of different species.

'Pollen prints' taken with an adhesive tape can also be used to study pollen grains. For this, a piece of cellotape/adhesive tape is applied onto the surface of the forager's body, gently peeled and placed on a slide and observed directly under a microscope for identification. Adhesive tapes peeled from different parts of the body can be used to produce pollen distribution map on the body of the animal. The involvement of floral visitor in removing the pollen in species bearing pollinaria such as orchids and asclepiads can be easily recognized, with naked eye or under a stereomicroscope, by the presence of pollinaria on the visitor's body or by the absence of pollinaria in the flower visited by the insect.

7.7.8.1 Special Requirements

Ethanol (95 %)

A suitable stain for staining pollen grains such as acetocarmine

A watch glass

7.7.8.2 Procedure

Trap the insect and immobilize it as described in Protocol 7.7.7.

For insects with low pollen load:

1. Hold the insect with a pair of forceps and wash it with droplets of 70 % ethanol to remove the pollen from its body. Carefully collect the drippings falling from the insect into a watch glass. Preserve the insect with all the records for identification and as voucher specimen.
2. Count pollen grains present in ethanol. Follow any of the procedures described in Protocol 5.5.1 or 5.5.2 to count pollen grains in ethanol.

7.7.8.3 Modification

If fluorescence microscope is available in the laboratory, it would be more convenient to mount the pollen in a drop of auramine O (see Appendix A.1 for preparation), a fluorochrome that induces bright fluorescence of pollen exine. Observe the slide under the fluorescence microscope using UV combination filters and count them. Pollen grains show bright yellow fluorescence and permit easy identification of pollen grains from debris.

For insects with heavy pollen loads

Additional requirement: rotary shaker/vortex mix:

1. Take immobilized insect in a clean, pollen-free vial. Add 70 % ethanol enough to cover the insect.
2. Put the vial on a rotary shaker for 2–4 h. We have found vigorous shaking of the vial manually or using a vortex mix for a few minutes is sufficient to release all the pollen from insect's body.
3. Take out the insect from the vial and preserve it for record.
4. Centrifuge the liquid under low speed (ca 2,000 rpm) and remove the supernatant. Use the pollen pellet for counting pollen grains using any of the procedure described under Protocol 5.5.1 or 5.5.2.

The methods described above are not suitable for birds, bats and other vertebrates. Pollen loads can be removed from vertebrate pollinator by using cubes of glycerin jelly (*see* Appendix A.3) taken with a pair of forceps and gently swabbing the cubes on the body parts of the animal that come in contact with the anthers during their visit to the flowers. The cubes are then mounted on a slide (*see* Protocol 5.5.5). Slides can be observed soon or stored for later observation. Pollen grains can also be removed through cellotape peeling and observed under the microscope.

7.7.9 Estimation of Density and Diversity of Insect Pollinators

The density and diversity of floral visitors is important in understanding pollination efficiency and pollination limitation across locations and seasons. This is also important in long-time studies aimed at understanding the decline of pollinator populations as a result of human activity such as habitat degradation, fragmentation and application of non-eco-friendly agrochemicals such as pesticides and fungicides.

The most common method used to estimate pollinators' density and diversity is by net sweeping along the line transects to collect pollinators. Generally, the investigator walks

steadily (about 10 m per min) while sweeping with backward and forward strokes at uniform speed. This needs some practice to familiarize the speed of the sweep and pace of the walk before making systematic collection for record. The length of transects varies depending on the density of insects.

The sweeping is done along transects at fixed time intervals during the day or for a selected period. Several investigators have done sweeping along several 50–100 m transects at specific times of the day (at intervals of 1 or 2 h during the peak activity of pollinators). Depending on the need, the sampling is repeated 1 day per week for 3–4 weeks. For crop plants, it is better to sweep the net between the rows of plants.

7.7.9.1 Special Requirement

Standard entomological sweeping net
Polyethylene bags
Screw cap bottles

7.7.9.2 Procedure

1. Select the area to be studied and fix transects.
2. Sweep the net at selected timings with a steady walk along the transect. After the sweeping is complete, twist the net to retain the collections inside the net.
3. The specimens collected are identified either in the field itself or labelled as morphotypes. Count the number of each species/morphotypes.
4. Fix representative samples of all the identified species/morphotypes for documentation or later identification.
5. Release the remaining specimens to avoid reducing the local population of pollinators. It is desirable to deposit the fixed samples in a recognized entomological collection.

7.7.9.3 Modifications

1. Instead of sweeping, pollinators may be recorded through visual observation. In this method, it may not be feasible to record individual species of insects; instead the identification may be assigned to guilds (honeybees, small solitary bees, etc.). This is particularly useful to get abundance data in the canopy of trees in tropical forests. One may use binoculars/telescope, if needed. Recording of each guild in fixed areas in relation to the known number of flowers/inflorescences and scoring each guild over specific time periods give a crude estimate of insect abundance.
2. Different types of insect traps such as sticky traps, water traps, Malaise trap or light traps and window traps have been used to collect insects (*see* Kearns and Inouye 1993) to get a rough estimate of insect abundance. However, the use of such traps is less useful in studies on pollination ecology as they also collect non-pollinators. Also, all insects present in the area are not trapped as the sensitivity of different insect types to the traps is variable. However, their use may be necessary under some situations particularly if the collections have to be made during night-times (Kearns and Inouye 1993). The use of insect traps permits simultaneous sampling of several locations. They are particularly suitable for long-term studies.

7.7.10 Demonstration of Nocturnal Pollination Based on Day/Night Exposure of Flowers

It is necessary to check for nocturnal pollination in species in which flowers open during the night and those in which flowers remain open during the day and night. Protocols for studying nocturnal pollination are basically similar to those described for diurnal pollination (Protocol 7.7.2), except that the observations have to be made during night-time.

Major difficulties to study nocturnal pollination are visibility and logistics. Also studies on plants growing in reserve forests and sanctuaries require special permission from the authorities to stay in the forest during the night. The presence of wild animals in the forests is another difficulty. Because of the safety reasons, it is always better to study nocturnal pollination with a minimum of two investigators.

One of the indirect methods to check whether pollination takes place only during the day and/or night is by exposing flowers for pollinators only

during the day or only during the night and checking their stigmas for pollen load.

7.7.10.1 Special Requirements

Isolation bags and tags

7.7.10.2 Procedure

1. Select flower buds 1 day before anthesis and bag them. Make them into two sets (1 and 2) and label them. It would be convenient to use tags of different colours for the two sets so that they can be easily distinguished. There should be as many buds as possible for each set, preferably 50 each. If the flowers are bisexual, flower buds have to be emasculated before bagging to prevent possible autogamous self-pollination.
2. When the flowers open, remove the bags of set 1 early morning soon after the daybreak to expose them to the pollinators during the day. Rebag them in the evening before it gets dark.
3. In the dusk before it becomes dark, remove the bags from the second set of flowers to expose them during the night. Rebag them in the early morning.
4. Repeat day and night exposure (of the same set of flowers) as long as the flowers remain fresh.
5. When the flowers start senescing, excise the flowers of set 1 and set 2 separately and observe their stigmas individually for pollen load.
6. Calculate per cent pollinated flowers in the two sets. If possible, one can count the number of pollen grains on the stigma and calculate average number of pollen per stigma in both the sets of flowers.

Repeat the protocol for 2/3 batches of flowers to get dependable results.

If the species is nocturnally pollinated, only the flowers exposed during the night show pollen load on the stigma. If the species is diurnally pollinated, only the flowers exposed during the day show pollen loads on the stigma. If pollination occurs during the day as well as night, pollen loads are found on the stigmas of both the sets.

This protocol will not give any information on the nature of pollinators, frequency of their visits and foraging details.

7.7.10.3 Modification

Nocturnal and diurnal pollinations can be identified on the basis of fruit set in the two sets of flowers. Instead of excising them to study pollen load, allow them to develop into fruits and then calculate per cent fruit set in both the sets.

7.7.11 Demonstration of Nocturnal Pollination Based on Direct Observations

As in the diurnal pollination, this protocol also requires some preliminary studies to identify floral visitors and the timing of their visits. Observations to record the details of pollinators' visits may have to be carried out continuously or at 15/30 min time slots each hour. Usually nocturnal pollination occurs during the crepuscular period (late evening, 18–20 h, and just before dawn, 04–06 h). Depending on the visitation time, observations may be confined to the late evening or early mornings or extended to the whole night. Lack of sufficient light makes it difficult to observe and record the details of nocturnal visitors to flowers. The use of intense artificial light may repel nocturnal visitors. Many investigators have used torch/lantern covered with red cellophane paper for nocturnal observations (Nagamitsu and Inoue 1997). However, the most convenient method to study nocturnal pollination is to use night vision glasses/binoculars which amplify minimal available light to be able to see night floral visitors with reasonable clarity. For larger animals, camera traps can be set up. Camera traps will only confirm the floral visitors at night but do not provide much detail. Video recording is also useful to record nocturnal visitors.

7.7.11.1 Preliminary Investigations

Nocturnal pollinators are mainly represented by bats and moths and to a lesser extent cockroaches, lizards and rodents. Initially the types of flower visitors can be ascertained by using the combination of battery-operated torch and binoculars. After ascertaining the type of pollinator, a group of observers have to position in a

selected spot from where a suitable patch of flowers/inflorescences can be clearly seen. In rodent-pollinated plants, flowers are generally positioned closer to the ground. In case of tree species, we have used the canopy of adjoining tree or a collapsible bamboo ladder of suitable height for recording the observations. The details of observations and recordings elaborated in Protocol 7.7.2, for diurnal pollination, have to be followed.

Observations on bat pollination is relatively easier than the other nocturnal pollinators because in typical bat-pollinated plants, the flowers/inflorescences are generally placed outside the canopies of the plants, either as upright racemes or drooping bunches of inflorescences (e.g. Bignoniaceae, *Mucuna* spp.).

7.7.11.2 Special Requirements

Night vision glasses (goggles/binoculars)
Isolation bags
Hand-held battery-operated torch
At least two investigators and any other items needed for safety

7.7.11.3 Procedure

1. Select a patch of freshly opened flowers which can be clearly seen from the observation place.
2. Keep record of floral visitors and duration of their stay on each flower. Make at least 20 observations of each visitor to flowers (see sample Table 7.3 for details of recording).
3. Record the foraging behaviour of floral visitors and whether they forage nectar or pollen.
4. Excise flowers visited by nocturnal visitors and keep them in a beaker or Petri plate without causing disturbance.
5. Bring excised flowers to the lab in the morning and observe their stigmas for the presence of pollen grains (*see* Protocol 7.7.3 for details).
6. Plot a graph on visitation frequency (and if data is available, with the temporal details of nectar production).

7.7.11.4 Modifications

1. Nocturnal pollination on the basis of fruit set: Flowers visited by nocturnal agents can be bagged to study fruit set.
2. If pollinators happen to be small insects, they can be trapped by tying sticky traps (3 × 3 cm, available commercially or prepared by smearing nondrying glue, see Protocol 7.7.12 for details) near the flowers/inflorescences. Once the insects come in contact with the tape, they get stuck to the tape. Although it is difficult to remove the insects from the tape, they can be observed under the microscope for pollen load. However, it should be kept in mind that insects caught on the tape are not necessarily the pollinators as many of them may not even visit the flowers.
3. As insects are attracted towards light, they can be conveniently trapped by using a suitable setup near the focal plant when in bloom. One may use a setup comprising a small battery-operated torch covered with blue cellophane paper to impart blue light. The torch then can be placed in bigger size glass jar. Once the insects enter the jar, cover it with a lid. This trapping exercise may be repeated at several locations in the population.

   The collected insects can be immobilized and studied for pollen load using the method described in Protocol 7.7.8.

It may not be feasible to trap larger animals. Their role in pollination may be confirmed by allowing them to visit virgin flowers and either excising them to observe pollen load on their stigmas or bagging them to study fruit set.

7.7.12 Demonstration of Ant Pollination

Ants are found on flowers of a number of species. As pointed out earlier, ant pollination is rare and has so far been reported only in a limited number of species. It may be, to some extent, due to the perception of the investigators not to consider them as effective pollinators, even when they are present on the flowers. When ants are present on the flowers of the focal species, it would be necessary to study the details at least to rule out their role in pollination. When ants are present, they are generally present during the day as well as night.

7.7.12.1 Preliminary Studies

This protocol also requires some preliminary studies to understand the behaviour of ants on the flowers and to standardize a suitable method to prevent and permit ants to the flowers used for observation. If these studies clearly establish that the ants do not come in contact with the anthers or the stigma, their role in pollination can be ruled out. If there are possibilities of ants coming in contact with the stigma, the details have to be investigated to study their role in pollination.

Several investigators have used insecticides to prevent ant visits. In herbaceous plants, insecticide can be applied to the soil around the plants. If they are large shrubs or tree species, insecticide has to be sprayed on flowering branches bearing focal flowers. Also, it requires standardization of the concentration and frequency of sprays to prevent ants during the observation period. Some investigators have applied glue (Tanglefoot) around the stem suspending the inflorescences or on the plasticine placed around the whole plant on the ground to prevent ants visiting the flowers (Gomez et al. 1996); they have used nylon mesh bags (0.25 mm mesh) around the flowers to permit visits of only ants to the flowers.

We have used a nondrying strong glue commercially available (Stickem special/Stickem ribbon; Seabright laboratories, Emeryville, CA, USA) to prevent ants' visit to the flowers. This glue is commonly used to make sticky traps for small insects and it does not dry up for several days. The following is the detailed procedure we have followed to prevent ants from entering focal flowers and to permit only ants to visit the flowers:

Smear Stickem special glue around 2–3 cm of the stem of the flowering branch proximal to the flowers/inflorescences. Crawling ants get stuck to the glue and cannot pass through the glue region, thus preventing ants from reaching the flowers. As an additional precaution, we have bagged (made up of pollen proof cotton cloth) the flowering branch tightly around the stem (distal to the glued part) without leaving any space for the movement of ants into the bag. This dual method prevented ant visits to focal flowers completely.

To study the visits of other insects to focal flowers, we open the bags at their tips and pull them back during the day to expose diurnal pollinators to visit the flowers. The bags are closed in the evening. For allowing only ants to visit the flowers but not any flying insects, we have found that conventional pollination paper bag loosely tied, leaving sufficient gaps around the stem of flowering branches, allows ants to visit the flowers readily.

7.7.12.2 Special Requirements

Tags

Pollination bags (made up of pollen proof cloth and of paper)

Nondrying glue

7.7.12.3 Procedure

1. Prepare the following four sets of flower buds one day before opening:
   - (i) To prevent ants from entering the flowers by using the method explained above. If individual flowers are used for bagging, smear the nondrying glue around the lower part of the pedicel or on the stem suspending the flower and bag them. If the inflorescence/flowering branches are used for bagging, smear the glue around the stem of the branch/inflorescence and remove opened flowers, very young buds and fruits, if any, before bagging and retain only those buds that would open the next day. Also remove any ants present on the flowers/inflorescences. This set will prevent ants as well as any other insects entering the flowers and indicates the extent of autogamy, if any, in bisexual flowers.
   - (ii) To allow only ants to enter the flower but not any other flying insects as explained above (by conventional loose bagging *without* applying nondrying glue).
   - (iii) To prevent ants but permit other flying insects to enter the flowers (treatment similar to set i), but the bags have to be opened at the tip and pulled back in the morning to allow diurnal insect visits and again closed in the evening. This has to be repeated every day until the flowers in the bag remain fresh.
   - (iv) To allow all floral visitors by tagging the flowers without bagging them or

smearing nonadhesive glue around the stem/pedicel.
2. Maintain the above sets until the flowers start senescing.
3. After initiation of senescence, excise all four sets of flowers and maintain them separately in Petri plates. Handle the flowers carefully to prevent disturbance and possible autogamous pollination (in bisexual flowers) during handling. Take the flowers to the laboratory.
4. Observe the stigma of each flower of each set and record the presence of pollen on the stigma. If possible, count the number of pollen grains on each stigma. Calculate per cent of flowers pollinated and average number of pollen grains present on the stigma of each set.

Ants can be easily lifted with the help of forceps and dipped in 70 % ethanol to study pollen load on their body as described in Protocol 7.7.8.

The presence of pollen on the stigma in significantly greater per cent of flowers in set (ii) than in set (i) demonstrates the role of ants in pollination. Results of set (iii) indicate the role of flying insects in pollination. The results of set (iv) indicate pollination efficiency under field conditions from all the pollinators combined.

The above treatment and observations of the stigma should be replicated using different branches and plants to get a clear information on the role of ants in pollination.

7.7.12.4 Modifications

1. *Ant pollination on the basis of fruit set:* Retain focal flowers on the plants until fruit development and analyse per cent fruit set and mean number of seeds per fruit in each set of the treatments.
2. Frequency of visits and pollination efficiency of each pollinator can be studied by recording the visits of each visitor (*see* Protocol 7.7.2 for details). To determine the frequency of ants, observe a set of open flowers and score the frequency of ant visits; chase away any other flying insects from visiting the focal flowers. The frequency of nonflying visitors can be scored in set iii flowers when the bags are opened during the day.
3. It would be useful to make nocturnal observations also if ants turned out to be effective pollinators. Check if ants are present on the flowers during night-time also and if so whether the species present is the same or different. If necessary, their role in pollination can be confirmed by setting up treatment i and ii described above under the 'Procedure'.

7.7.13 Demonstration of Wind Pollination

Next to biotic pollination, wind is the major pollinating agent. A standard method used to demonstrate wind pollination is to use mesh bags that permit airborne pollen but not any insects.

7.7.13.1 Special Requirements

Insect- and pollen-proof pollination bags

Pollination bags of desired mesh size. Bags of mesh size 1 or 2 mm^2 are considered suitable to allow entry of air borne pollen but not the insects. Many workers have used double layered insect netting for preparing such bags. We have found the bags stitched from mosquito net cloth are satisfactory to demonstrate wind pollination. Although mesh bags are effective in preventing insects from bringing about pollination, they do, to some extent, reduce the efficiency of wind pollination as some air borne pollen is likely to get trapped in the mesh and also they may disrupt air currents around the flowers.

7.7.13.2 Procedure

1. Select older flower buds about one day before anthesis. If the flowers are small and cannot be bagged, select inflorescences or flowering branches containing as many buds of suitable stage as possible (see Sect. 2.2). If inflorescences or the flowering branches are selected, remove young buds, opened flowers and fruits, retaining only the flower buds about one day before anthesis.
2. Make them into the following three sets:
   (i) Flowers allowed to open pollinate
   (ii) Flowers enclosed in insect- and pollen-proof bags (to exclude air- and insect-borne pollen)

(iii) Flowers enclosed in bags of suitable mesh size that exclude insects but not airborne pollen

Emasculate buds of set (ii) and (iii) before bagging them to prevent autogamy.

Label and record the number of flower buds in each set.

3. Remove the bags in sets (ii) and (iii) after the stigmas lose receptivity but keep the tags intact. This period may vary between species.
4. Keep track of the three sets of flowers until abscission or fruit development. Maintain the record of abscised flowers.
5. Collect mature fruits before their dehiscence from each set, and count the number of seeds from each fruit.
6. Calculate per cent fruit set and average number of seeds per fruit in each set of flowers. Compare fruit and seed set in the three sets of flowers.

Flowers isolated with pollen- and insect-proof bags should not set any fruits unless the species is an apomict (which does not require pollination). Fruit set in flowers isolated with mosquito net bags (but not in insect-proof bags), if any, represents fruits developed exclusively as a result of wind pollination. As the mosquito net bags also prevent a proportion of pollen from reaching the stigma, fruit set may be marginally lower in wind-pollinated flowers when compared to open-pollinated flowers.

7.7.13.3 Modification

The protocol can be modified to assess wind pollination on the basis of pollen load on the stigma. Instead of allowing the three sets of flowers for fruit development, excise senescing flowers from each set, and study pollen load on the stigma (see Protocol 7.7.3).

7.7.14 Use of Slide Traps for Airborne Pollen

The release of pollen grains to ambient air and their movement in the air is the basic requirement for wind pollination. This can be easily demonstrated by trapping pollen grains present in the air by using various devices. Although the presence of pollen on slide traps does not demonstrate wind pollination, it provides strong evidence for the involvement of wind in pollination. The use of slide traps is one of the simplest pollen traps.

7.7.14.1 Special Requirements

Prepared microscopic slides: The slides used to trap pollen have to be prepared before they are exposed to collect air borne pollen. As pollen grains trapped on slides need to be quantified generally as number of pollen grains per cm^2, marking the 1 cm^2 squares on the slide with a marking pen before exposing would make it convenient to count. Mark 3 or 4 squares on one surface of the slide. These squares have to be divided into smaller rectangles by marking about 1 mm parallel lines across each square. This is to facilitate counting of pollen grains between two adjacent lines after trapping pollen grains (see Protocols 5.5.1 and 5.5.2). On the other side of marked slide (which is not used for marking), rub glycerin jelly (*see* Appendix 13.3 for preparing glycerin jelly) or petroleum jelly (available with chemists) as a thin film. This will help pollen grains adhere to the slides. It is convenient to prepare 20–30 slides at a time and use them as and when needed. Dry the slides in pollen-free and dust-free room and keep them in slide boxes to prevent contamination with pollen.

Thread to hang slides

7.7.14.2 Procedure

1. Tie prepared slides with a thread on one end (for vertical exposure) or both the ends (for horizontal exposure). Label the slides with permanent ink on one side of the glycerin jelly-coated surface. Hang one set of slides horizontally (keeping the glycerin-coated surface up) and another set vertically on and around the plant at various distances from the flower. If the flowers are unisexual, the slides have to be exposed with reference to the male as well as the female flowers. If it is a tree species, place the slides at different levels in the canopy and known distances away from the canopy. Record the placements with reference to each numbered slide.
2. Collect exposed slides individually after 24 h, and keep them in a slide box until used for observation.

3. Observe the glycerin-coated surface of the slides under a microscope for the presence of pollen grains of conspecific species. If pollen grains cannot be identified easily, put a drop of acetocarmine, and lower a cover glass before observing. We have found that mounting the slides in a drop of auramine O and observing them under a fluorescence microscope (UV excitation) is very convenient. Auramine O induces bright fluorescence in pollen exine making it easier to distinguish pollen grains from the dust/debris. If pollen grains are present, count the number of pollen grains in each pre-marked cm^2 (*see* Protocol 5.5.1 for pollen counting).

7.7.14.3 Modifications

1. If there is any difficulty in identifying the presence of conspecific pollen on the slide or if there are pollen grains belonging to different species, prepare reference pollen slides of the conspecific and co-flowering species by mounting their pollen grains in glycerin jelly (see Protocol 5.5.5). Identify conspecific pollen grains present on exposed slides with the help of pollen on the reference slides. It would be convenient to prepare reference slides of focal and co-flowering species at the beginning of the study, and keep them in a slide box as permanent records, and use them whenever required.
2. Standard pollen traps can also be used to demonstrate the presence of pollen grains of a given species in the air. Burkard Portable Pollen Sampler, which can be operated by a battery, is the most convenient. The details of these traps can be found in Kearns and Inouye (1993) and Dafni et al. (2005). Instruction manual gives the details of the procedure to be followed.

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Pollen–Pistil Interaction

8

Pollen–pistil interaction is unique to flowering plants. This is one of the critical postpollination phases that determine reproductive success. Following conspecific compatible pollination, pollen grains germinate on the receptive stigma and the resulting pollen tubes grow through the tissues of the stigma and style and enter the ovules where they deliver the sperm cells for fertilization. Pollen–pistil interaction involves a series of sequential events from pollination until the pollen tube enters the embryo sac. A break at any level in these sequential events results in the failure of fertilization. There is a continuous dialogue between the pollen grain and later the pollen tube and the tissues of the pistil. Successful completion of pollen–pistil interaction is a prerequisite for fertilization and subsequent fruit and seed development.

Pollen–pistil interaction was taken for granted by the early investigators, partly because of lack of suitable techniques to study pollen germination and pollen tube growth in the pistil and partly because of their preoccupation with the development of the male and female gametes and the embryo and endosperm. However, during the last three to four decades, there has been an increasing realization of the importance of pollen–pistil interaction in reproductive biology of flowering plants, and considerable amount of information is now available (Shivanna 2003; Higashiyama and Hamamura 2008; Chapman and Goring 2010; Kawashima and Berger 2011).

8.1 Evolutionary Significance of the Pistil

The main function of the pistil is to screen pollen grains for compatibility and quality during pollen–pistil interaction. Only the right type (compatible) of pollen grains are facilitated to complete pollen–pistil interaction, and the wrong (interspecific pollen as well as self-pollen in self-incompatible species, *see* Chap. 9 for details) types are inhibited during their germination or pollen tube growth (*see* Shivanna and Johri 1985; Shivanna 2003; Lord 2003; Cheung et al. 2010).

Generally, the number of pollen grains deposited on the stigma is many times more than the number of ovules that are available in the ovary for fertilization. This creates intense competition even amongst the compatible pollen grains during pollen–pistil interaction. Pollen grains which germinate early and the pollen tubes which grow faster in the style enter the ovules earlier and effect fertilization; pollen grains which germinate later and pollen tubes which are slow growing are eliminated in this competition (Mulcahy 1979, 1984; Mulcahy and Mulcahy 1983; Armbruster et al. 2002; Shivanna 2003; Lankinen and Madjidian 2011). There is no scope for such a competition amongst the male gametes in lower groups of plants; any compatible male gamete that comes in contact with the female gamete, irrespective of its quality, fuses with the female gamete. There are evidences to indicate that pollen

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competition in flowering plants increases the fitness of the progeny; the progeny resulting from rapidly growing pollen tubes are superior over the others that result from slow-growing pollen tubes (Mulcahy et al. 1988; Ottaviano et al. 1980; Davis et al. 1987; Snow and Timothy 1991). Evolutionary success of flowering plants over the other groups of plants has been suggested to be the result of the evolution of the most efficient outbreeding system (based on self-incompatibility) and intense pollen competition (Whitehouse 1950; Mulcahy 1979; Armbruster et al. 2002), both of which are the attributes of the evolution of the pistil.

8.2 Pollen Germination and Pollen Tube Growth

The stigma is the recipient of the pollen. The major events that occur on the stigma surface are pollen adhesion, pollen hydration and pollen tube entry into the stigma. Each of these events is a prerequisite for the subsequent event (Lord 2003; Shivanna 2003; Chapman and Goring 2010; Cheung et al. 2010). Pollen adhesion is not critical in species with wet type of stigma but is critical in those with dry type of stigma. The adhesive carbohydrates, lipids and proteins present on the pollen surface and/or the stigma surface play a crucial role in pollen adhesion and hydration. In members of Brassicaceae, the presence of pollen coat substances seems to be essential for pollen adhesion and hydration. Pollen coat substances are released onto the pellicle surface and form a meniscus in the interface which enables not only pollen adhesion but also facilitates pollen hydration by signalling movement of water from the stigma to the pollen. Many mutants with defects in pollen coat substances as well as pollen grains, from which pollen coat substances have been removed, fail to hydrate (Doughty et al. 1993; Dickinson 1995; *see* Zinkl et al. 1999; Shivanna 2003). In *Arabidopsis* also, pollen grains which have defects in lipid biosynthesis cannot hydrate on the stigma surface. Flavonoids which form a part of the pollenkitt have been shown to play an important role in pollen germination and pollen tube growth (Preuss et al. 1993). Flavonoid-deficient mutants of maize and *Petunia* produce flavonoid-deficient pollen which cannot function on the stigma of mutants. However, exogenous supply of nanomolar concentration of kaempferol, a flavonoid aglycone, either to pollen or to the stigma restored germination of mutant pollen (Mo et al. 1992).

Many earlier studies have shown that the stigma provides boron and calcium which are required for pollen germination in several species. In *Gladiolus* washing of the stigma with a detergent removed the ability of the stigma to support pollen germination, and in *Raphanus* enzymatic digestion of pellicle reduced pollen germination and totally inhibited pollen tube entry into the stigma (*see* Shivanna 2003). Thus, the pellicle seems to have many components, some of which are involved in germination and others in the entry of pollen tubes.

Pollen tube entry into the stigma is another critical step. In wet stigma types, the cuticle of the stigma surface is disrupted during secretion of the exudates, and thus there is no physical barrier for the pollen tube entry into the intercellular matrix of the stigmatic tissue. In dry type of the stigma, the cuticle forms a physical barrier for pollen tube entry. The pollen tube has to erode the cuticle by activation of cutinases. Cell wall hydrolases are present in the pollen coat. There are evidences to indicate the involvement of pollen xylanase in pollen tube penetration. Suen and Huang (2007) produced transgenic lines (*xyl-less*) containing little or no xylanase in the pollen coat of maize pollen. *xyl-less* pollen germinated as efficiently as wild-type pollen in vitro but less so on the silk (stigma) of corn. Once germinated, the *xyl-less* and wild-type pollen tubes elongated at comparable rates. However, pollen tubes of *xyl-less* pollen did not penetrate into the silk as efficiently as the tubes of wild-type pollen, and this lower penetration efficiency could be overcome by the addition of xylanase to the silk. These results clearly indicate that maize pollen coat xylanase facilitates pollen tube penetration into silk via enzymatic xylan hydrolysis.

In species with solid style, pollen tubes grow through the extracellular matrix (ECM) of the

stigma and eventually enter the ECM of the style. In hollow-styled species such as *Lilium*, in which the stylar canal is filled with secretion products from the canal cells, pollen tubes grow down on the surface of the canal cells. In species such as *Gladiolus* and *Crocus*, in which the mucilaginous substances accumulate between the cuticle and the canal cells, pollen tubes enter the cuticle and grow through the mucilage. The amount of nutrients present in the pollen is limited and not sufficient to support pollen tube growth in the style until it reaches the ovary. Obviously, the stylar tissue has to provide nutrients to the growing pollen tubes. Earlier studies using labelled sugars in the pistil have demonstrated that sugars are taken up by the growing pollen tubes from the style (*see* Shivanna 2003). Although the details of chemical interaction between pollen tubes and stylar components are not clearly understood, the ECM obviously plays a critical role in these interactions. Some studies have indicated that transmitting tissue-specific (TTS) proteins present in the ECM play an important role in pollen tube growth (*see* Lord 2001; Cheung et al. 2010).

8.3 Pollen Tube Guidance

Pollen tubes follow a predetermined path in the pistil from the stigma to the ovary. Traditionally it was thought that the pollen tubes are guided from the stigma to the ovary by a gradient of chemotropic substance present in the pistil. For some time it was believed that calcium is the chemotropic agent (*see* Shivanna 2003). However, most of the later evidences did not support the concept of the presence of a chemotropic substance in the pistil. Many investigators suggested that the architecture of the transmitting tract in the stigma and style with elongated, cylindrical cells interconnected at their ends to form vertical files, surrounded by the ECM, provides a continuous pathway of least mechanical resistance for growing pollen tubes. When once the pollen tubes enter the transmitting track, they follow this path unidirectionally from the stigma to the ovary (*see* Heslop-Harrison and Heslop-Harrison 1986). Although many experimental studies have confirmed lack of chemotropic gradient in the style, some evidences have indicated that TTS proteins may provide a chemotropic gradient in the style in *Nicotiana* (Cheung et al. 1995).

Irrespective of the presence or absence of a chemotropic gradient in the style, there has to be some factor(s) at the level of the stigma to guide the pollen tubes into the stigma from its surface and in the ovary where in they have to change the direction of growth from the placenta to the ovule by about 90°. There has been significant progress in understanding these factors (Lord 2003; Shivanna 2003). In *Nicotiana*, characterized by the wet stigma, there are convincing evidences to show that the lipidic stigmatic exudate establishes a water gradient which ensures directional growth of pollen tubes into the stigma. The composition of the stigmatic exudates is such that it is permeable enough for pollen hydration to occur but not so permeable for water supply to become non-directional (Wolters-Arts et al. 1998). However, in *Brassica* and *Arabidopsis* with dry type of stigma, the lipids present on the pollen grain surface are involved in pollen tube guidance. They are released soon after pollination and come in contact with the stigma surface to form a contact zone, which provides a medium to establish directional water gradient for pollen hydration, germination and pollen tube guidance into the stigma (Wang et al. 1993; *see* Shivanna 2003).

Several studies, using in vitro assay, in *Arabidopsis* and *Torenia* have provided strong evidences to show that synergids of the embryo sac are involved in directing the pollen tubes from the placenta into the embryo sac (Hulskamp et al. 1995; Higashiyama et al. 2001; Punwani and Drews 2008). The filiform apparatus of the synergid seems to play an important role in guiding the pollen tube. A number of synergid-expressed genes and their proteins which are secreted into filiform apparatus have been identified (*see* Polanivelu and Preuss 2006; Okuda et al. 2009; Li et al. 2011; Kawashima and Berger 2011). These proteins have been suggested to play a role in the formation and/or function of filiform apparatus and possibly in pollen tube guidance.

Pollen–pistil interaction has now become an active area of research. Advance techniques of molecular biology and genetics are being used to unravel the mysteries of pollen–pistil interaction. In the coming years, significant advances are likely to be made in our understanding of pollen–pistil interaction.

8.4 Double Fertilization

Following the entry of pollen tubes into one of the synergids, pollen tube discharges the two male gametes into the synergid. One of the sperm cells approaches the egg and fuses with it to give rise to the zygote. The other sperm cell moves towards the central cell and eventually fuses with the fused polar nuclei (secondary nucleus) to give rise to the primary endosperm nucleus (Russell 1992; Russell and Dumas 1992). Although cytoskeletal elements have been implicated in the movement of the sperm cells, recent studies have indicated that the cytoplasmic flow of the pollen tube content generated by pollen tube discharge is enough to move sperm cells into the intercellular region between the egg cell and the central cell (*see* Kawashima and Berger 2011). The zygote gives rise to the embryo and the primary endosperm nucleus to the endosperm. Fertilized ovules develop into seeds and the ovary into fruit.

8.5 Protocols

8.5.1 Aniline Blue Fluorescence Method to Study Pollen Germination and Pollen Tube Growth in the Pistil

Details of pollen germination and pollen tube growth in the pistil are required to understand a number of aspects of reproductive ecology such as pollen viability, stigma receptivity and breeding system (Fig. 8.1). Most of the earlier studies depended on staining pollinated pistils in acetocarmine or aniline blue in lactophenol, making a squash preparation and observing them under a light microscope. Although pollen germination could be easily observed in such preparations, pollen tube growth could not be seen clearly in many species with thicker stigma and style. Some investigators used clearing of the pistil or dissecting the transmitting tissue of the pistil before staining. Lack of suitable techniques was one of the major limitations for studies on pollen germination and pollen tube growth by early investigators. Following the standardization of the technique of aniline blue fluorescence (Linskens and Esser 1957; Martin 1959; Shivanna and Rangaswamy 1992), it has become a standard method for studying various aspects of pollen

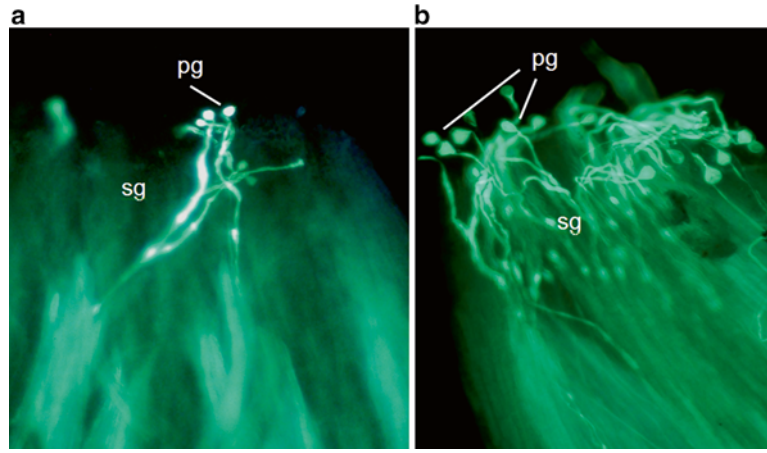

Fig. 8.1 Stigma receptivity in *Syzygium* sp. (**a**) Fluorescence micrograph of a cleared stigma stained with decolourized aniline blue, pollinated soon after anthesis. Stigma (*sg*) is not fully receptive and has supported germination of a few pollen grains (*pg*). (**b**) Stigma similar to **a** but pollinated 24 h after anthesis. Stigma is fully receptive and supported profuse pollen germination

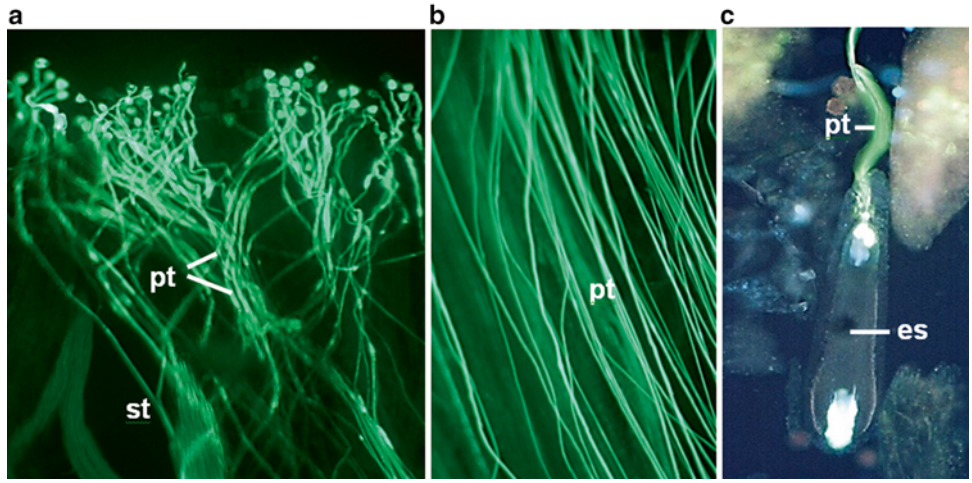

Fig. 8.2 Fluorescence micrographs of cleared aniline blue-stained preparations. (**a**, **b**) The stigma (**a**) and style (**b**) of pollinated pistil of *Syzygium* sp. to show profuse germination of pollen grains (*pg*) on the stigma and growth of pollen tubes (*pt*) in the style (*st*). (**c**) An ovule of *Tribulus terrestris* to show pollen tube (*pt*) entry into the embryo sac (*es*)

germination and pollen tube growth and considerable literature has accumulated on this aspect. Aniline blue is a callose-specific fluorochrome. As pollen tubes invariably contain callose along the wall and also as callose plugs across older region of pollen tubes, aniline blue-stained preparations show bright fluorescence under fluorescence microscope with UV illumination (Fig. 8.2).

8.5.1.1 Special Requirements

Decolorized aniline blue stain (*see* Appendix A.1).

Sodium hydroxide 4–8 N.

Fixative: Many investigators have used FAA or acetic alcohol or LA (*see* Appendix A.1).

Fluorescence microscope.

8.5.1.2 Procedure

1. Fix the pollinated pistils in a fixative for about 24 h and store them in 70 % ethanol until use.
2. Transfer the fixed pistils to 4–8 N NaOH for clearing depending on the thickness of the pistil. For delicate pistils, 4 N NaOH is sufficient, while the robust pistils require 8 N NaOH. The period of clearing also varies based on the thickness of the pistils. At laboratory temperature, clearing is generally done overnight. The period of clearing may be reduced by increasing the temperature to about 60 °C. After clearing, the pistils become rather soft and need careful subsequent handling. When LA is used as fixative, clearing may be done using 4 N NaOH or 4 N KOH. Lactic acid simultaneously fixes and also clears the tissue to some extent. KOH has been found to be useful in removing the phenolics.
3. Transfer cleared pistils to water taken in a Petri plate and rinse them carefully at least twice.
4. Mount the pistils in 1:1 mixture of aniline blue and 10 % glycerin.
5. Apply gentle pressure on the cover glass to spread the tissue.
6. Observe the preparations under a fluorescence microscope with UV filter combination (refer to Appendix A.8). Sieve plates of the phloem and often xylem elements also show some fluorescence and confuse the identity of the pollen tubes. The sieve plates and xylem elements can be identified by observing the same field under bright light; the thickenings of the xylem elements and the position of sieve plates along the xylem tissue help in their identification. The preparations can be stored for a few weeks in a refrigerator; however, care should be taken to prevent them from drying.

8.5.1.3 Modifications

1. Some investigators leave cleared and rinsed pistils in the stain for a few hours or overnight for staining and then mount in glycerin.
2. Resin-embedded sections or wax-embedded serial sections of the pistils or ovary along with ovules may be stained directly with aniline blue to localize pollen tubes in the transmitting tissue of the pistil or in the micropyle of the ovules.

8.5.2 Temporal Details of Pollen Germination and Pollen Tube Growth

In several studies, it becomes necessary to determine the rate of pollen germination and pollen tube growth. This is particularly important in self-incompatible species. In some self-incompatible species, pollen tubes may reach the base of the style in both self- and cross-pollinated pistils, but the growth of self-pollen tubes is much slower when compared with cross-pollen tubes; in such species even when pollinations are carried out with mixed pollen (self and cross), most of the seeds are sired by cross-pollen. Such studies are also needed in interspecific crosses. In this protocol, pollinated pistils are fixed at various time intervals after pollination and the extent of pollen tube growth is determined.

8.5.2.1 Special Requirements

Tags and pollination bags
Other requirements as in Protocol 8.5.1.

8.5.2.2 Procedure

1. Tag sufficient number of flower buds before anthesis and anther dehiscence. Divide them into several sets and label each set as i, ii and so on. The number of sets depends on the frequency of time intervals needed; to some extent this depends on the time taken for the pollen tubes to reach the base of the style. The number of flower buds for each set should be at least six.
2. Emasculate all the flower buds and bag them.
3. On the day of anthesis or when the stigma becomes receptive, open the bags and carry out pollination with required pollen sample in all the sets of flowers and rebag them. Note down the time of pollination.
4. Excise pollinated pistils at preselected intervals (starting with set i) and fix them in a fixative. Generally, interval of 2–4 h is sufficient in short-styled flowers; in long-styled flowers such as tobacco, 6–12 h may be suitable.
5. Process each set of pistils separately to study pollen germination and pollen tube growth through aniline blue fluorescence method following Protocol 8.5.1.
6. Observe each pistil from each set and record the extent of pollen germination and the length of pollen tube growth in the pistil. One of the standard methods to measure the length of pollen tube growth in the pistil is to put a dot (on the mounted cover glass) with a marker at the region on the pistil up to which the pollen tubes have traversed, and then measure the length (mm/cm) from the tip of the stigma up to the dot.
7. After recording the extent of pollen germination and pollen tube growth in all the pistils of all the sets, calculate, for each set, average length up to which pollen tubes have grown in the pistil. Calculate the rate of pollen tube growth using the data from all the sets. Growth rate in the pistil is generally expressed as μm/h.

8.5.2.3 Modifications

1. Pollen tube growth can also be studied by pollinating excised flowers/pistils maintained in a beaker or a Petri plate (*see* Protocol 9.6.1).
2. To compare the rate of pollen tube growth in self- and cross-pollinated pistils, divide emasculated flower buds into two sets. Carry out self-pollinations on flowers of one set and cross-pollinations on the other set. Divide each set into subsets for fixing at regular intervals. And follow later steps as described above.
3. For studying the speed of pollen germination, fix pollinated pistils at 15 min intervals and score percent pollen germination/number of pollen grains germinated on the stigma (*see* Protocol 6.3.5 for details).

8.5.3 Semi-Vivo Technique to Study Pollen–Pistil Interaction

This method is a combination of in vivo and in vitro methods. The method can be used for studies on pollen vigour, temporal details of pollen tube growth and the division of the generative cell in 2-celled pollen systems. The flowers are pollinated in situ on the plant or after implanting them in a beaker containing water or agar medium set in Petri plates (*see* Protocol 9.6.1). Pollen germination and pollen tube growth are allowed to proceed in the pistil up to a desired length and the style is cut in front of the growing pollen tubes and implanted in a liquid or agar medium for the pollen tubes to emerge in vitro into the nutrient medium through the cut end of the style. This method has been used in several species with robust pistils such as *Oenothera, Nicotiana* and *Lilium*. Some of the aspects studied using semi-vivo technique are self-incompatibility (Niimi 1982), effect of ovary on pollen tube growth (Mulcahy and Mulcahy 1985), isolation of sperm cells in two-celled pollen species (Shivanna et al. 1988), pollen vigour (Shivanna et al. 1991) and the factors responsible to attract pollen tubes into the micropylar region of the ovules (Higashiyama et al. 2001; Higashiyama and Hamamura 2008).

This exercise needs some preliminary studies to standardize suitable medium for pollen tube emergence in vitro and to get some idea about the rate of pollen tube growth in the pistil.

8.5.3.1 Special Requirements

A suitable nutrient medium that permits satisfactory pollen tube growth. As a general guideline, a medium standardized for pollen germination would be suitable. For several species, we have used the medium containing sucrose (10 %), boric acid (100 mg/ml) and calcium nitrate (300 mg/ml) – either semisolid (0.8 % bacto agar) or liquid. Addition of polyethylene glycol may enhance pollen tube growth (*see* Appendix A.4).

Petri plates or beakers of suitable size.

8.5.3.2 Procedure

1. Emasculate mature flower buds before anther dehiscence and bag them.
2. On the day of anthesis or when the stigma becomes receptive, pollinate the emasculated flower buds with fresh pollen grains and rebag them.

   The number of sets of pistils to be used for pollination depends on the objective of the study. For example, if the study is designed to compare the rate of pollen tube growth in self- and cross-pollinated pistils, two sets of pistils (one for self- and one for cross-pollination) have to be pollinated. Each set should have at least 6 pistils.

   Also, the period for which pollinated flowers have to be left in situ depends on the length of the style through which pollen tubes have to grow in vivo and the nature of the study. For several studies, the growth of pollen tubes for 0.5–1.0 cm in the style is suitable; this usually takes 8–24 h depending on the species. However, the required period has to be checked.
3. Prepare suitable liquid/solidified agar medium for pollen tube growth and have the agar medium set in Petri plates or beakers depending on the size of the explant to be used.
4. At the end of the selected period, excise the pollinated pistils and bring them to the laboratory.
5. Cut the style transversely with a sharp blade in the region just ahead of the growing pollen tubes in the style.

   This has to be done carefully without smudging the cut end. In hollow-styled species, hold the stylar part to be cut immersed in the liquid germination medium (used for pollen tube emergence) and then only cut the style. Otherwise air bubbles may get trapped in the stylar canal which may affect pollen tube emergence.
6. Immediately after cutting the style, implant the cut end in the liquid medium/agarified medium set in Petri plate/small beaker. If a liquid medium is used, keep the cut end of the

pistil dipped in the medium through small holes made in aluminium foil or parafilm used as lid for the beaker/Petri plate.

7. Maintain the implanted pistils for 12–24 h at 22 ± 2 °C or at laboratory temperature.

   Incubation time may vary depending on the species, the length of the style retained and the objective of the study.

8. Observe periodically the cut ends of the style until pollen tubes emerge. Note the time of pollen tube emergence (from stylar implantation) and allow pollen tubes to grow 1–2 mm into the medium.
9. After sufficient growth of the pollen tubes, gently pull out the style along with the emerged pollen tubes; place the cut end in a drop of the liquid medium; count the number of pollen tubes emerged and measure the length of pollen tubes. If the number of pollen tubes is too much, measure the length of randomly selected pollen tubes. If necessary, emerged pollen tubes along with a very short length of the cut end of the style may be cut from the remaining part of the style and used for mounting.

By staining pollen tubes with a DNA fluorochrome, such as DAPI, generative cell/male gametes and vegetative nucleus may be observed in the pollen tubes.

8.5.3.3 Modifications

1. Instead of carrying out pollinations using flowers maintained on plants, pollinations can be carried out on flowers/inflorescences/flowering branches excised and maintained with their cut end dipped in water in the laboratory (for details *see* Protocol 9.6.1).
2. Pollen vigour can be effectively assessed through semi-vivo method by timing the emergence of the pollen tubes after pollinating each set of the pistils with different pollen samples.
3. This method can also be used to study stigma receptivity. Pollinate flowers of different ages/stages, allow a fixed time for pollen germination in all of them, cut the style a few mm below the stigma and implant in a medium. Pollen tubes emerge earliest in the pistils with receptive stigma. In others pollen tubes may not emerge or emerge much later than that in receptive stage.

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Breeding Systems

9

Genetic variation in the population is the basis of adaptation and evolution. Genetic variation is assessed on the basis of the extent of heterozygosity. Heterozygosity enables the species to cope under changed habitat and also its establishment when migrated to new areas. The extent of heterozygosity in the population is dependent on a number of reproductive traits, particularly the sexuality of the species (see Chap. 4) and the breeding system.

Breeding system is the mode of transmission of genes from one generation to the next through sexual reproduction. It largely reflects the extent of selfing/crossing and thus determines genetic variation of the population and the species. Some investigators have distinguished breeding system from mating system (Kevan 1997; Harder and Barrett 2006; Dudash and Murren 2008). Breeding system is defined as 'all aspects of sex expression in plants that affect the relative genetic contributions to the next generation of individuals within a species' (Wyatt 1983). The mating system is defined as 'those factors that determine the pattern of gene inheritance between generations' (Kearns and Inouye 1993). However, most of the investigators do not seem to distinguish between these two terms as revealed in their publications. We have used breeding system to cover both the breeding and mating systems.

The breeding system in flowering plants is highly variable and flexible. The plants have evolved a whole range of options starting from autogamy to obligate outcrossing. The system is responsive to different selection pressures, both intrinsic and environmental. Amongst the intrinsic factors that determine the breeding system are floral morphology and sexuality of the flowers, plants and populations (see Chap. 4) and prevalence of several outbreeding devices. Breeding system provides an insight into the potential of gene flow within and between plants in the population. Studies on the breeding system also help in the estimation of appropriate population sizes needed to maintain genetic diversity of the species.

9.1 Outbreeding Devices

Unlike animals which are mostly unisexual, a majority of plants are bisexual, and also there is ample scope for self-pollination. Because of the advantages of outbreeding, plants have evolved a number of devices to avoid inbreeding. The following are the major outbreeding devices:

Dichogamy: Temporal separation of pollen release and stigma receptivity in bisexual flowers.

Protandry: Anthers release pollen before stigma becomes receptive.

Protogyny: Stigma becomes receptive before pollen release.

Herkogamy: Spatial separation of the anthers and the stigma in bisexual flowers so that the chance of autogamy is remote.

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Self-incompatibility: Inability of self-pollen grains to effect fertilization upon self-pollination.

Dicliny: Flowers are unisexual.

Monoecious: Male and female flowers are borne on the same plant.

Dioecious: Male and female flowers are borne on different plants.

Herkogamy, dichogamy and monoecy prevent autogamy but not geitonogamy (pollination between flowers of the same plant). Self-incompatibility (SI) and dioecy prevent selfing completely. However, self-incompatibility in many species is partial, and in such species self-pollinations do result in some seed set. Similarly, in several dioecious species also, male and female plants may produce some bisexual flowers (androdioecy and gynodioecy) that permit some degree of selfing. There is obligate selfing in cleistogamous flowers (which do not open); however, many such species produce chasmogamous (open) flowers also so that there is ample scope for cross-pollination. Thus, in a majority of species with hermaphrodite flowers, it is a combination of selfing and crossing, although the extent of each varies greatly between and within the species.

9.2 Self-Incompatibility

Self-incompatibility (SI) is an important outbreeding mechanism prevalent in a large number of species. SI is a prefertilization barrier and results in the inhibition of pollen germination and/or pollen tube growth in self-pollinated flowers. Extensive studies have been carried out on the structural and functional details of SI (*see* Richards 1986; de Nettancourt 2001; Shivanna 2003; Takayama and Isogai 2005). SI is of two types.

9.2.1 Heteromorphic Self-Incompatibility

Flowers produced on different plants within the species are of two (dimorphic/distylic) or three (trimorphic/tristylic) types (Fig. 9.1), but each plant produces only one type of flower. The major difference between the morphs is in the relative position of the stigma and anthers. In dimorphic flowers, the thrum (short-styled) morph has long anthers and short stigma, and the pin (long-styled) morph has short anthers and long stigma. Self- as well as intramorph pollinations are incompatible, and intermorph pollinations are compatible. Distyly (e.g. species of *Linum, Primula* and *Limonium*) is controlled by a single gene complex, S, with two alleles S and s. The allele for short style (S) is dominant over that of long style (s). The pin morphs are homozygous recessive (ss), and the thrum morphs are heterozygous (Ss).

In trimorphic species (e.g. species of *Oxalis and Lythrum*), floral morphs can be distinguished as short-styled, mid-styled and long-styled morphs (Fig. 9.1). The stamens are generally at two levels in trimorphic species. In short-styled morph, the stamens are at mid- and long levels; in mid-styled morph, the stamens are at short and long levels; and in long-styled morphs, the stamens are at mid- and short levels. Intramorph pollinations are incompatible. For compatibility, pollen grains have to come not only from flowers of the other morph but also from anthers corresponding to the same level as that of the stigma. Genetic studies have indicated that trimorphy is controlled by two genes: S and M with two alleles each. S is epistatic over M. Long-styled morphs are homozygous recessive for both the genes (ssmm), mid-styled morphs are homozygous recessive for S and heterozygous or homozygous dominant for M (ssMM/ssMm), and short-styled morphs are heterozygous for S and M may be in any form (Ssmm/SsMM/SsMm).

9.2.2 Homomorphic Self-Incompatibility

In homomorphic SI, flowers produced on all plants of a species are morphologically similar. Homomorphic SI is controlled by multiple alleles termed S alleles (S_1, S_2, S_3…S_n) at a single locus. Pollen grain carrying an S allele identical to the one or both the S alleles present in the pistil is inhibited at the level of germination or during pollen tube growth in the pistil (Fig. 9.2). For a successful pollen–pistil interaction, S allele present in the pollen has to be different

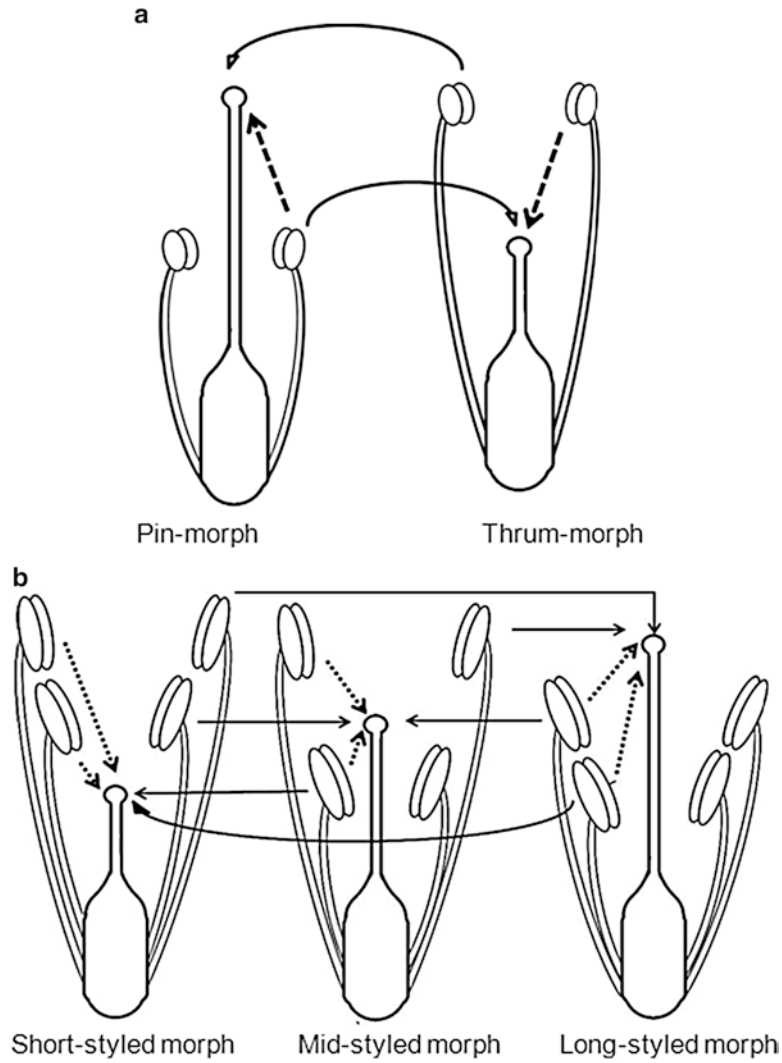

Fig. 9.1 Diagrammatic representation of heteromorphic self-incompatibility in distylous (**a**) and tristylous (**b**) systems. *Solid arrows*, compatible pollinations; *broken arrows*, incompatible pollinations

than those present in the pistil. In members of Poaceae, however, SI is controlled by multiple alleles at two loci, S and Z; allelic matching of both the genes is required for SI. There are a few species in which self-pollen tubes enter the ovary but do not result in seed development (Tandon et al. 2003, *Acacia*; Sunnichan et al. 2005, *Boswellia*). In some species, SI is due to post-fertilization abortion of fertilized embryos (Seavy and Bawa 1986; Lee 1988). Many investigators consider post-fertilization abortion of selfed embryos as a separate phenomenon caused by the accumulation of lethal genes, rather than self-incompatibility (see Mulcahy and Mulcahy 1983). Homomorphic SI is of two types:

Gametophytic (GSI): The incompatibility phenotype of the pollen is determined by the genotype of the individual pollen (some examples: members of Solanaceae, Liliaceae and Rosaceae).

Sporophytic self-incompatibility (SSI): The incompatibility phenotype of the pollen is determined by the diploid genotype of the pollen-producing parent (some examples: members of Asteraceae and Brassicaceae).

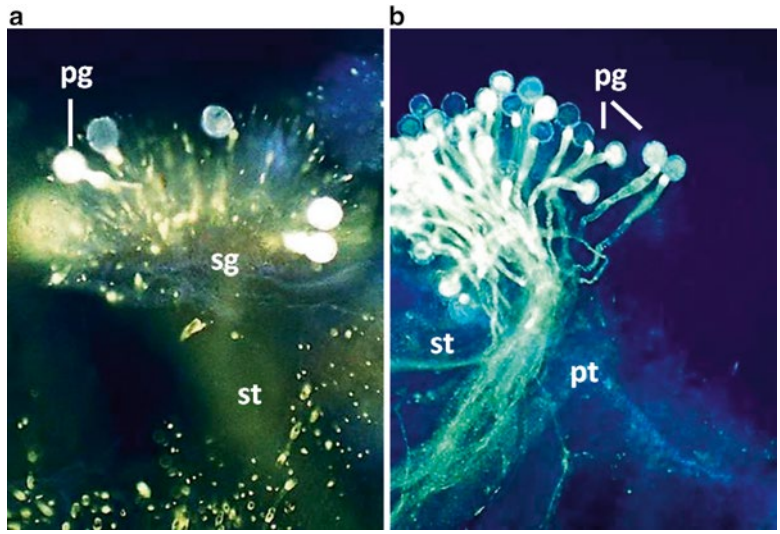

Fig. 9.2 Responses of self- and cross-pollinations in self-incompatible *Azadirachta indica* (*After* Vikas and Tandon 2011). Fluorescence micrographs of the stigma and a part of the style in self-pollinated (**a**) and cross-pollinated (**b**) pistils. Pollen tubes (*pt*) have not grown beyond the stigmatic zone (*sg*) in (**a**), whereas the pollen tubes have grown normally in the style (*st*) in (**b**)

Table 9.1 Correlations between some phenotypic characters and the genetics of self-incompatibility (SI)

Phenotypic characters	Gametophytic SI	Sporophytic SI
Pollen cytology[a]	2 celled	3 celled
Zone of inhibition of pollen	Style	Stigma surface
Morphology of the stigma	Wet/dry	Dry
Development of callose plug in the stigmatic papillae in selfed pistils	Absent	Present

[a]Major exception: In members of Poaceae, pollen grains are 3 celled, but SI is of the gametophytic type

The determination of the genetics of SI is laborious and time consuming. It involves carrying out a large number of controlled pollinations, raising the progeny and their analyses often extending to several generations (*see* Wallace 1979). However, there are several correlations between the genetics of SI and the phenotypic manifestation of SI (Table 9.1). These studies give some indication on the genetics of SI. However, one has to be careful in concluding the genetics of SI based on the phenotypic features as there are many exceptions to the correlations (see Shivanna 2003).

Extensive studies have been carried out on the cytology, physiology, genetics and molecular biology of homomorphic self-incompatibility. In several species, particularly in members of Solanaceae, Papaveraceae and Brassicaceae, SI genes and their products have been identified and characterized, and the basis of recognition and inhibition of self-pollen has been established. Going into these details is beyond the scope of this manual. There are several comprehensive reviews on these aspects (*see* de Nettancourt 2001; Shivanna 2003; Takayama and Isogai 2005; Chen et al. 2010; Wheeler et al. 2010; Rea et al. 2010).

Self-pollination decreases the opportunities for outcrossing as a result of pollen and ovule discounting. Pollen discounting refers to the reduction in the available pollen, that can be exported to other plants in the population, as a result of self-pollination. Pollen discounting is more prevalent in species particularly tree species which produce larger number of flowers on each plant at a given time; this permits considerable geitonogamy. Ovule discounting refers to the reduction in the number of ovules available for outcrossing as a

result of fertilization of ovules by self-pollen. This occurs in self-compatible or mildly self-incompatible plants in which autogamy and/or geitonogamy results in fertilization of some ovules. Many investigators have quantified self-incompatibility by calculating index of self-incompatibility (ISI) by applying the formula given by Zapata and Arroyo (1978, see Protocol 9.6.4).

9.3 Apomixis

Apomixis, often referred to as agamospermy, is a non-sexual mode of breeding system prevalent in some species. Seed development generally involves two important events, meiosis and fertilization. Apomixis is the development of seeds without fertilization (for details see Richards 1986, 2003; Asker and Jerling 1992; Koltunow 1993; Bicknell and Koltunow 2004; Barcaccia and Albertini 2013). Apomixis has been reported in over 400 species belonging to over 40 families. It is more prevalent in members of Asteraceae, Poaceae and Rosaceae. Seedlings raised from apomictic seeds are the clones of the parent. Although many earlier investigators included all forms of asexual reproduction including vegetative reproduction under apomixis, most of the recent definitions of apomixes include only the asexual seed pathway. Apomixis has been classified into different types based on the details of seed development. The following are the major categories:

9.3.1 Nonrecurrent Apomixis

Embryo sac is formed through normal meiosis, and the embryo arises from the unfertilized egg parthenogenetically. As the resulting embryo is haploid, it does not perpetuate from generation to generation.

9.3.2 Recurrent Apomixis

Apomixis perpetuates from generation to generation. Recurrent apomixes are again characterized into:

Generative apospory/diplospory: Megaspore mother cell does not undergo meiosis, or it may enter meiosis but forms restitution nucleus. Both the pathways result in diploid embryo sac. The embryo develops from the unreduced egg or any other cell of the embryo sac (*Ranunculus*, *Ixeris, Erigeron*).

Apospory: A diploid somatic cell of the nucellus gives rise to unreduced embryo sac and the egg develops into diploid embryo parthenogenetically (species of *Poa, Panicum, Pennisetum, Hieracium*).

Adventive embryony: Several embryos develop from the sporophytic cells. In most of the species with adventive embryony, nucellar cells develop into embryos (*Citrus, Mangifera, Opuntia, Garcinia*). In a few species, embryos develop from integumentary cells.

Many of the apomictic species are pollination dependent for the development of embryos. In such species, pollen tubes grow through the stigma and style, enter the embryo sac and discharge the two male gametes. One of them fuses with the secondary nucleus (fused polar nuclei, pseudogamy) and gives rise to the endosperm. The second male gamete does not fuse with the egg. It is difficult to identify apomixis in species which are pollination dependent. The only effective method in such apomicts is through the use of morphological or genetic markers. There are a few apomictic species which are pollination independent (e.g. *Commiphora wightii*, Gupta et al. 1996). In these species, the endosperm develops from the secondary nucleus autonomously (non-pseudogamous) without the fusion of one of the sperms. This type of apomixis can be easily identified from field studies; even emasculated and bagged flowers in such species develop seeds. As endosperm development is necessary for continued growth of apomictic embryos, even nucellar or integumentary embryos protrude into the endosperm for their continued development. In some apomicts such as *Citrus*, zygotic embryo also develops normally in addition to adventive embryos originating from the nucellus. Thus, out of several embryos from a seed, one is the zygotic embryo and all others are adventive embryos. For clonal propagation, only nucellar embryos are needed, while

for genetic improvement of the species, only zygotic embryo is needed.

Apomixis imparts several advantages to the species. Apomicts combine the benefits of vegetative propagation with the advantage of seed-dependent dormancy and dispersal. Apomictic species are able to fix extremely fit genotypes rapidly for a given niche. Non-pseudogamous apomicts which are independent of pollination are able to avoid costs of pollen development and also the resources needed to attract and sustain pollinators inherent in sexual reproduction. Some of the disadvantages of apomicts are that they are able to occupy a narrow niche, burdened by the accumulation of nonadaptive mutants, and their inability to recombine novel advantageous mutants.

Apomixis has important potential application in hybrid seed industry (see Barcaccia and Albertini 2013). One of the major constraints in hybrid seed technology is the need to produce hybrid seeds every year and distribute them to the farmers. Transfer of apomictic trait to the hybrids would result in fixation of hybridity. As apomictic seeds do not show segregation, farmers can use the seeds collected from the hybrids, generation after generation. Although extensive studies are being carried out to transfer apomictic trait to the hybrids in several laboratories, it has not yet been possible to achieve the objective.

9.4 Pollen:Ovule Ratio and the Breeding System

According to Cruden (1977), the number of pollen grains produced for each ovule in a flower tends to reflect the breeding system of the species. The general assumption of this concept is that the number of pollen grains produced per flower in a species is inversely proportional to pollination assurance. Based on his work on 86 species, Cruden (1977) proposed pollen:ovule ratios for species showing various breeding systems (Table 9.2). Although this concept has often been criticized (Charnov 1979), the pollen:ovule ratio does indicate to some extent the breeding system in several species, and many investigators continue to present pollen:ovule ratios in their publications.

Table 9.2 Pollen:ovule ratio in species showing different breeding systems (Cruden 1977)

Pollen:ovule ratio	Breeding system
2.7–5.4	Cleistogamy
8.1–39	Obligate autogamy
31.9–396	Facultative autogamy
244.7–2,588	Facultative xenogamy
2,108–195,525	Obligate xenogamy

9.5 Reproductive Assurance Through Autogamy

Outcrossing species are dependent on pollinators for seed and fruit set. Such species often experience pollen limitation because of the scarcity of pollinators and/or conspecific partners. Outcrossing species tend to evolve spontaneous autogamy (self-pollination within the flower)/apomixis/vegetative propagation as a means of reproductive assurance. Reproductive assurance has been considered to be the main driving force for the evolution of autogamous selfing from outcrossing species, and this has been one of the most frequent transitions recorded in the breeding system of flowering plants (Kalisz and Vogler 2003; Eckert et al. 2006). One of the important features of autogamous flowers is that their floral traits do not prevent outcrossing completely; most of them, although capable of seed set in the absence of pollinators, permit outcrossing wherever suitable pollinators are present. Thus, the system is dynamic. The evolution of autogamy is frequent in monocarpic (produce fruits only once in their life and die) species particularly in annual weed species (Shivanna 2014) as it facilitates not only their survival under pollinator scarcity but also in colonizing new areas and thus contributes to their evolutionary success (Baker 1974; Barrett 2011).

Reproductive assurance through autogamy enhances the fitness of the species in several ways: (1) It increases reproductive efficiency as partially or fully autogamous species are able to set seeds even under unfavourable pollination

environments. (2) They can exploit seed-dependent dormancy and dispersal advantages. Low density of conspecific plants and pollinator scarcity are common limitations during the initial stages of colonization of new areas. Autogamous species are able to colonize new areas more effectively than outcrossing species; even one individual is sufficient to establish viable population in the new location irrespective of the presence or absence of pollinators. Outcrossing species on the other hand have to have several conspecific individuals and effective pollinators to colonize new areas. This feature is of particular importance in marginal and extreme habitats. (3) Autogamous species can reduce resource allocation to flowers; they do not have to produce large and showy flowers to attract pollinators. The amount of nectar, one of the important rewards for pollinators, may be limited or even absent, and they can also economize on pollen production as limited amount of pollen is enough to bring about autogamy.

One of the major limitations of autogamy is that it results in inbreeding depression (Charlesworth and Charlesworth 1987) which generally results in the progeny with reduced vigour. Homozygosity in the population results in loss of evolutionary potential to cope with changed environment. Inbreeding depression is more pronounced in outbreeding species when compared to inbreeding species. This is explained on the basis of purging of recessive deleterious alleles and thus increasing the frequency of adaptive genes in continuously inbreeding populations.

9.6 Protocols

9.6.1 Controlled Pollinations

Controlled pollinations often termed manual pollinations are important components of studies on pollination ecology and the breeding system. Pollinations are carried out manually with the pollen of the known type. An essential part of controlled pollinations is to isolate individual flowers to prevent contamination with a suitable device. Enclosing flowers/inflorescences/flowering branches with suitable bags is the most convenient method (see Sect. 2.2 for details).

Pollination biologists have used a variety of devices to carry out manual pollinations. The simplest method is to hold dehisced anther(s) on their filaments with a pair of forceps and gently rub the dehisced anther onto the surface of the stigma. Alternately, pollen grains may be collected at the tip of a needle or toothpick or pointed paper wick and used for pollination.

One of the important *precautions* needed for controlled pollination is to scrupulously avoid contamination of pollen. The use of brush for pollination needs to be avoided as it is difficult to decontaminate the brush before reusing it for any other type of pollination. When the forceps/needle is used for pollination, it has to be decontaminated by dipping in 70 % ethanol and drying it before using for another type of pollination. Many investigators flame the instruments after dipping in ethanol and cool them before reuse. If a filter paper strip is used for pollination, use a fresh one for each type of pollination.

For studying controlled pollination, some preliminary studies on temporal details of anthesis, anther dehiscence, stigma receptivity and pollen viability are required (see protocols under Chaps. 3 and 6).

9.6.1.1 Special Requirements

Tags and isolation bags of suitable size
Ethanol (70 %) to sterilize instruments
Requirements for aniline blue fluorescence studies on pollen germination and pollen tube growth (see Protocol 8.5.1).

9.6.1.2 Procedure

1. Select mature flower buds before anthesis and anther dehiscence and tag them. In a majority of species, the flower buds 1 day before anthesis are suitable. However, in some species, the anthers may dehisce earlier, and in such species select buds at an earlier stage before anther dehiscence.
2. Open the flower buds carefully causing minimal disturbance to the floral parts and remove their anthers (emasculation) with a pair of

forceps. Make sure that anthers are not damaged during emasculation; damaged anthers may spill pollen grains onto the stigma. It is better to handle anthers by holding their filaments rather than anthers directly. Bag emasculated flowers/inflorescences.

3. When flowers open and the stigma becomes receptive, open the bag and pollinate emasculated flowers with fresh pollen of the *desired type*. Make sure that adequate amount of pollen grains are deposited on the stigma. Rebag the flower. Record the date, time and the type of pollination on the label and in the notebook.
4. Remove the bags after the stigma loses receptivity or when the flower is no more fresh (2–7 days depending on the species) but retain the labels.
5. Observe pollinated flowers at suitable intervals until their abscission or fruit development. Initially it is better to observe them every day for 3–7 days; after fruit initiation, they can be observed at weekly intervals. Record the fate of each pollinated flower.
6. Excise mature fruits before dehiscence. When comparing different pollination treatments, measure the size of each fruit (diameter or length and width, depending on the shape).
7. Collect seeds from each fruit. Count the number of seeds in each fruit.
8. Calculate per cent fruit set and average number of seeds/fruit.

   In some treatments, it may be necessary to measure seed size or to determine seed weight to calculate average seed size or seed weight for each treatment.

   Depending on the size of individual seed, seeds can be weighed individually or in lots of 10/100 seeds.

9.6.1.3 Modifications

1. If the aim is to study pollen germination and pollen tube growth only, excise pollinated pistils 24–48 h after pollination and fix them in a suitable fixative (see Appendix A.1). Process the fixed pistils for aniline blue fluorescence method (see Protocol 8.5.1).
2. Several investigators have used excised pistils/flowers to study pollen germination and pollen tube growth. Excise flower buds just before anthesis/anther dehiscence and maintain them with their cut end dipped in water. When the pistils/flowers are large, they can be maintained in a beaker with their cut end dipped in water. If the pistils/flowers are small, they can be maintained on agar plates (see Appendix A.3) by inserting their cut ends into the agar (Fig. 9.3). Emasculate the flowers carefully. When the stigma becomes receptive, carry out manual pollinations as described above. Fix the pistils 24–48 h after pollination and process them for aniline blue fluorescence method to study pollen germination and pollen tube growth following the procedure as described in Protocol 8.5.1.

9.6.2 Identification of Dichogamy (Protogyny and Protandry)

In several species, pollen release and stigma receptivity are not synchronous. In protogynous species, the stigma becomes receptive before anther dehiscence. In protandrous species, the anthers dehisce before the stigma becomes receptive. The time gap between male and female phases may range from a few hours to a few days. In some species, the flowers pass through a sequence of male, bisexual and female phases (in protandrous flowers) or female, bisexual and male phases (in protogynous flowers).

9.6.2.1 Special Requirements

See protocols on pollen viability (5.5.10 and 5.5.11) and stigma receptivity (6.3.5).

9.6.2.2 Procedure

1. *Male phase*:
   (a) Observe freshly opened flowers carefully and record whether the anthers have dehisced or not. In many species, anthers dehisce along with or soon after anthesis. In some species, anthers may have dehisced even before anthesis. In species in which anthers have dehisced at or before anthesis, the male phase is considered to have begun along with anthesis.

Fig. 9.3 Controlled pollinations using excised flowers of *Tribulus terrestris*. A setup to show excised flowers implanted in agar medium dispensed in Petri plates for pollination

However, in protogynous species, anthers dehisce after the stigma becomes receptive or after the stigma loses receptivity and thus the male phase starts later.

(b) If the anthers have remained undehisced at or soon after anthesis, the flower is not in the male phase. Keep observing at regular intervals (2–6 h depending on the species) and record the day (with reference to the day of anthesis) and time of anther dehiscence; this indicates the beginning of the male phase.

(c) Determine the period of male phase. Most of the pollen grains may be removed by pollinators or fall off from the anthers on the day of anthesis itself, or they may be available for pollinators for several days. If they are present for more than a day, their viability has to be assessed at suitable intervals using one of the viability tests (see Protocols 5.5.10 and 5.5.11).

(d) Alternately they can be used for pollination of flowers (intact/excised) with receptive stigma and pollen viability assessed on the basis of pollen germination in manually pollinated pistils by following the procedures described in Protocols 6.3.5 and 9.6.1.

(e) On the basis of these studies, fix the onset and duration of the male phase of the flower with reference to the day of anthesis.

2. *Female phase*:

(a) The determination of the female phase of the flower takes more time and efforts. Observe the stigma carefully in freshly opened flowers. In some species, the stigma receptivity is indicated by morphological changes of the stigma. For example, in species with folded stigma lobes, the lobes open and spread out when the stigma becomes receptive. In some species, the stigma receptivity is indicated by the projection of the stigma beyond the level of the anthers. In species with wet stigma, the onset of receptivity

may be indicated by the accumulation of stigmatic exudates on the stigma surface. However, the association of morphological changes of the stigma with stigma receptivity needs to be confirmed at the beginning in the study species by conducting a suitable test for stigma receptivity (see Protocols 6.3.5 and 6.3.6 for details); if morphological stages of the stigma are confirmed with stigma receptivity, they can be used routinely to recognize onset of stigma receptivity for later studies.

(b) However, in species in which there are no morphological differences associated with stigma receptivity, stigma receptivity has to be determined by carrying out tests for stigma receptivity at suitable intervals after anthesis following the procedure described in Protocol 6.3.5 or 6.3.6.

3. Duration of stigma receptivity has to be assessed through manual pollinations on emasculated and bagged flowers at different intervals after anthesis, until the stigma does not support pollen germination any more.
4. On the basis of the onset and duration of the male and female phases, determine if the species shows protogyny or protandry or synchrony in pollen dispersal and stigma receptivity. If the species is protandrous and protogynous, estimate the time difference between the two phases.

9.6.3 Estimation of Pollen:Ovule Ratio in Bisexual and Monoecious Species

The number of pollen grains produced for each ovule of a flower reflects, to some extent, the breeding system of the species. Many investigators on reproductive ecology present the data on pollen:ovule ratio and its relevance to the breeding system.

9.6.3.1 Special Requirements

As in Protocols 5.5.1, 5.5.2, and 6.3.2.

9.6.3.2 Procedure

Bisexual species: The estimation of pollen:ovule ratio in bisexual flower is straightforward:

1. Estimate the mean number of pollen grains per flower (see Protocol 5.5.1 or 5.5.2 depending on the number of pollen grains in the anther).
2. Estimate the number of ovules per flower (either by dissection of the ovary or following Protocol 6.3.2, depending on the number of ovules).
3. Calculate pollen:ovule ratio as the mean number of pollen grains per flower to the mean number of ovules per flower.

Monoecious species: The determination of pollen:ovule ratio in monoecious species is more elaborate. This is because the number of male and female flowers produced in monoecious species is not equal; the number of male flowers generally tends to be higher than that of female flowers. Thus, apart from calculating the mean number of pollen grains and ovules produced in male and female flowers, the ratio of male to female flowers also has to be calculated. Counting the total number of male and female flowers produced in the entire plant may not be feasible particularly in tree species. In such species, the counting of male and female flowers has to be done by selecting suitable random sampling units such as the inflorescences or branches of known size with sufficient number of replicates.

For the purpose of calculation, let us consider the proportion of male to female flowers as 2. The following procedure may be followed for calculating pollen:ovule ratio with arbitrary values for pollen and ovule numbers:

Mean no. of ovules per flower: 10

Mean no. of pollen grains per flower: 10,000

The ratio of male to female flowers: 2

Mean no. of pollen grains per flower for determining pollen:ovule ratio = $10{,}000 \times 2 = 20{,}000$

Pollen: ovule ratio = $20{,}000/10 = 2{,}000$

In dioecious species, xenogamy is the only possible mode of breeding system; information on pollen:ovule ratio is not of much relevance.

9.6.4 Analysis of the Breeding System of the Species

In strictly dioecious species, xenogamy is the only possible breeding system. In all other species, the breeding system has to be determined through controlled pollinations. Even in gynodioecious and androdioecious species, there is a possibility of autonomous and geitonogamous self-pollinations, and the breeding system needs to be investigated. Similarly in self-incompatible species also, self-incompatibility is often leaky and self-pollinations result in some seed set; controlled pollination is the only effective method to analyse the breeding system of the species.

When seed set in self-pollinated flowers is less than that in cross-pollinated flowers, the values generated in Table 9.3 can be used for calculating the *index of self-incompatibility* (ISI) (Zapata and Arroyo 1978) as follows:

$$\text{ISI} = \frac{\text{fruit set in self pollinated flowers}}{\text{fruit set in cross pollinated flowers}}$$

Fruit set can be assessed on the basis of per cent fruit set or no. seeds per fruit/pollination. The species is considered fully self-compatible when the ISI is 1 or >1, partially self-compatible when ISI is >0.2 but <1 and fully self-incompatible when ISI is <0.2 or 0.

Estimation of selfing rates (Charlesworth 1988):

$$\text{Selfing Rate} = \frac{P_x - P_o}{P_x - P_s}$$

where P_x = average seed set in cross-pollinated flowers, P_o = average seed set in open-pollinated flowers, P_s = average seed set in self-pollinated flowers.

9.6.4.1 Special Requirements

Tags and isolation bags
Ethanol 70 %

9.6.4.2 Procedure

1. Select five sets (**i**–**v**) of flower buds of suitable stage (just before anthesis and anther dehiscence). Make sure that they are selected randomly from the same and different plants. The number of buds has to be sufficient for the five sets of treatments. Label the five sets as **i**–**v** (using flower buds from different plants for each set). Label each set as follows:
   Set i: autogamy
   Set ii: apomixis
   Set iii: geitonogamous self-pollinations
   Set iv: xenogamous pollinations
   Set v: open pollinations

Table 9.3 Expected fruit and seed set in various pollination treatments in species showing different breeding systems

Breeding system / Pollination treatment	Autogamous and self-compatible species	Non-autogamous and self-compatible species	Non-autogamous and partially self-compatible species	Species with strong self-incompatibility (autogamous/ non-autogamous)	Apomictic species
Set i (bagged without emasculation)	+	–	–	–	+
Set ii (bagged after emasculation)	–	–	–	–	+/–[a]
Set iii (self-pollination)	+	+	(+)	–	+
Set iv (xenogamous pollination)	+	+	+	+	+
Set v (open pollination)	+	+	+	+	+

(+) Significantly less seed set when compared to xenogamous pollinations
[a] + Pollination-independent and – pollination-dependent apomictic species

2. Treat different sets as follows:
   Set i: bag without emasculation.
   Sets ii–iv: bag after emasculation.
   Set v: no treatment.
   Sets i, ii and v are not subjected to any further treatments.
3. On the day of anthesis/stigma receptivity, open the bags from flowers of sets iii and iv and carry out the following types of pollinations using pollen grains from freshly dehisced anthers (open the bag of one flower at a time, pollinate it as mentioned below and rebag before opening the bag of the next flower for pollination).
   Set iii. Geitonogamous pollinations (pollen from other flower of the same plant)
   Set iv. Xenogamous pollinations (pollen from flower of another plant)
   Take all the precautions needed to prevent contamination during pollinations (see Protocol 9.6.1). Rebag each flower soon after pollination.
4. After the flowers start senescing or the stigma loses receptivity, open the bags from all the sets but retain the labels.
5. Keep the flowers of all sets under observation until their abscission or development of fruits. Maintain the record for each flower of each set.
6. Collect mature fruits before they dehisce. Determine per cent fruit set and average number of seeds per fruit for each type of pollination. Tabulate the results.
7. Interpret the results on the basis of expected responses given in Table 9.3. Fruit set in set i indicates the presence of autogamy and that in set ii apomixis. The extent of fruit set in sets iii and iv indicates the presence and the extent of self-incompatibility in the species. Fruit set in set v indicates efficacy of open pollination.
8. Check if the breeding system is confirms with the results of the pollen:ovule ratio (see Protocol 9.6.3 for determining pollen:ovule ratio).

9.6.5 Estimation of Reproductive Assurance Through Autogamy

In several species with bisexual flowers, autogamy occurs to various degrees. Autogamy provides reproductive assurance and enables the populations to set seeds even in uncertain pollination environment. The extent of autogamy is highly variable; it may be marginal or exclusive depending on the morphology of the flower and the availability of the pollinators. This protocol gives details for estimating the extent of autogamy.

9.6.5.1 Special Requirements

Tags and isolation bags

9.6.5.2 Procedure

1. Select five sets of flower buds (i–v) and label them as follows:
   Set i. Bagged
   Set ii. Emasculated and bagged
   Set iii. Emasculated but left without bagging
   Set iv. Open pollination
   Set v. Manual cross-pollination
2. Bag the flower buds of set i. Emasculate and bag the buds of set ii and emasculate the buds of set iii but *leave them without bagging*. No other treatment is given to buds of set iv. Carry out manual cross-pollination of the flowers of set v, when their flower buds open.
3. Keep track of all sets of flowers until their abscission or fruit development and record.
4. Collect mature fruits before dehiscence and count the number of seeds from each fruit.
5. Calculate per cent fruit set and mean number of seeds per fruit in all the treatments. Compare fruit and seed set in different sets.

Fruit and seed set in bagged flowers (set i) indicate the extent of autogamy. Set ii should not yield any fruits and seeds unless the species is apomictic. The development of fruits and seeds in set iii indicates the extent of exclusively biotic pollination. Fruit set in open pollination (set iv) indicates the combination of autogamy and non autogamous pollinations. Fruit set in manual pollinations (set v) indicates maximum capacity of the species to set fruits without pollination limitation.

The comparison of fruit and seed set in sets i and iii indicates the extent of reproductive assurance through autogamy. If there is no seed formation in set iii, the seed set in the population studied is exclusively through autogamy, and animals do not have a role in pollination. If seed

development is recorded in set iii flowers also, the population show mixed mating with both autogamy and biotic pollination.

9.6.5.3 Modification

This protocol can be suitably combined with observations of insect visitation to the flowers, their frequency, pollen load on their body and their pollination efficiency.

9.6.6 Estimation of Inbreeding Depression

In cross-pollinated species, inbreeding (selfing) results in inbreeding depression (ID) which lowers the fitness of the progeny. ID operates at different levels – the number of seeds produced, their rate of germination, vigour of the seedlings, survival of the seedlings up to the flowering stage and reproductive effort of the progeny in terms of the production of number of flowers and seeds (Eckert and Barrett 1994; Husband and Schemske 1996; de Jong and Klinkhamer 2005). ID is calculated on the basis of the performance of selfed seeds over that of outcross seeds by using the following formula:

$$\mathrm{ID} = 1 - \frac{\text{performance of selfed seeds}}{\text{performance of outcrossed seeds}}$$

When the performance of selfed seeds is equal to that of crossed seeds, inbreeding depression is zero. When the selfed seeds perform poorly, ID increases depending on the extent of reduction in the performance of selfed seeds. ID can be determined for the whole population or for individual plants.

9.6.6.1 Special Requirements

Tags and isolation tags
Ethanol 70 %

9.6.6.2 Procedure

1. Select 6–8 plants randomly in a population and label them.
2. Select mature flower buds from each plant and bag them. The number of buds selected on each plant should be sufficient to carry out self- as well as cross-pollinations. If the flowers are bisexual, emasculate them before bagging.
3. When the flower buds open and the stigma becomes receptive, self-pollinate (with pollen of the same plant) half the number of flowers from each of the selected plants and cross-pollinate (pollen from another plant) the remaining half. Label the type of pollination on each flower. Make sure that there is no contamination of pollen. Use sufficient amount of pollen for pollination so that there is no pollen limitation.
4. Remove the bags (but retain the labels) after the flowers start senescing/stigma loses receptivity in all the flowers.
5. Keep the labelled flowers under observation until fruit maturity; record abscission of any of the pollinated flowers.
6. Collect mature fruits before dehiscence. Collect seeds from each fruit, count them and measure their size/weight.
7. Calculate per cent fruit set and mean number of seeds per fruit and mean seed size/weight in selfed and crossed treatments. Compare the values and check if there is any inbreeding depression in fruit and seed set. Calculate the extent of inbreeding depression, if any.
8. Mix all seeds from selfed flowers and those from crossed flowers separately.
9. Sow the seeds of both the sets for germination (see Protocol 10.5.3 for details). Maintain sufficient sample size and replicates for each set of seeds.
10. Record seed germination at regular intervals until they do not show any more germination. Tabulate the results and prepare a graph or a histogram to show temporal details of germination of the two samples. Calculate if there is any ID in seed vigour. Seed vigour is assessed on the basis of the speed of germination rather than their ability to germinate (Association Official Seed Analysis; Seed Vigour Test Committee 1983).
11. Allow the seedlings to grow and take growth measurements at suitable intervals (such as height, number of leaves/nodes, general

vigour, etc.) of the two sets of seedlings and assess ID, if any, in seedling vigour.
12. Transplant the seedlings of both the sets into larger polybags/nursery beds and maintain them under uniform conditions and record the number of plants that survive until reproductive phase. The vigour of the plants during their growth may be monitored by measuring some morphological parameters such as height, number of branches, etc. and compared.
13. Estimate reproductive efforts of both the sets of plants in terms of the flowering phenology, the number of flowers and fruits produced and the number of seeds/fruit and seed size in selfed and crossed progeny.
14. Calculate ID for each of the parameters by using the above formula.

9.6.6.3 Modifications

1. If sufficient number of seedlings is available, one can determine the wet and dry weights of seedlings of both the sets at a fixed time (number of days after sowing). This can be done either for shoot only (by cutting shoots at the ground level) or for the whole seedling by carefully lifting the seedling from the soil so that most of the root system is recovered. It would be better to raise the seedlings in polybags filled with loose sand so that the root system can be easily lifted.For determining fresh weight of only shoots, remove the debris if any from the shoot and weigh each shoot in an electronic balance. For fresh weight of seedlings along with the root system, wash the root system gently to remove sand particles, dab them on blotting paper to remove surface water and weigh. For dry weight determination, keep the material in a preweighed container such as glass Petri plate lid/aluminium foil and maintain them in an oven at 60 °C. Take them out at intervals of one to several days, allow the container to cool and weigh them. (If the humidity in the ambient air is high, keep the dishes covered soon after they are taken out of the oven to prevent their rehydration until weighing.) Repeat this until the weight remains constant for consecutive readings. Calculate the mean fresh wet and dry weights of seedlings for each treatment and calculate ID, if any, in seedling vigour.
2. Instead of completing the protocol completely, ID can be calculated using one or more parameters such as per cent seed set, seed germination rate and seedling vigour for a given period.

9.6.7 Confirmation of Self-Incompatibility and Identification of Inter-compatible Individuals

Self-incompatibility (SI) breaks down in some populations/individuals, even in species reported to be SI, due to mutation of S allele or due to environmental conditions. Also, SI may be absolute (without yielding any selfed seeds) or partial (yielding some selfed seeds). Cross-pollinations between individuals of self-incompatible population may also be incompatible if they carry the same S alleles. Therefore, in several studies dealing with SI, it is necessary to confirm self-incompatibility in the plants/populations used for investigation and also to identify individuals which are inter-compatible for cross- pollinations.

9.6.7.1 Special Requirements

Tags and isolation bags
Ethanol 70 %

9.6.7.2 Procedure

1. Select some plants randomly and label them as A, B, C, D and so on. The number of plants depends on their availability and the objective of the study.
2. Bag and tag as many flower buds/inflorescences/flowering branches as possible depending on convenience (see Protocol 9.6.1) from each of the selected plants. When bagging inflorescence/flowering branch, make sure to remove opened flowers, fruits and very young flower buds, retaining only those suitable for pollination.
3. Emasculate flower buds before anthesis and anther dehiscence and rebag them.

4. Divide them into two sets from each plant. Label set i as self-pollination and set ii as cross-pollination. Some investigators do not emasculate the flower buds to be used for self-pollination; pollen is used from the same flower to carry out self-pollination. However, this may induce some experimental error as the flower buds used for cross-pollination would suffer some physical injury when compared to buds used for self-pollination.
5. When the flower buds open and the stigma becomes receptive, carry out pollinations as follows taking all precautions elaborated under Protocol 9.6.1:
   Set i: Self-pollination using pollen grains from the same flower or the same plant and rebagging.
   Set ii: Cross-pollinations (pollen from other identified labelled plant). Ideally, flowers of each plant have to be cross-pollinated with pollen of each of the selected plants. There should be enough number of flowers (at least 6–10) for each combination. Although reciprocal crosses may be necessary between selected individuals to determine the genetics of SI, for the purpose of identification of SI and inter-compatible individuals, each selected plant may be used either as male or female parent with all other selected individuals. For example, using four plants, A–D, the following combinations may be tested:

   A×B, A×C, A×D, B×C, B×D, C×D

   The number of cross-combinations will increase as more number of plants are selected.
   Soon after cross-pollination of each flower, label each of them. Make sure that the instruments used for pollination are decontaminated by dipping in 70 % ethanol and drying before using for other crosses.
6. Keep all the pollinated flowers under observation until they abscise or develop into fruits. Keep a record of the responses of each flower.
7. Collect mature fruits before they dehisce, measure them and count the number of seeds in each.
8. Calculate per cent fruit set and average number of seeds per fruit.
9. Analyse the results. If the selected plants are fully or partially self-incompatible, there would be no fruit set or significantly lower fruit set in self-pollinated flowers. If they are self-compatible, there is no significant difference in fruit set between selfed and crossed flowers. When the plants used for cross-pollination are *inter-compatible*, fruit set in the cross-pollination is significantly higher when compared to self-pollinations (if the plants are self-incompatible). If the cross-combination is *inter-incompatible*, fruit set is similar to that obtained in self-pollinations.

   Many investigators represent SI in terms of mean number of seeds per pollination (rather than per cent fruit set) in selfed and crossed pistils.

9.6.7.3 Modifications

1. These studies can be carried out by studying pollen germination and pollen tube growth in selfed and crossed pistils. Excise the pistils 24–48 h after pollination and process them to study pollen germination and pollen tube growth (Protocol 8.5.1).
2. Instead of carrying out pollinations on flowers retained on plants, flowers can be excised before opening and implanted in beaker or agar plates and used for pollination and subsequent studies on pollen germination and pollen tube growth.

9.6.8 Analysis of Interspecific Incompatibility

Reproductive isolation is the basis of speciation. However, species isolation may not be strong enough to prevent the formation of hybrids completely between related species. Often natural hybrids can be seen growing amidst two sympatric species. The pollinator may visit

flowers of related co-flowering species and deposit heterospecific pollen on the stigma. It may be necessary to check if the related co-flowering species are reproductively isolated. Reproductive isolation between cultivars and wild relatives is also important in studies associated with the release of genetically modified plants. Strong reproductive isolation between the transgenics and its wild relatives is crucial for preventing escape of engineered genes to the wild species. The generation of detailed information on the prevalence of wild species around the location where transgenics are grown and their compatibility with the wild relatives and the possibility of introgression of transgenes into wild populations is required before transgenics are considered for release. This protocol requires some preliminary studies on anthesis, anther dehiscence and stigma receptivity of both the parents. All the precautions to control the contamination from unwanted pollen are described under controlled pollinations (Protocol 9.6.1), need to be followed.

9.6.8.1 Special Requirements

Isolation bags and tags
Ethanol 70 %

9.6.8.2 Procedure

1. Select suitable number of flower buds (before anther dehiscence and anthesis) from each parent species. Divide them into two sets, i and ii. Label set i for *intra*specific pollination and set ii for *inter*specific pollination.
2. Emasculate and bag the flowers of both the sets.
3. When the flowers open/stigma becomes receptive, collect fresh pollen from dehisced anthers of the two parents separately in suitable containers (watch glasses/Petri plates).
4. Open the bag from bagged flowers of set i and pollinate their stigma with the pollen of the same parent (intraspecific pollination) and rebag pollinated flowers. This set will act as control. Similarly pollinate set ii flowers with the pollen of the other parent (interspecific pollination) and rebag the flowers. Make sure that the pollen of the same parent will not contaminate the stigma during interspecific pollinations.
5. Remove bags when the flowers start senescing or the stigma loses receptivity but retain the labels.
6. Continue observations of pollinated flowers at suitable intervals and record all the abscised flowers and those developing into fruits in both the sets on both the parents.
7. Collect mature fruits before they dehisce and measure their size. Collect the seeds from each fruit, count them and measure their size (length × width). Compare per cent fruit set, fruit size and the number of seeds per fruit and seed size/weight between the two sets. If the cross is strongly incompatible, no fruits will develop in interspecific crosses. If the cross is partially compatible, there may be some fruit and seed set; more often seeds from such fruits may be smaller when compared to those from intraspecific pollinations.
8. Confirm the presence of embryos in hybrid seeds by dissecting both sets of seeds or germinating them under suitable conditions (see Protocol 10.5.3). Often the hybrid seeds would be empty/the embryo may or may not germinate.

9.6.8.3 Modifications

1. The extent of compatibility/incompatibility can also be studied by fixing the pistils 24–48 h after pollination and comparing pollen germination and pollen tube growth (see Protocol 8.5.1) in both sets of pollinations. However, it is difficult to quantify the results. Occasionally, even crosses which do not show good pollen germination and pollen tube growth may produce a few hybrid seeds.
2. Often, interspecific pollinations are carried out one way only (by using one of the parents as the male and the other as the female). In such pollinations, select two sets of flower buds in only the female parent and carry out emasculation, bagging and intra- and interspecific pollinations.

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Fruit and Seed Biology

10

Except in apomictic species, seeds are the products of fertilization. Fertilized ovules develop into seeds and the ovary into the fruit. In a few species, accessory structures such as thalamus (apple) or pedicel (cashew nut) become the predominant parts of the fruit. The fruits are made up of the pericarp developed from the ovary wall and centrally located seed(s). The fruits are adapted to perform three functions: protection, nourishment and dispersal of their seeds. Extensive data are available on fruit and seed biology; some of the books and reviews include: Herrera (1985, 2002), Jordano (1992), Turner (2001), Fenner and Thompson (2005), Dennis et al. (2007), Cousens et al. (2008) and Schaefer and Ruxton (2011).

The seed represents a miniature plant in the form of an embryo protected by the seed coat. It also has a source of nutrients to sustain the embryo during germination and early seedling growth. The embryo is made up of the cotyledon(s) (two in dicotyledons and one in monocotyledons) and the embryonal axis made up of the plumule, the hypocotyl and the radicle. The embryo is surrounded by the endosperm that provides nutrition to the developing embryo. In many species, the endosperm is consumed during development and is absent in the mature seed; such seeds are called non-endospermous seeds. In others, the endosperm is present in mature seeds also; such seeds are called endospermous seeds, and the endosperm provides nutrition to the embryo during germination also. In several species, particularly those with non-endospermous seeds, the cotyledons are massive and store food material. In such seeds, cotyledons provide nutrition to the embryo during its germination and early seedling growth. In a few species, the remains of the nucellar tissue of the ovule are present in mature seeds just below the seed coat, and this persistent nucellar tissue is referred to as the perisperm; it may also provide nourishment to the embryo. The seed coat, derived from the integuments of the ovule, gives protection to the seed.

There is great variation amongst the plant species in the extent of fruit and seed set. In many species, particularly in tree species, only a very small proportion of flowers develop into mature fruits. The failure of flowers developing into fruits is due to a combination of factors – pollination failure (*see* Chap. 7), abortion of young fruits due to genetic defects, predation by insects and vertebrates and microbial attack of fruits and seeds. Developing fruits have to compete amongst themselves for the available resources. Resource limitation is another major factor that induces abortion of developing fruits. In spite of sufficiently higher percentage of successfully pollinated flowers, the plant terminates the developing fruits before investing in their full development due to limited amount of available resource (Bhardwaj and Tandon 2013). The fruits that develop earlier in the season tend to be considerably larger by forming stronger sinks to draw nutrients than those that develop later. Also, fruits produced towards the end of the season often contain

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reduced number of seeds. This appears to be due to the decline in the available resources as the season progresses. In many of the multiovulate species, the proportion of ovules that develop into seeds is also limited. This is the result of either failure of fertilization or the abortion of some of the young seeds (Bawa and Webb 1984; Nakamura 1988). In species such as *Dalbergia*, *Phaseolus* and *Leucaena* in which the ovules are arranged linearly, the ovules located near the stylar end are the first to get fertilized and are more likely to develop into mature seeds, while those located near the pedicel are fertilized later and are more likely to abort (Ganeshaiah and Shaanker 1988; Rocha and Stephenson 1991). This again has been explained on the basis of competition for resources.

10.1 Types of Fruits

There is great variation in the morphology of the fruits. The fruits may be of dry or fleshy type. In dry fruits, the pericarp is dry and thin, but in fleshly fruits, the pericarp is thick and often elaborated into two or three layers. Both dry and fleshy fruits are divided into several types based on their structure. As studies on reproductive biology involve description on the type of fruits in the focal species and the community, a brief description of the major types of fruits is given below with examples.

10.1.1 Simple Fruits

Only one fruit develops from the ovary. It may be of dry or fleshy type.

10.1.1.1 Dry Fruits

Legume/pod: Monocarpellary fruit developing from one chambered ovary dehiscing from both the sutures (fruits of Fabaceae).

Follicle: Similar to the legume but dehisces by one suture only (*Calotropis*).

Siliqua: Long narrow fruit developing from bicarpellary ovary dehisces from below upwards on both the sutures (fruits of Brassicaceae).

Capsule: Many seeded fruit developing from a syncarpous ovary dehisces in various Ways (poppy, cotton, *Hibiscus*).

Schizocarp: Developing from an inferior bicarpellary ovary. When mature, the fruit splits into individual carpels called mericarps (fruits of Apiaceae).

Caryopsis: A small one-seeded fruit developing from a superior monocarpellary ovary in which the pericarp is fused with the seed coat (fruits of Poaceae).

Achene: A small one-chambered and one-seeded fruit developing from a monocarpellary ovary, but the pericarp is free from the seed coat (*Mirabilis, Fagopyrum*).

Nut: One-seeded fruit with hard and woody pericarp (cashew nut, chestnut, oak).

Cypsela: One-seeded fruit developing from inferior ovary with the seed coat and pericarp free (fruits of Asteraceae).

Lomentum: It is a legume, but the fruit is partitioned between the seeds into a number of one-seeded chambers (*Acacia, Cassia, Mimosa*).

Samara: Dry indehiscent fruit with flattened wing-like outgrowths from the pericarp (maple, *Acer, Dioscorea, Shorea*).

10.1.1.2 Fleshy Fruits

Drupe: One or many seeded and one or many chambered fruit developing from monocarpellary or syncarpous ovary. Pericarp is differentiated into a thin epicarp, fleshy mesocarp and hard endocarp (mango, coconut, *Terminalia*, peach).

Berry: Many seeded fleshy fruit in which the pericarp remains soft and is not differentiated into a hard endocarp (tomato, grape).

Pome: A syncarpous fruit developing from an inferior ovary surrounded by the thalamus (apple, pear).

Pepo: Many seeded fruit similar to berry developing from inferior ovary with parietal placentation (fruits of Cucurbitaceae).

Hesperidium: Many celled fleshly fruit developing from a syncarpous ovary with axile

placentation; the exocarp is leathery and the endocarp protrudes inwards to form distinct chambers (fruits of Rutaceae: *Citrus*).

10.1.2 Aggregate Fruits

Aggregation of simple fruits developing from apocarpous (free carpels) pistil.

An etaerio of follicles: An aggregation of two (*Calotropis, Vinca*) or many (*Michelia*) follicles.

An etaerio of achenes: Several achenes aggregated together (strawberry).

An etaerio of drupes: Small drupes aggregated together on a fleshy thalamus (raspberry).

An etaerio of berries: Berries remain together embedded in fleshy thalamus (custard, apple).

Multiple/composite fruits: Develop from multiple flowers/inflorescences (jackfruit, mulberry, fig, pineapple)

10.2 Seed Viability and Germination

Following maturation, the seeds undergo dehydration and enter natural dormancy; they remain dormant until they get suitable conditions for germination. Viability is the ability of the seeds to germinate under favourable conditions. The period for which seeds remain viable varies greatly, from a few weeks to several years. It also depends on the storage conditions. In general, seeds stored under low temperature and humidity retain viability for a considerably longer period than those stored under room conditions. This feature is dependent on the ability of the seeds to withstand desiccation. One category of seeds, termed orthodox seeds, is dispersed at low moisture level (<20 %) and can withstand drying; such seeds can be stored under low temperature and humidity in the seed banks for prolonged periods without the loss of viability. However, seeds of several species are dispersed under higher moisture content (>30 %) and lose viability within a short period; they do not withstand desiccation and cannot be stored in the seed banks for longer periods. Such seeds are referred to as recalcitrant seeds. The seeds of many species growing in the humid tropics and whose seeds mature during rainy season are generally recalcitrant. They start germinating soon after dispersal or often while they are still on the plant (vivipary). Seeds of many fruit and plantation crops such as litchi, mango, citrus, walnut, cocoa and jackfruit belong to recalcitrant category and are not amenable for long-term storage.

Seed germination is an irreversible process; once initiated it cannot be reversed (Fenner and Thompson 2005). Under suitable conditions of moisture, temperature and availability of oxygen, the seeds get hydrated and result in the activation of the embryo leading to germination. The process of germination is associated with a rapid increase in respiratory activity and mobilization of nutrient reserves. Following germination the radicle emerges from the seed and grows into the soil. This is followed by the emergence of the plumule which grows away from the soil. During germination, the cotyledons may remain below the soil (hypogeal germination) or pushed above the soil by elongation of the hypocotyl (epigeal germination). Eventually, the radicle grows into the root system and the plumule into the shoot system. In epigeal species, the cotyledons generally turn green and become photosynthetic. In the early phases of germination, the seedling makes use of the nutrients stored in the cotyledons/endosperm/perisperm. Soon, new leaves emerge and turn green and the seedling becomes independent.

10.3 Seed Dormancy

Seed is a desiccated, dormant and dispersal unit, similar to pollen grains. This has important ecological significance, as it prevents seeds from germinating while they are still on the plant or soon after shedding when the conditions for seedling establishment may not be optimal (Fenner and Thompson 2005). Seed dormancy is the basis of the evolution of agriculture. Seeds of several species particularly of crop species (although dormant under unfavourable conditions) germinate when they are exposed to

favourable conditions. Seeds of many other species, however, do not germinate even under favourable conditions; this condition is termed seed dormancy. Seed dormancy is an adaptation to survive for longer periods; it prevents seeds from germinating when conditions are suitable for germination but may not be suitable for subsequent survival and growth of the seedlings. The following are the main causes for seed dormancy.

10.3.1 Impermeable Seed Coat

Seed coats are impermeable and prevent the entry of water and/or oxygen, which are essential for germination, to the seed. Such seeds do not imbibe water when placed in aqueous media for germination. The seeds of many legumes are well-known examples of impermeable seed coat. Continued weathering action by microorganisms or passage through the digestive tracts of animals softens the seed coat sufficiently to permit water imbibition and germination.

10.3.2 Mechanically Resistant Seed Coat

In some species such as walnut, cashew nut and olive, seed coats are very hard and prevent the expansion of the embryo and emergence of the radicle and plumule. Seed coat resistance decreases over a period of time due to the action of microorganisms and permit seed germination.

10.3.3 Presence of Inhibitors

Seeds of several species may contain inhibitors in the seed coat and/or the embryo and inhibit seed germination.

10.3.4 Rudimentary Embryos

In some species such as *Ranunculus, Fraxinus* and *Anemone*, the embryo is not fully mature at the time of seed shedding. They undergo continued growth after shedding and become competent to germinate.

10.3.5 Physiological Immaturity of the Embryos

In several species such as members of Rosaceae, the embryos are morphologically mature at the time of seed shedding but require a period of physiological maturity before they can germinate. Some of them require low-temperature stratification for physiological maturity.

10.4 Methods to Overcome Seed Dormancy

For testing seed viability and for raising plant nursery, it is necessary to overcome seed dormancy. The effective methods depend on the cause(s) for dormancy.

10.4.1 Scarification of Seed Coat

This is one of the most common and effective treatments to overcome dormancy imposed by impermeable or mechanically resistant seed coat. This treatment results in damage to the seed coat and facilitates their imbibition of water and oxygen to germinate. Scarification can be achieved either by mechanical means or through acid treatment. In mechanical scarification, the seed coat is cut in a corner or abrased against sand paper. The extent of scarification depends on the species. In acid treatment, seeds are treated with strong acids such as concentrated sulphuric acid for a desirable period (15 min to 3 h) depending on the species with occasional stirring. Acid treatment should be sufficient to allow imbibition but should not damage the inner tissues of the seed, especially the embryo. The seeds are then removed from the acid, washed in running water for 10–15 min to remove all traces of the acid and sown for germination.

10.4.2 Soaking Seeds in Water

For many species, soaking of dormant seeds in water helps in overcoming dormancy. Often exposure of seeds to running water by tying them in a bag made of muslin/cheese cloth and fixing it to a tap to allow water to tickle through the bag is more effective than soaking in water. Water soaking/washing in running water is particularly effective if the seed coat or the inner tissues contain any inhibitors, which are leached out from the seed during the treatment.

10.4.3 Stratification

In this method, the seeds are subjected to chilling temperature (0–10 °C) for the required period (vernalization). This is generally required for seeds of temperate species such as apple, peach and cherry in which seeds are exposed in nature to chilling winter conditions before they germinate in the spring. The seeds are soaked in water for about 24 h until they fully imbibe before transferring them to chilling temperature either in the refrigerator or natural outdoor winter cold. The period of treatment varies with species. Chilling brings about certain physiological changes in the embryo, particularly the level of growth regulators. The level of growth promoters such as gibberellins and cytokinins increases and that of the inhibitors, particularly abscisic acid, decreases during stratification.

10.4.4 Chemical Treatments

The treatment of seeds with various chemicals is one of the effective methods to overcome seed dormancy. A number of chemicals such as growth substances (gibberellic acid, kinetin and ethylene), potassium nitrate, thiourea and hydrogen peroxide have been used.

10.4.5 Light Treatment

In positively photoblastic seeds (species in which seeds are light dependent for germination), they have to be exposed to light of suitable intensity and wavelength to induce germination. In most of the photoblastic species, red light promotes and far-red light inhibits germination.

10.5 Protocols

10.5.1 Estimation of Per Cent Fruit Set

Per cent fruit set indicates the proportion of flowers that set fruits. The number of fruits developed is generally much less than the number of flowers produced by a plant. This is particularly true in tree species.

10.5.1.1 Special Requirements

Tags for labelling flowers (they should last until the fruits mature; in some species, fruit maturity may take months)

10.5.1.2 Procedure

1. Label flowers, selected randomly from different plants, in the population. The number depends on the availability of flowers; the larger the numbers, the more dependable the results.
2. Keep them under observation until they abscise or develop into mature fruits. Continue to monitor developing fruits as some of them may abort at different stages of fruit development.
3. Keep record of all the labelled flowers in terms of abscised flowers, abscised young fruits and those developing into mature fruits.
4. Calculate per cent fruit set (total number of mature fruits/total number of flowers × 100).

10.5.1.3 Modifications

1. The extent of fruit set can also be expressed as flower-to-fruit ratio by calculating the proportion of flowers with that of fruits.
2. In multiovulate species, all ovules may not develop into seeds. This can be expressed as ovule-to-seed ratio. Calculate mean number of ovules per flower (see Protocol 6.3.2). Count the number of seeds in each fruit and calculate the mean number of seeds per fruit. Calculate ovule-to-seed ratio based on the mean number of ovules present in the ovary and mean number of seeds present in the fruit.

10.5.2 Estimation of Moisture Content of Seeds and Its Relation to Loss of Viability

Moisture content of seeds refers to the amount of water present in the seeds at the time of dispersal. It is generally expressed as percentage of the fresh weight of seeds. As pointed out earlier, seeds of most of the species are dispersed at low moisture level (orthodox seeds). However, refractory seeds are dispersed at higher moisture level. Desiccation of such seeds beyond a critical moisture level is detrimental to their viability. Even a small change in the moisture content, therefore, may affect their germinability and storability. Thus, moisture content of the seed plays an important role in seed biology.

Prevailing humidity and temperature at which the seed samples are maintained affect seed moisture level. For crop species, seed biologists recommend equilibration of seeds at the prevailing humidity and temperature (generally in seed bank laboratories) for a few days before estimating moisture level. The moisture level of seeds (equilibrated at different humidity levels) of most of the crop species have been estimated and are available in the literature (Roberts 1972). However, for the wild species, the moisture level needs to be estimated. The collection of data on the moisture content of seeds at the time of shedding and its relation to viability is very useful for the management and conservation of the focal species.

The number of seeds taken for determining moisture level depends on the size of the seeds. Seeds of known weight are taken for smaller seeds and of known number are taken for large-size seeds. Many of the reports of seed moisture content of crops are based on smaller quantity of seeds; samples of 5–10 g or 15–20 seeds form the desirable sample size. At least two sets of seeds for each sample are recommended for moisture estimation.

10.5.2.1 Special Requirements

Oven set around 60–70 °C for drying seed samples

Analytical balance

Suitable containers to keep seeds for weighing and desiccation

Facilities for seed germination (germination trays with germination paper/Petri plates with filter paper discs or polybags filled with suitable substrate such as garden soil/soil and sand mixture)

An incubator set at about 22 °C, if the laboratory temperature is too low

10.5.2.2 Procedure

1. Take fresh seeds of a given species and mix them into one uniform lot. Divide them into six samples containing equal number/weight of seeds in each sample. The required number of samples in experimental set up depends on how long the experiment has to be run. The number of seeds in each sample should be sufficient to conduct germination tests with enough number of replicates.
2. Label each seed sample serially. Sample 1 is used to calculate moisture content of fresh seeds and sample 2 is used to score per cent germination of fresh seeds. Samples 3–6 are used to desiccate them to different levels before testing for germination. Two or three replicates should be taken for each sample. The humidity and temperature conditions of the laboratory where this work is being carried out should be similar to the field conditions where the seeds were collected. If the conditions of the laboratory are different than those in the field, it is better to collect seeds in airtight containers and carry out moisture estimation in the laboratory as soon as possible.
3. Weigh each seed sample in a preweighed container (such as small Petri plate/aluminium cup) and record their weight.
4. Take seeds of sample 1 and keep it in an oven set around 60 °C. This is for estimating moisture level of fresh seeds.
5. Take seed sample 2 and sow them for germination (treatments of samples 3–6 are explained from step 9 onwards) (*see* Protocol 10.5.3).
6. Estimate the moisture content of fresh seeds (kept in step 4) as follows:

Table 10.1 Sample tabulation of data to determine moisture content of seeds and its relation to germination (figures given are arbitrary for samples 3–6)[a]

Seed sample no.	3	4	5	6
Period of drying in desiccator (days)	1	2	3	4
Weight of seed samples after drying (g)	9	8	7.5	7.3
Loss of weight (compared to weight of fresh seeds 10 g; see step 7)	1	2	2.5	2.7
Loss of moisture during desiccation	1/10 × 100 = 10 %	2/10 × 100 = 20 %	2.5/10 × 100 = 25 %	2.7/10 × 100 = 27 %
Moisture content of seeds at the time of sowing for germination (total moisture level in fresh seed sample, see step 7) = 30 % − loss of moisture during desiccation	30 %−10 % = 20 %	30 %−20 % = 10 %	30 %−25 % =5 %	30 %−27 % = 3 %
% germination (germination of fresh seeds = 75 %; see step 8)	20	0	0	0

[a]According to the results of this arbitrary sample table, loss of moisture below 30 % is detrimental for germination; seeds lose germinability completely when the moisture level is <20 %

Take seed sample kept in the oven (sample 1) each day or once in 2/3 days depending on the rapidity of desiccation, allow them to cool to laboratory temperature, weigh the sample up to mg and put them back in the oven for continued drying. If the laboratory condition is too humid, keep seed samples closed (to prevent reabsorption of moisture from the atmosphere) during cooling period.

Repeat this weighing procedure at selected intervals until recording consistent value for two or three consecutive readings. This indicates that the seed sample will not lose any more moisture.

7. Calculate the moisture content of the fresh seed sample by using the formula:
   Moisture content = weight lost by seeds/initial weight × 100. For determining seed weight, make sure to minus the weight of the container (seed + container weight − container weight = seed weight).

   For the purpose of calculation, let us say that the initial weight of seeds was 10 g and the final weight was 7 g; loss of weight is 10−7 = 3 g. The moisture level of fresh seeds is 3/10 × 100 = 30 %.
8. Score for per cent germination of fresh seeds (sample 2 sown in step 5) by recording the number of seeds germinated at regular intervals (depending on their speed of germination). Continue this until all viable seeds have germinated. Calculate per cent germination by using the formula:
   No. seeds germinated/no. seeds sown × 100.
   Let us say that their germination score was 75/100 × 100 = 75 %.
9. Keep the remaining samples (3–6) of seeds in a desiccator (containing desiccating agent such as silica gel) maintained under laboratory conditions to allow desiccation.
10. From the seed samples kept in the desiccator, take seed sample 3 after 1 day or 2 days (this depends on the rapidity of desiccation of seeds). Record its weight (as shown in Table 10.1), and sow the seeds for germination (similar to fresh seeds).
11. At subsequent selected time intervals, take the next seed sample (sample 4), weigh the sample (and record its weight as shown in Table 10.1), and sow them for germination. Complete this step with the remaining seed samples and record.

At the end of the treatment, seed samples desiccated to different levels in the desiccators (samples 4–6) are weighed and sown for germination.

12. Keep observing all the samples sown for germination at regular intervals and score for germination. The scoring for each sample should be with reference to the day from sowing. Continue this until all viable seeds have germinated.
13. Record the germination scores of all seed samples as shown in the sample Table 10.1.
14. Calculate the moisture content (%) of each seed sample in comparison to fresh weight of the fresh sample (sample 1) and correlate with their germination percentage.

(Note: This procedure is for one replicate of seeds. For each set you have to maintain enough replicates to get valid data.)

10.5.3 Germination Test for Seed Viability

The assessment of seed viability is an important aspect of seed biology. Testing the viability of seeds is an essential requirement in assessing soil seed banks. Germination test is the most authentic test for seed viability. For crop species, this test is routinely used to assess the quality of seeds in seed banks and those sold to farmers for sowing. It is necessary to carry out some preliminary studies to standardize suitable germination conditions for the given species and method to break dormancy if the seeds are dormant.

10.5.3.1 Special Requirements

Petri plates lined with moist filter paper or germination trays with germination paper

An incubator with temperature set at around 22 °C, if lab temperature is not suitable for germination

10.5.3.2 Procedure

1. Collect suitable number of seeds from the stock of seed sample and sow them for germination in selected containers. It is advisable to place at least 25 seeds for each replicate and to raise three or four replicates. A general practice of sowing seeds is to leave space about five times the normal seed width or its diameter, between seeds.
2. Add to each container sufficient amount of water. Keep the containers under suitable conditions of light and temperature. Except light-sensitive seeds, seeds of most species germinate readily in dark. The period of incubation may vary from a few days to weeks depending on the species. When the period is more than a few days, it may be necessary to surface-sterilize the seeds with 70 % ethanol for a few minutes before sowing; otherwise they get infected with bacteria and fungi.
3. Keep observing the seed containers at intervals of 2–3 days and make sure that the containers will not dry up. Add sufficient amount of water periodically to prevent drying.
4. When there is no more germination, score the seeds for germination and calculate per cent germination.

10.5.3.3 Modifications

1. Although the use of Petri plates or germination trays with paper are convenient and have been used routinely, many seed biologists have used other containers and substrates. Sterilized sand or vermiculite has been used in Petri plates/germination trays. Some investigators have used polybags or sand beds as germination medium.

10.5.4 Tetrazolium Test for Seed Viability

This is a simple biochemical test (see Protocol 5.5.9 for the basis of this test) that can be used for nondormant as well as dormant seeds. This test permits quick estimation of viability of seed samples.

1. Soak the seeds in water for 24–36 h to activate the embryo.
2. Cut soaked seeds lengthwise into two halves to expose the embryo. Place the halved seeds in 1.0/0.5 % aqueous solution of 2,3,5-triphenyl tetrazolium chloride (TTC) or any other

tetrazolium salt at room temperature for several hours depending on the species. As the reaction occurs satisfactorily at neutral pH, TTC solution should be maintained around pH 7. Also, TTC is light sensitive, and therefore, the incubation should be carried out under dark. If the laboratory temperature is too low, seeds have to be incubated in an incubator around 22 °C.

3. After sufficient time of incubation, observe halved seeds, particularly the embryonal part. The embryos of viable seeds turn red and those of dead seeds do not take up the colour. The embryos in a proportion of seeds stain partially; such seeds create difficulty in interpreting their viability. An experienced researcher, however, would be able to interpret the viability of such seeds with reasonable accuracy based on colour differences and health of the tissues.
4. Score seeds for per cent viability based on colour development.

It may be helpful to carry out both germination and TTC test on the seed sample at the beginning. If both the tests respond similarly, TTC may be used routinely in subsequent tests.

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Seed Dispersal 11

Dispersal of seeds is one of the most critical aspects of reproductive ecology. In general, a majority of seeds fall below the parent plant. A proportion of them are dispersed away from the parent. Most of the plants depend on external agents for seed dispersal (Corlett 1998; Dennis et al. 2007; Cousens et al. 2008). Seed dispersal provides many advantages to plants: escape from specialist predators and pathogens prevalent under the parent, prevention of intraspecific competition, location of seeds in ecological safe sites where they can successfully germinate and establish seedlings (Janzen 1970; Connell 1971), colonization of new habitats and enhanced seed-mediated gene flow (Howe and Smallwood 1982; Peres et al. 1997; Fenner and Thompson 2005; Pinto et al. 2009). The locations of seed deposition by dispersers are generally random and unpredictable. However, some dispersers may deposit seeds predictably at sites favourable for seedling establishment. This has been referred to as directed dispersal (Wenny and Levey 1998; Wenny 2000). For example, in *Ocotea endresiana*, a shade-tolerant Neotropical montane tree in Costa Rica, four of the five species of birds involved in seed dispersal disperse seeds randomly, but the fifth species, a bellbird, predictably disperse seeds under song perches located in the canopy gaps (Wenny and Levey 1998). Seedling survival was higher from bellbird dispersed seeds than from those dispersed by the other species. Thus, seed dispersal enables plants to escape from the sources of mortality that are concentrated around the parent and increases the probability of colonizing suitable habitats.

11.1 Dispersal Agents

There are four main agents of seed dispersal – gravity (autochory), wind (anemochory), water (hydrochory) and animals (zoochory). Generally, seeds of non-fleshy fruits adapt the former two agents for seed dispersal. In addition to the dispersal of the seeds by primary dispersal agents, there may be secondary dispersal by another dispersal agent from the primary site. Dung beetles and ants are the important secondary seed dispersal agents for small seeds that are trapped inside the bird droppings and dung of browsing animals. Dung beetles, particularly the 'roller' and 'tunneler' groups, carry the trapped seeds either away from the dung pat or place them beneath the dung pat deeper inside the soil. The larvae consume only the dung, leaving the seeds for germination. Ants carry small seeds (myrmecochory) that are dispersed by primates and birds. Rodents also bring about secondary seed dispersal; they hoard dispersed seed and forget some of the hoarded sites (Forget 1993).

Fruits and seeds have adopted a range of devices for effective dispersal. One of the major difficulties of studying seed dispersal, particularly in bird- and bat-dispersed seeds, is in

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identifying the location of dispersed seeds, as it is difficult to follow these animals and locate the deposition sites.

11.1.1 Autochory

Autochory includes those seeds that fall to the ground beneath the parent plant without any other special means of dispersal. This also includes those with ballistic dispersal mechanism such as *Bauhinia*, balsam and *Ruellia*, in which the fruits dehisce with some force and hurl the seeds. However, the distance for which the seeds are dispersed by ballistic means is, at the most, limited to a few metres.

11.1.2 Anemochory

Fruits and seeds of several species have adapted for wind dispersal. They show various adaptations such as wings (seeds of *Cinchona, Moringa, Dioscorea*, fruits of *Acer, Dipterocarpus,* and *Shorea*) and hairs (fruits of *Asteraceae*, seeds of *Calotropis* and cotton) on their surface. The wings and hairs help them to float in the air and facilitate their dispersal. Some wind-dispersed seeds do not develop any special structures but show great reduction in their size and weight (such as orchids and some grasses). These seeds are thin and extremely light and are easily carried by the wind. Most of the wind-dispersed seeds are carried only for short distances. Long-distance wind dispersal occurs rarely as a result of stronger winds (Fenner and Thompson 2005).

11.1.3 Hydrochory

Water dispersal is a secondary dispersal mechanism after the fruits/seeds have fallen to the ground. Water dispersal is common in riverine and coastal species. Water-dispersed fruits usually develop floating devices and can float long distances without injury (coconut, lotus). Many riverine species do not have any buoyancy mechanism but they are carried by flowing water along the river/stream to various distances; the seeds germinate in the crevices of the river/stream and develop into seedlings. As they generally do not have opportunities to spread to other areas, such species remain confined to riverine habitat. The natural recruitment occurs along the seasonal rivulets in the desert. The role of water in seed dispersal in tropical forests seems to have been underestimated. Seeds of even those species which are not specially adapted to water dispersal and do not grow in the riverine habitat are carried by runoff water during heavy rains to various distances from the parent plant. For example, in *Acacia senegal* (Tandon et al. 2001), the dehisced fruits (diaspore) containing the seeds are first dispersed by wind and then carried by the seasonal rains (ombrohydrochory). This mechanism breaks the seed coat dormancy through mechanical scarification, and timely availability of water facilitates imbibition and germination (Parolin 2006).

11.1.4 Zoochory

Animals are the major seed dispersers. In tropical forests, over 70 % of the species are dispersed by animals (Howe 1990; Tadwalkar et al. 2012). Zoochory is of two types: epizoochory in which the seeds are passively carried on the animal's body and endozoochory in which the seeds are carried by the animals in their mouth or gut. Those fruits which are dispersed passively tend to be of dry type and develop many devices such as hooks, spines, bristles, stiff hairs and sticky glands on their surface (*Xanthium, Boerhavia*, spear grass, *Tribulus*) by means of which they adhere to the body of passing animals and are effectively dispersed. The epizoochorous fruits generally do not have any reward for the dispersal agents. Agricultural practices involving a range of implements, contamination of seeds during the harvest and use of manure also bring about extensive dispersal of seeds.

Fruits are rich in nutrients and minerals when compared to vegetative parts of the plant and attract a number of frugivores. The endozoochorous seeds provide some reward generally in the

form of fleshy expendable part of the fruit to attract the frugivorous animals. Colour and odour are the major attractants for diurnal dispersers, and odour is the main attractant for nocturnal dispersers. Fruits dispersed by diurnal animals show bright colours, while those dispersed by nocturnal animals are dull coloured but develop strong odour. Neotropical bats also use their echolocation ability to locate fruits (*see* Schaefer and Ruxton 2011). Frugivores consumes the pulp of the fruit through piecemeal (picking the fleshy part of the fruit) without plucking the fruit from the tree. Small birds, such as sunbirds, hummingbirds, white eyes and some flycatchers, belong to this group.

Seeds of some species which produce dry fruits can also be dispersed by animals; in such species, the seed itself acts as the reward for granivorous animals which hoard seeds. Most of their dispersal is secondary. Rodents (such as mice and squirrels) disperse mostly larger dry fruits/seeds as a consequence of their seed hoarding activities either singly or in clumps. Often the animal either dies or forgets the sites of hoarding and thus facilitates dispersal and germination of undamaged seeds. They can act as primary dispersers by collecting fruits/seeds directly from the plant or as secondary dispersers by collecting fallen fruits/seeds.

Vertebrates, especially birds and mammals (Jordano 1995; Herrera 2002; Tiffney 2004; Bronstein et al. 2006; Schaefer and Ruxton 2011; Sinu 2012), are the main animal dispersers in tropical rain forests. Amongst the vertebrates, birds, bats, primates and rodents are the most important dispersal agents. Bird- and bat-dispersed fruits are fleshy. In large fruits, the pulp is consumed either on the plant itself or after carrying the fruit to their roosting sites where the seeds are discarded (ectozoochory). Smaller fruits are swallowed (endozoochory), and eventually the seeds are discarded through regurgitation or defecation. The seeds which pass through the gut are generally protected by thick and smooth seed coat so that they are not destroyed during the passage. Ungulates, bears, lemurs, monkeys and tortoises have also been shown to be seed dispersers to a limited extent (Ghazoul and Sheil 2010). They are opportunistic foragers on a variety of pods and nuts. They generally eat the entire fruit and defecate the seeds elsewhere. Endozoochory results in the removal of the flesh, scarification of the seed coat and deposition of other materials in the form of dung/faeces along with the seeds. These processes may favour seed germination and seedling establishment; the removal of flesh may remove germination inhibitors, scarification may remove seed dormancy, and other materials deposited may act as fertilizers (Traveset et al. 2007). Seeds of some riparian species are dispersed by frugivorous fishes (ichthyochory) (Reys et al. 2009). There are also records of dispersal by frugivorous tree frogs and lizards (*see* Ghazoul and Sheil 2010).

Many plant species which use vertebrates as seed dispersers are mycorrhizal; they form mycorrhizal associations which promotes seedling establishment. Seed-caching rodents may improve seedling establishment by dispersing mycorrhizal inoculums along with seeds (Schupp 2007). Many vertebrates are also reported to deposit viable mycorrhizal spores in their faeces, thus favouring plant–mycorrhizal symbiosis (Theimer and Gehring 2007).

11.1.5 Myrmecochory

Ants are important secondary seed dispersers particularly in the temperate and arid regions; their role in seed dispersal is limited in tropical forests. This mutualism of seed dispersal between plants and ants is well investigated (Bronstein et al. 2006; Giladi 2006). Apart from seeds fallen below the canopy of plants, ants also remove seeds from the faeces/droppings of frugivores. Ants bring the seeds to their nest and bury them in shallow nests or deposit them near the entrances to the nest. These areas of seed deposition are usually aerated and enriched with faeces and discarded animal body parts. Ants are not effective for long-distance dispersal as they can only move seeds in the distance range of a few metres. Many ant-dispersed seeds develop special fat bodies termed elaiosomes. Ants consume elaiosomes and the undamaged embryonate

seeds are discarded in the nest or near the entrance of the nest. Germination success of such seeds and their seedling growth is improved owing to reduced infection by fungal pathogens and relocation of seeds to favourable sites.

There are hardly any reports of dispersal of seeds by other insects. However, there are a few reports of wasp-mediated seed dispersal (*see* Manohara 2013). In a recent study, a yellow-banded wasp (*Vespa affinis*) has been reported to be an effective dispersal agent of seeds of *Aquilaria malaccensis*, a tree species from the tropical forests of North-East India (Manohara 2013). Wasps are attracted by brightly coloured caruncle of the seed and carry the seeds for varying distances; after consuming the caruncle, they discard undamaged seeds.

11.2 Seed Rain

Spatial deposition of dispersed seeds over a distance from the parent plant is referred to as seed rain/seed shadow. Various environmental factors affect dispersal pattern of seeds. Seed rain determines the abundance and diversity pattern of the species in the habitat by affecting population and community structure. In isolated plants, the density of seeds deposited is generally high below the parent and declines with increasing distance. The density is also affected by the distance between the neighbouring conspecific plants.

11.3 Soil Seed Bank

Following fruit ripening, seeds fall on the soil beneath the canopy or away from the canopy of the parent following dispersal. They may germinate soon after dispersal or may remain dormant for varying periods before germination. Some of them get covered with leaf litter and eventually get buried in surface layers of the soil by the action of animals, drainage water and by seeds falling in the holes and cracks. The viable seeds present on or in the soil form the soil seed bank (Fenner and Thompson 2005). Extensive studies have been carried out on soil seed banks of temperate regions (Thompson et al. 1997). However, there is very little information on soil seed banks of tropical region.

Refractory seeds, which germinate within a few days or weeks after falling on the soil, are generally absent in the soil seed bank. The study of seed banks of smaller seeds is more difficult than those of larger seeds. Occasionally, soil seed bank may contain seeds of plants which are no more growing in the region. An interesting example of longevity of seeds in soil seed bank is *Nelumbo nucifera*. Several seeds recovered from dried bed of a former lake in North-East China germinated, and radioactive carbon dating of the oldest germinated seed showed that it was 1,288 ± 250 years old (Shen-Miller et al. 1995).

11.4 Protocols

11.4.1 Estimation of the Proportion of Fruits Dispersed

In general, only a small proportion of seeds are dispersed away from the parent plant. The remaining seeds fall to the ground underneath the canopy of the plant. The determination of the proportion of fruits/seeds dispersed would indicate the efficacy of dispersal.

11.4.1.1 Special Requirements

Suitable tags to label some branches, if necessary.

11.4.1.2 Procedure

1. Randomly select a suitable number of fruit-bearing plants that are not overlapping.
2. Count the number of developing fruits on each of them. Clear the ground below the selected plants.
3. Count and record the number of seeds/fruits that are fallen under the plant at suitable intervals (every day or once in 2 days/a week), and remove the fruits after each count. These are non-dispersed fruits.
4. After all the fruits have fallen/dispersed from the plant, count the total number of fruits collected below each plant over the period.

Total number of seeds produced on the plant minus the total number of seeds collected below the plant would give the number of fruits removed by the dispersal agents. Calculate the per cent fruits/seeds dispersed.

11.4.1.3 Modification

In larger shrubs and trees, counting the total number of fruits/seeds would be difficult. In such plants, label some isolated fruiting branches and count the number of fruits in each branch. Make a platform below the selected branch(es) with a cloth/net to collect fallen fruits. If there are fruiting branches above the selected branches, make a cloth/net platform above the fruiting branches also to prevent fruits falling from other branches onto the fruit collecting platform.

The total number of fruits in the labelled branches minus the total number of fruits collected from these branches gives the number of fruits dispersed. Calculate per cent fruits dispersed. If necessary, the data can be extrapolated to cover the whole plant by counting the number of branches on the plant.

11.4.2 Identification of Seed Dispersal Agents

This is one of the critical phases of reproductive ecology. Although animals are the most important dispersal agents, seeds and fruits of a considerable number of species are dispersed by water and wind. It is important to identify the dispersal agents and the details of their dispersal activity. These studies take a lot of time, efforts and patience. For seed dispersal study, observation period has to be selected, based on preliminary studies on animals visiting the focal plants. Dispersal animals may be active diurnally or nocturnally. Diurnal dispersal is generally more prevalent in the morning hours and/or evening hours. Many investigators have selected observation period for some time in the morning (06.00–08.00 h), afternoon (12.00–02.00 h) and evening (04.00–06.00 h) in a time window of 15/30 min each hour. The observations are extended for several days covering different plants. If the frequency of visitations is low, one may have to observe continuously for the whole day. If the dispersal agents are nocturnal (largely bats), observations have to be carried out during the night, either at selected times and intervals or the whole night. A foraging bout is the time a frugivore spends on a plant during each visit. During each bout, it may consume/handle just one fruit or several fruits.

11.4.2.1 Special Requirement

A pair of binoculars

If the dispersal agents are nocturnal, a pair of night vision binoculars would greatly help the observations. One can also install camera traps close to fruit-bearing inflorescences/branches.

Tabulated record book.

11.4.2.2 Procedure

Preliminary studies: This study requires some preliminary investigation to identify the visitors to the plant, their visiting time and frequency. Prepare a timetable for observing each labelled plant depending on the frequency of the visitor (morning and/or evening hours or the whole day in pulses). Each investigator has to focus one plant at a time, at least for a day, and extend the studies to several other plants one by one; these studies last for several days. The studies become easier if two or three investigators can score on dispersers.

1. Select plants bearing ripened fruits randomly from the population and label them.
2. Each day select one plant or a group of plants close to each other (in herbs and shrubs) or some branches (tree species) depending on the convenience. Select a place at a short distance away from the focal plants/branches from where you can observe all the visiting animals. If they are bird species, you have to sit farther from the selected plant/branch, preferably behind a camouflage as they get disturbed by the presence of humans and fly away without landing on the plant.
3. Record the visits of all the species of animals to the selected plant or branches. Also record whether the visitor (i) handles the fruit or not and if yes, whether just one fruit or many fruits during each bout; (ii) harvests the fruit

and forages only the pulp, scattering the seeds below the plant; (iii) pecks the fruit intact on the plant (without harvesting) and leaves the seed intact in the fruit; (iv) swallows the fruit and flies away or carries the entire fruit (without swallowing) to a breeding site or a feeding perch; and (v) whether visits in singles or in groups. Birds and bats tend to carry fruits to another roosting perch/plant and consume the fruit, scattering the seeds on the ground below the roosting plant. When they swallow the fruits, they may discard the seed after regurgitation, or the seeds may enter their digestive tract and are extruded during defecation far away from the original plant.

4. Record the details of the visitation bout and departure pattern of visitors. During each bout, they may fly to handle another fruit on the same plant/branch or fly away from the selected plant/branches.
5. Repeat these observations over several days. Select different plant or groups of plants each day.
6. Calculate the average frequency of each visitor to the plant and the number of fruits it handles during the observation period, and describe the details of seed dispersal.

11.4.2.3 Extended Studies

1. *Identification of the location of dispersed seeds*

   The distance up to which the seeds are dispersed and the site of dispersal are important parameters to assess dispersal efficiency. One of the standard methods is to follow the dispersal agents and locate the sites of seed deposition. This is a difficult task particularly in bird- and bat-pollinated species. Generally, the dispersal agents carry fruits in the mouth during their breeding season. Locating the breeding site of the visiting species and searching for the seeds below the site would enable the investigator to assess the dispersal distance for such seeds. Feeding perch sites are often used by small birds such as barbets; these perches are generally located very close to the fruit-bearing plants. In such species, identifying their perching sites and searching for dropped seeds would help in locating sites of seed deposition. The other method is indirect by making a survey of the surrounding area, particularly below the trees which are used by birds/bats as perches, and looking for dispersed seeds of the focal species. These seeds may have fallen down after the disperser consumes the fruit and discards the seeds or after regurgitation or in the faecal matter. This is the only method for locating seeds dispersed through faecal matter. The time gap between fruit consumption and seed ejection through faecal matter varies between animals. After the dispersed seeds are located, record the number of seeds dispersed on each site and the distance from the nearest fruiting tree. Also look for any evidences for secondary dispersal particularly by ants.
2. *Effects of passage of seeds through the gut*

   Often the passage of seeds through the gut of an animal may help in breaking dormancy of seeds. One can isolate the seeds from the faecal matter and study their germination responses by sowing them along with control seeds (*see* Protocol 10.5.3). Before doing so, one has to count the number of intact and broken seeds in the faecal matter and use only intact seeds for germination studies.
3. *Responses of dispersed seeds in situ*

   One can extend the studies to follow the responses of dispersed seeds in situ by monitoring them over a period of time and recording the proportion of seeds that get predated by vertebrates or insect attacks and those that germinate. If necessary, comparative studies can be made with seeds fallen beneath or below the canopy. Depending on the need, the responses of seedlings in terms of their mortality (through predation or other causes) and survival can also be studied in comparison to the seeds fallen below the canopy.

11.4.3 Analysis of Seed Rain

As the mortality of seeds and seedlings, to a large extent, is density and distance related, considerable data has accumulated on seed rains, particularly in tree species.

11.4.3.1 Special Requirements

Seed traps

11.4.3.2 Procedure

1. Select 5–10 isolated individuals, and place seed traps of suitable size from the trunk of the parent extending to various distances away from the trunk. Investigators have used seed traps of various sizes. Seed traps are generally in the form of aluminium/plastic trays or quadrats of required size. Some have laid quadrats along transects. The gap between transects and the distance between seed traps are variables according to the requirements. The distance up to which the seed traps are laid depends on the potential distance of seed dispersal. When there is predation of dispersed seeds by larger vertebrates, it is desirable to cover each quadrat by a wire mesh to prevent seed loss by vertebrates, particularly rodents but not to prevent seeds from falling into the seed traps.
2. Count the number of seeds in each seed trap at regular intervals until the end of the fruiting season. It is better to sample seed stations at intervals of 2–3 days to prevent seed loss to insect predators. Remove seeds from traps after each sampling.
3. At the end of the observation period, add all the seeds collected from each trap and analyse the distribution pattern with reference to the distance to the parent plant.

11.4.3.3 Modification

Seed counting quadrats can be combined with vertebrate seed predation by laying duplicate quadrats side by side and by covering one with wire mesh and leaving the other open.

11.4.4 Analysis of Soil Seed Bank

In most of the species, seeds persist in the soil for a considerable length of time, often up to several years, and thus contribute to population recruitment and persistence. Refractory seeds lose viability rapidly and do not persist in soil seed bank. Although seeds in the soil are subjected to different levels of predation and fungal attacks, which to some extent depends on edaphic conditions, a proportion of dormant seeds do survive for considerable time and are able to germinate. Sampling of seed banks is important in understanding the population dynamics.

11.4.4.1 Special Requirements

Soil sampler
Seed germination trays
Sieves of suitable mesh

11.4.4.2 Procedure

1. Select suitable sites depending on the objective of the study. One may study soil seed bank in a particular habitat or compare soil seed banks of different habitats/communities/vegetations or in areas following a change in soil use (e.g. converting a forest land to agricultural land) or to analyse the effect of fire.
2. Collect seed samples at different locations within each sampling site by taking cylindrical cores of suitable size (researchers have used samples of 4–8 cm diameter and 4–8 cm depth depending on the objective and study sites). Keep each sample in separate labelled bags.
3. Take soil samples to the laboratory and air-dry. Weigh known amount of soil (depending on seed density in the soil) from each sample, and spread on a plastic tray of suitable size. Some investigators have used trays containing 2 cm layer of sterilized sand for spreading the soil sample. Many investigators have sieved the soil using sieves of different mesh size to enrich seeds in the sample before spreading on the soil. This will reduce the soil volume and include more seeds.
4. Incubate soil trays under suitable laboratory conditions (either to mimic field conditions or to provide ideal conditions for germination in incubators or growth chambers depending on the objective). Irrigate the trays at regular intervals. The period of incubation varies according to the germination conditions.
5. Observe soil trays at regular intervals and record the number of seeds germinated.

6. Continue observation until the seedlings gain sufficient size so that they can be assigned to specific plant species.
7. Analyse the number of seeds of each species that have germinated and determine species richness in the soil seed bank.

11.4.4.3 Modification

Some investigators have buried seeds of different species in the soil for varying periods to study the effects of edaphic factors on their persistence (*see* Pakeman et al. 2011 and references therein).

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Seedling Recruitment

12

The stability of any species in its natural habitat is dependent on its optimal reproduction and recruitment of new individuals to sustain populations. Seedling recruitment is the ultimate step in a series of sequential events involved in successful reproduction of a species. Although habitat degradation, overexploitation and climate change are the proximate causes for vulnerability of the species, the ultimate driving force that pushes the species to endangered category and eventual extinction is reproductive constraint in the species induced by proximate causes. Because of these constraints, plant species are unable to produce optimal number of seeds and recruit new individuals to sustain populations. The following are the major steps involved in seedling recruitment that affect recruitment success:

- Optimal seed production
- Effective seed dispersal
- Seed germination
- Establishment of seedlings and their growth into adult plants

Each of these steps is associated with a number of hazards that induce seed/seedling mortality. Detailed studies carried out by several investigators have shown that the major recruitment constraints operate at the level of pollination and seed dispersal, both of which depend, to a large extent, on mutualistic interactions with animals (Corlett 2007).

12.1 Constraints for Seed Production

Only a small proportion of ovules develop into seeds, and only a tiny fraction of seeds produced on a plant develop into new adults. Seedling recruitment is more critical in tropical forests. Only one seed out of 100,000 is estimated to result in seedling establishment (Harms et al. 2000; Ghazoul and Sheil 2010). Even under ideal conditions, therefore, the number of seeds that result in seedling recruitment is very limited. When the seed production goes down, the chances of seedling recruitment are proportionately reduced. The following are the major constraints for seed production.

12.1.1 Predation of Flower Buds and Flowers

A proportion of flower buds and flowers are grazed by several herbivores and infested by insects. Grazing animals consume whole flowers/inflorescences. In the dry season, Brazilian scaly headed parrot has been reported to consume up to 38 % of the flowers (Galletti 1993). Generalist insects such as beetles consume floral parts. Many such flowers may remain functional, unless the insect consumes or damages sexual parts also. Some insects enter the flower buds and

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consume the inner floral parts; such flower buds do not open and appear faded and often show signs of drying. Specialized insects lay their eggs in the ovary/ovules in the flower bud or flower. Such flowers can be recognized by the presence of a hole on the flower bud or the floral parts, representing oviposition sites. The larva may emerge in the bud stage itself and consume internal parts of the bud or may emerge during the later stages of fruit development and consume seeds.

12.1.2 Pollination Limitation

As pointed out in Chap. 7, pollination is a prerequisite for seed set. Pollination limitation (reduced seed set as a result of inadequate pollination) is a major constraint for optimal seed production (*see* Protocol 7.7.6 for the estimation of pollination limitation). A great majority of plants depend on biotic pollinators. Adequate pollination is essential for the production of sufficient number of seeds for recruitment of new individuals. Pollination limitation is widespread (Burd 1994; Larson and Barrett 2000; Wilcock and Neiland 2002; Knight et al. 2005) and may often act as a driving force in making a species vulnerable. This is more prevalent in self-incompatible and dioecious species as the seed set in these species is dependent on the efficacy of interplant cross-pollination.

Anthropogenic threats to pollinators in their natural habitats and their impact on sustainability of biodiversity have been highlighted by many investigators (*see* Potts et al. 2010; Obutu 2010). Several recent studies have shown a significant decline in the density and diversity of pollinators (Biesmeijer et al. 2006; Potts et al. 2010; Burkle et al. 2013; Tylianakis 2013; Garibaldi et al. 2013). Based on the national entomological databases for native bees and hover flies of Britain and the Netherlands before and after 1980, a decline by around 30 % in species richness of these insects in both the countries has been reported (Biesmeijer et al. 2006). Interestingly, many of the plant species pollinated by these insects have also shown a parallel decline. Plant species that were obligately dependent on declining insect pollinators have shown a decline, but those dependent on abiotic pollination have shown an increase; self-pollinating plant species have shown an intermediate response. Similarly, the relative abundance of several species of bumblebees in the USA also has shown a decline by up to 96 %, and their geographic ranges have contracted by 23–87 % (Cameron et al. 2011). Pollination limitation is expected to be more acute in tropical rain forests which are characterized by low density of conspecific plants and higher incidence of dioecy and self-incompatibility (Bawa and Opler 1975; Wilcock and Neiland 2002). Even wind-pollinated species may also show pollination limitation when the density of plant population becomes low. In response to worldwide decline in pollinators' diversity and density, the 'Conference on Parties to the Convention of Biological Diversity' has established an International Initiative for the Conservation and Management of Pollinators (Anonymous 2002).

In India also, there are some indirect evidences to indicate that pollination limitation may be a factor for low recruitment in some species in fragmented forests. In Pondicherry region of Southern India, Nayak and Davidar (2010) studied the effects of pollination limitation on reproductive success of ten species covering both self-compatible and self-incompatible species. They recorded a higher level of pollination limitation in self-incompatible species leading to a lower fruit set than in the self-compatible species. This is obviously because self-compatible species are less dependent on pollinators than the self-incompatible ones. Further, the densities of sapling and adult plants of self-compatible species in the fragmented habitats were significantly greater than those of the self-incompatible species indicating that lower fruit set as a result of pollination limitation would lead to lower rate of recruitment.

12.1.3 Pre-dispersal Seed Predation

Pre-dispersal seed predation is a major constraint for seed production. The fruits are predated by a range of insects such as weevils, beetles, bruchids, scolytids and Lepidopteran caterpillars. The damage

to fruits/seeds by insect predators is quite severe and may reach >90 % (Toy et al. 1992; Fenner and Thompson 2005). Insects lay their eggs in the flower buds/flowers/young fruits, and their larvae, after emergence, feed on the developing seed. Many of the predated fruits often abscise before maturity. Birds and arboreal mammals also consume a considerable number of seeds before dispersal. The seeds are reported to form up to 70 % of the diet of scaly headed parrot in Southern Brazil (Galletti 1993). The rate of seed predation varies in time and space, and site-specific factors determine the density and diversity of seed predators (Kolb et al. 2007).

Pre-dispersal seed predation may often act as a driving force for rarity of the species as the seeds of some rare species are more susceptible to insect damage (Hegazy and Eesa 1991; Combs et al. 2011) when compared to species that are more common. For example, seed predation in a rare endemic, *Astragalus sinuatus,* has been shown to be significantly higher than that in two sympatric common congeners (*A. leibergii* and *A. purshii*) in a semideciduous forest (Combs et al. 2011, 2013).

12.1.4 Abortion of Ovules and Developing Fruits

Many ovules in multiovulate species abort as they do not get fertilized. Many of the fruits containing developing seeds also abort at different stages of development due to genetic defects or resource limitation (*see* Fenner and Thompson 2005). Abiotic stresses particularly of moisture and temperature also induce abscission of developing fruits.

12.2 Constraint for Seed Dispersal

Janzen–Connell Model: This model explains the features of recruitment in tropical forests. High diversity of tree species and long distances between conspecific adults are characteristic features of tropical forests. Density- and distance-dependent mortality has been put forward as a mechanism to explain the coexistence of many tree species in the tropical rain forests; this is generally referred to as the Janzen–Connell model (based on the authors who independently proposed the hypothesis – Janzen 1970 for seeds and Connell 1971 for seedlings). Subsequently, seedling mortality due to fungal pathogens was also shown to act in a density- and distance-dependent manner (Augspurger 1983). Seed dispersal is a determining factor in maintaining high tree species diversity in tropical forests. Seed density declines rapidly with increasing distance from the parent. The mortality of seeds and seedlings due to predation and competition from parents or siblings is the greatest near the maternal parent. The mortality factors decrease away from the parents, and seedling survival increases with dispersal distance. A low recruitment near the parent reduces the potential for single species dominance in the community and thus maintains the high tree species diversity. Although the model works to some extent, a number of other interacting mechanisms also seem to determine the diversity in tropical forests (Howe and Smallwood 1982; Bustamante and Simonetti 2000; Burkey 1994; Turner 2001; Fenner and Thompson 2005; Hansen et al. 2008; Ruiz 2008; Clark 2009; Bagchi et al. 2014).

Seed dispersal confers several advantages to plants (*see* Chap. 11). In the absence of effective seed dispersal, a large proportion of seeds fall below the canopy and are subjected to density-dependent mortality factors. Animals are the major seed dispersers. About 70 % of the species are dispersed by animals in tropical forests (Howe 1990; Tadwalkar et al. 2012). Scarcity of seed dispersers is the main causative factor for ineffective dispersal. Anthropogenic disturbances as a result of direct persecution of vertebrate seed dispersers, change in land use and fragmentation have greatly reduced animal dispersers (*see* Anonymous 2007; Wright et al. 2007; Corlett 2007). Direct persecution is through hunting for their meat, hide, medicinal/ornamental value or as game species; some vertebrates are also hunted as they are agricultural pests/predators of domestic animals. The collection of live animals for pet trade may also reduce the populations of dispersers. Large birds, large fruit bats, primates, civets and terrestrial frugivores, which are the major dispersers

in large-fruited plant species, are subjected to more severe human persecution. Some of them have been almost eliminated from many of their natural ranges.

The consequences of hunting on recruitment success of the species are considerable (*see* Anonymous 2007). Several investigators have documented a decrease in seed dispersal in hunted habitats, when compared to habitats protected from hunting in Neotropical forests (Wang et al. 2007; Beckman and Muller-Landau 2007). In Cameroon, studies on seed dispersal of a mammal-dispersed species, *Antrocaryon klaineanum* (Anacardiaceae), showed that only 2 % of the seeds got dispersed in heavily hunted forest, while it was 42 % in protected forest (Wang et al. 2007). In another study in Central Panama (Beckman and Muller-Landau 2007), a significant reduction in seed removal has been reported in two other species, *Oenocarpus mapora* (Arecaceae) and *Cordia bicolor* (Boraginaceae) in hunted forests when compared to protected forests. Interestingly, a dramatic increase in lianas, whose seeds are largely dispersed by wind, has been recorded in heavily hunted forests as a consequence of reduced seed dispersal by vertebrate-dispersed species (Wright et al. 2007). In Southeastern Peru also, hunting has reduced large- and medium-size primates by >80 % leading to a marked reduction in the dispersal of large-seeded trees when compared to protected forests (Nunez-Iturri and Howe 2007). Consequently, the density of tree species dispersed by these animals has reduced by 55 % in hunted sites, while the density of abiotically dispersed species and those dispersed by non-game animals has increased.

In India also, there are some evidences to indicate the effect of habitat disturbances on animal-dispersed plant species. Tadwalkar et al. (2012) reported that the density of plants of animal-dispersed species in disturbed areas in northern Western Ghats is less when compared to anemochorous and autochorous individuals. They categorized the Cumulative Disturbance Index (CDI) of the study area from 1 (lowest) to 13 (highest). As the CDI increased, the proportion of zoochorous individuals decreased, whereas the number of anemochorous and autochorous individuals did not show any significant pattern. This is apparently the result of dispersal limitation since animal dispersers are more severely affected by forest fragmentation and other disturbances.

12.3 Constraints for Seed Germination

A majority of the dispersed seeds as well as those fallen below the parent are subjected to post-dispersal hazards – predation by various granivores particularly rodents and insects, infestation by soil bacteria and fungi and loss of viability through natural aging process.

12.3.1 Post-dispersal Seed Predation

Rodents and insects are the major post-dispersal seed predators (Figs. 12.1 and 12.2). In riverine species, even slugs, crabs and fishes can cause predation to a limited extent. The extent of seed predation varies greatly between species, locations and years. In species which show mast seeding (production of synchronized bumper crops in some years but little or no crop in the intervening years), seedling survival is high during mast years since the predators are satiated allowing at least a proportion of seeds to escape predation (Janzen 1971, 1974; Nakagawa et al. 2005). There is hardly any recruitment of seedlings in the lean years since most of the seeds are consumed by the predators. Cache-hoarding and scatter-hoarding animals act as seed dispersers as well as predators. Most of the hoarded seeds are eaten, and only a small proportion of them survive. Apart from animal predators, dispersed seeds are attacked by soil microorganisms. Forest soil is rich in fungi and bacteria; they infest seeds deposited on the soil and result in seed mortality.

12.3.2 Lack of Safe Sites for Seed Germination

Most of the dispersed seeds may not be able to germinate because of lack of safe sites. Safe sites

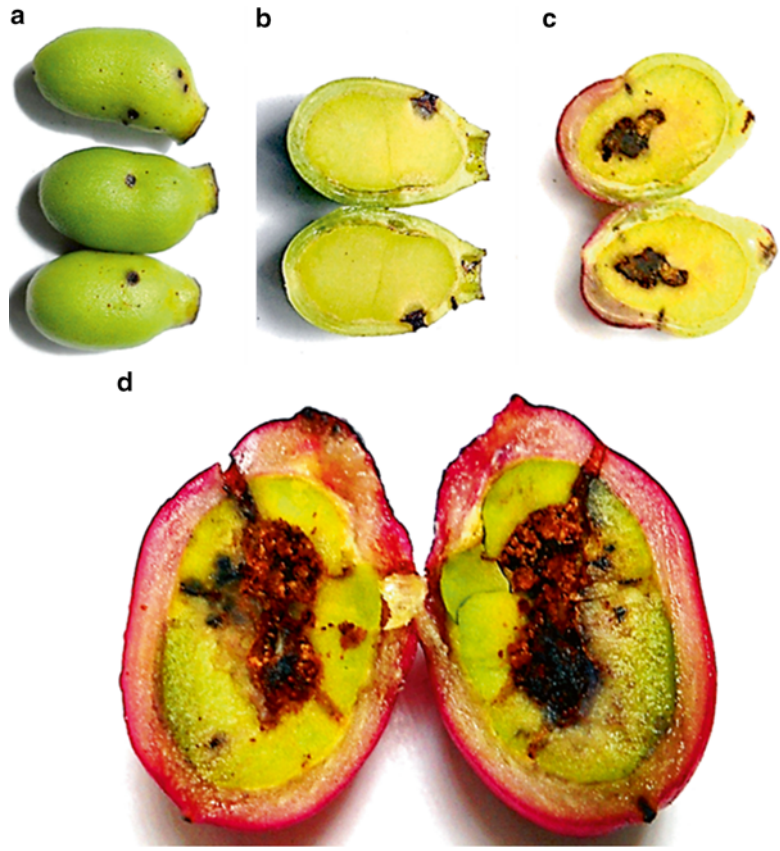

Fig. 12.1 Insect seed predation in *Syzygium cumini*. (**a**) Infested immature fruits with black spots representing oviposition sites. (**b**–**d**) Predated fruits at different stages of development cut open longitudinally to show infested parts. (**b**) Young fruit; the infestation is confined to fruit wall. (**c**) Slightly older fruit in which the central part of the embryo is infested. (**d**) Ripened fruit in which most of the embryonal part is infested

are places where the requirements for seed germination and seedling establishment are fulfilled. Generally, only a very small proportion of seeds produced by a plant are carried to locations suitable for seed germination and seedling establishment. The seeds that do not fall in safe sites do not germinate; they may get predated or lose viability over time. One of the major limitations in forests with closed canopy is lack of light gaps for germination of light-sensitive seeds (*see* Ghazoul and Sheil 2010).

12.3.3 Loss of Seed Viability

Even those seeds that escape predation or infestation by microbes in the soil seed bank may not be able to germinate because of the loss of viability through natural process of aging (see Fenner and Thompson 2005). As seeds present in the soil are subjected to frequent wetting and drying cycles as well as fluctuations in temperature, loss of viability tends to be faster in the soil when compared to those stored in the laboratory or low-temperature seed banks. Tropical soil seed banks are generally smaller than those in temperate forests (Skoglund 1992); the former are also subjected to higher levels of seed mortality due to higher rate of predation. Often seeds may germinate in ephemeral conditions such as occasional rains in dry season that induce seed germination but cannot sustain seedling growth.

The period for which seeds remain viable in the soil seed bank varies greatly, from a few

Fig. 12.2 Fruit predation in *Terminalia bellirica* (*after* Sinu 2012). (**a**) Cross section of the fruit to show a late instar weevil larva (*arrow*) consuming the kernel part of a fruit. (**b**) Some fruits predated by Malabar giant squirrel. Irregular teeth marks on the fruit wall are evident. (**c**) Three fruits predated by Malabar spiny dormouse. Fruits show finely cut, large orifice. (**d**) A cache of consumed seeds by a rat species

weeks to several years. This depends largely on intrinsic nature of the seeds and prevailing physical conditions of the soil. Recalcitrant seeds do not form soil seed bank as they lose viability within a few weeks after dispersal. Recalcitrant seeds are generally larger and are dispersed when the soil moisture is suitable for seed germination and seedling establishment. Because of the presence of sufficient amount of nutrients in the seed, such seedlings can survive for longer periods even when the light conditions are not favourable for photosynthesis. As a large number of seeds of recalcitrant species germinate soon after dispersal, they form a blanket of seedlings which are capable of surviving for considerable time; these are often referred to as 'seedling bank'. Orthodox seeds retain viability for longer periods and contribute to soil seed bank. Specific favourable environmental cues trigger germination of dormant seeds and thus maximize seedling establishment. Although we have extensive data on seed viability and storage of cultivated species, there is very little information on species growing in the wild particularly in tropical forests. For seedling recruitment success, viability of soil seed bank is more important than their viability under laboratory conditions.

12.4 Constraints for Seedling Establishment

Seedling establishment that culminates in the recruitment of new adult individual is the final hurdle in a series of events starting from seed

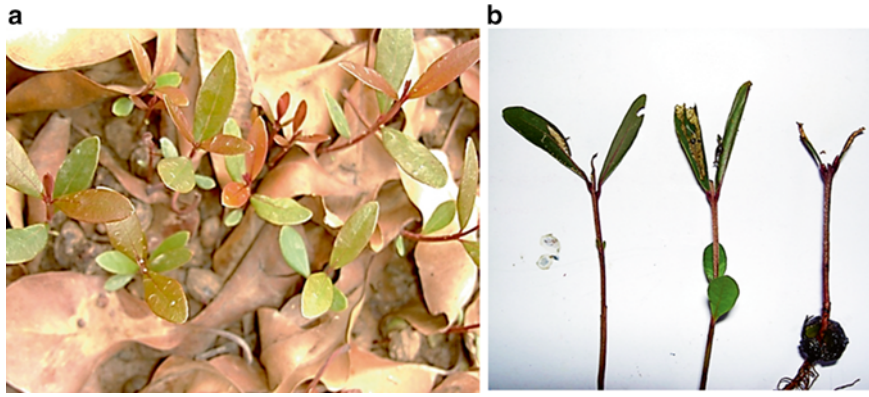

Fig. 12.3 Seedling predation by insects. (**a**) A group of young healthy seedlings that have come up below the canopy. (**b**) Some of the infested seedlings in which the shoot tip has been consumed

production. Similar to the hazards faced by the seeds, the seedlings also face additional set of hazards. Seedling phase is considered to start following the emergence of the radicle that grows into the soil as the root and the plumule that grows away from the soil towards the light as shoot.

Plant recruitment is generally described in terms of seedlings developing into saplings and then into young adults. The use of the terms seedling and sapling is not consistent in the literature. According to some (Rey and Alcantara 2000), newly emerged plants still bearing the cotyledons/cotyledonary leaves are considered as seedlings. Thus, the seedling stage extends until seed reserves are exhausted (Hanley et al. 2004). Several others fix the cut-off point between seedling and sapling based on height. For example, Tang and Ohsawa (2002) considered plantlets <0.5 m tall as seedlings and those measuring 0.5–1.3 m as saplings, while Teegalapally et al. (2010) have considered plantlets up to 25 cm tall as seedlings and those between 25 and 100 cm as saplings. In a broad sense, many investigators consider young plants that have acquired vegetative adult characters about one year after germination as saplings. Here, no distinction is made between seedlings and saplings; the description refers to the growth of seedlings to young adults as a continuous process. The following are the major constraints in the growth of seedlings into young adults.

12.4.1 Herbivory

The predation of seedlings by vertebrates and infestation by insects (Fig. 12.3a, b) and pathogens are the major biotic seedling mortality factors (Coley and Barone 1996). Of these, insects are the most important herbivores in tropical forests (Fig. 12.3). As nutritional quality of young leaves is higher and also they lack any defence mechanism, most of the herbivores attack leaves while it is expanding (Kursar and Coley 2003). Thus younger seedlings are most susceptible to herbivory. As the seedlings grow, they develop morphological and/or chemical defences, and thus the intensity of herbivory is reduced (*see* Ghazoul and Sheil 2010). As seedlings of some species are more palatable to specific herbivores, such differential herbivory affects relative abundance of species in the community

In some forests vertebrates are important herbivores. Dirzo and Miranda (1990) compared seedling predation in a defaunated (absence or low abundance of floor dwelling and arboreal mammals) site with a site that harbours normal vertebrate population in the forests of Southern Mexico. The site with normal vertebrate population showed vertebrate damage of 29 % of seedlings and 30 % of the saplings, whereas none of the plants were damaged in defaunated site. Also the latter site showed 2.33 times higher seedling density than that in the former indicating the role of folivorous vertebrates in predation.

12.4.2 Competition and Availability of Resources

Competition from other seedlings and surrounding vegetation for resources particularly light, water and soil nutrients is one of the constraints for seedling establishment and its further growth (Gross 1980; Taylor and Aarssen 1989; Fenner and Thompson 2005). Many of the sites suitable for seed germination may not be suitable for further growth of the seedlings and their establishment. In seasonal forests, where wet and dry seasons alternate, unseasonal rains during the dry season may induce seed germination; such seedlings die as ephemeral conditions cannot sustain seedling growth (Skoglund 1992).

In forests with closed canopy, light is a major constraint (Hubbell et al. 1999; King et al. 2005), and the establishment of seedlings requires the availability of gaps in the canopy (Harper 1977). Light limitation markedly reduces the seedling establishment particularly of tree species (Lorimer et al. 1994). The gaps in the forests result largely as a consequence of abiotic causes such as falling of branches or whole trees, floods, fires and landslides. These gaps permit light to reach the ground and provide opportunities for seedling recruitment. Pioneer species (initial colonizers of disturbed or damaged areas facilitating ecological succession) generally produce smaller seeds with prolonged dormancy. The seedlings of pioneer species require light throughout their growth. They can exploit favourable conditions more competitively than climax species (those that remain stable in terms of species composition until the ecosystem becomes disturbed) and show faster growth rate. Climax species generally produce large seeds; they can germinate and the seedlings can persist in shade with very little growth, often for years. They grow rapidly when light becomes available and overtake the pioneers (Turner 1990; Leishman and Westoby 1994; Osunkoya et al. 1994; Hammond and Brown 1995; Fenner and Thompson 2005; Ghazoul and Sheil 2010). The creation of gaps itself causes considerable physical damage to seedlings and young plants (Clark and Clark 1989; Peters et al. 2004). Cyclonic storms and hurricanes also cause considerable damage in coastal forests not only to the trees but also to the regenerating seedlings and young plants at the ground level. Drought is another abiotic stress that affects the survival of the seedlings, particularly in seasonal forests in which dry season intervenes between rainy seasons (Delissio and Primack 2003).

12.5 Climate Change

Climate change is another important factor that affects reproductive success of the species. A shift in the phenological events and migration of species to higher latitudes and altitudes are two of the important consequences of climate change.

12.5.1 Uncoupling of Phenological Adaptations

One of the major consequences of climate change that affects reproductive success of the species is a change in the timing of phenological events. A shift in flowering phenology as a result of climate change has been recorded by several investigators (Hepper 2003; Bharali and Khan 2011; Dunnell and Travers 2011; Allen et al. 2013). Such changes, particularly in the timing of flowering and fruiting, may lead to uncoupling of phenological adaptations of the species to the prevailing climate over thousands of years. Flowering generally coincides with the availability of pollinators and fruiting with the availability of seed dispersers and onset of suitable conditions for seed germination and seedling establishment. For example, seeds of a large number of species in the Western Ghats are shed during the pre-monsoon or monsoon period (Tadwalkar et al. 2012); they germinate soon after the onset of monsoon rains, and their seedlings are established before the onset of post-monsoon drier months. Seeds of many of these species are refractory and loose viability within weeks after shedding. If they miss the monsoon season, there is zero recruitment for that year. Similarly, most of the wind-dispersed species shed their seeds in the drier months before the onset of monsoon

rains so that the prevailing drier conditions facilitate dispersal of their seeds (Aravind et al. 2013); subsequent wet monsoon season facilitates seed germination and seedling establishment. Any change in the timing of fruiting in such species as the result of climate change would uncouple this dispersal adaptation and may lead to serious recruitment constraint.

12.5.2 Species Migration

Climate change is the main driver for migration of species towards higher altitudes and latitudes (Parmesan 1996; Parmesan et al. 1999; Dunnell and Travers 2011; van der Putten et al. 2010). All species do not migrate at the same rate and time; species shift individually. This results in community reorganization affecting its composition (van der Putten 2012). These alterations affect ecosystem processes and services. Unless the pollinators also move with the plant species, pollination may become a serious limitation. The migration may affect species abundance; depending on the extent of predation, competition and availability of resources, pollinators and seed dispersers, the species may become rare or disproportionately abundant (van der Putten 2012).

Climate change may also alter weather pattern, particularly the frequency and intensity of rains. Severe drought caused by El Nino in Lambir Hills National Park in Sarawak, Borneo, during 1998 and its impact is one of the well-investigated examples of climate change. Drought induced defoliation of many fig species; some of them are endemic, and several dioecious figs failed to initiate new syconia which are necessary to sustain specific pollinators of these species; even pollinated syconia failed to develop into fruits (Harrison 2000). With the result, pollinators of such fig species became locally extinct. As the fig fruits are important resources for vertebrate fruit dispersers, this is likely to have important effects on fruit dispersers. Similarly, this severe drought reduced seedling recruitment when compared to non-drought years in these forests (Delissio and Primack 2003). When such droughts increase in frequency and intensity, they affect natural regeneration of the forests and lead to a change in their composition.

12.6 Raising of Plantlets in the Nursery

Afforestation and species-specific conservation programmes follow direct seeding or transplantation of seedlings to natural habitat. Direct seeding is generally not effective in drier habitats due to lack of safe sites for seed germination and early growth of the seedlings. In such areas, rising of plantlets in the nursery until they get established before their transplantation to the forest is more effective than direct seeding. This requires raising of a large number of seedlings in the nursery. This facilitates availability of proper conditions during critical stages of germination and growth of seedlings.

Seed beds for raising seedlings should be located preferably on a well-drained soil with suitable light conditions that permit good seedling growth. Seeds should be sown in rows in seed beds. The distance between seeds and the depth of sowing depends on the species and the period for which the seedlings have to be maintained in seed beds before transplantation. Seed beds should be watered carefully and optimally; overwatering increases seedling susceptibility to damping off diseases. A shading material over the beds may be needed to protect tender seedlings. However, excessive shading makes the plants etiolated. Similarly, overexposure to sun and also water deficiency leads to wilting of plants. The seedlings may remain in the bed for a few weeks or up to 1–3 years depending on the species and the requirement.

Plants raised in seed beds are either transplanted to permanent locations or to another experimental plot or pot before transferring them to the field. Root branching increases in the transplanted plants leading to the development of robust plants. The success of transplantation depends on the amount of root system retained during transplantation. It is better to retain as much soil around the roots as possible and to cause minimum disturbance to the root system

during transplantation. For transplantation, a planting hole of the required size is made using a suitable implement/machine, and the root system is inserted in the hole. The soil around the plant is firmly pressed and thoroughly watered immediately after transplantation.

When plants have to be transplanted directly to the field from the nursery bed, it is better to subject them to gradual hardening in the nursery bed before transplantation. Hardening is generally done by withholding the supply of water for about a week or two before the seedlings are taken out of the nursery. The leaves of hardened plants are deeper green and smaller than those of unhardened plants.

Seedlings can also be raised in plastic bags (polybags) of different sizes available in commercial nurseries. The size of the bag depends on the size of the seedlings and the period for which they have to remain in the bag. The polybags are generally filled with a mixture of soil, farmland manure and sand (2:1:1), and seeds are sown, generally one per bag, and watered. A few holes need to be pierced at the bottom of the bag for drainage of excess water. The seedlings raised in polybags do not require additional transplantation to nursery beds; seedlings can be maintained in polybags until they are transplanted to permanent locations.

12.7 Protocols

It is rather difficult to present protocols on seed and seedling predation, recruitment and regeneration pattern. The approaches used to study these aspects and the protocols used by different investigators are highly variable. The type of sampling units laid, their size and placement and other details are largely dependent on the objectives of the investigation and the type of questions asked by the investigators. The protocols given below are only for guidance. The researchers have to go through original papers in the field to become familiar with the details of different protocols described and to select or modify the most suitable protocol for his/her studies.

12.7.1 Estimation of Predation of Flower Buds and Flowers by Insects

Predation of flower buds and flowers by several herbivores and insects is one of the constraints for seedling recruitment. Grazing animals consume whole flowers/inflorescences/branches particularly in herbs and shrubs and can be easily observed and identified. It is more difficult to identify insect infestation and needs careful studies.

12.7.1.1 Special Requirements

Hand lens
Sharp blade/razor

12.7.1.2 Procedure

1. Carefully observe a considerable number of flower buds, and open flowers selected randomly from different plants for infestation by generalized insects/caterpillars, which usually consume the floral parts, and record their number. Identify the insect(s). If it cannot be identified in the field, collect and preserve them as morphospecies for later identification. If the larvae are present, collect such infested buds/flowers, and maintain them in a container under room conditions until the adult insect emerges. Identify the insect.
2. Observe flowers and flower buds for infestation by specialized insects by carefully looking for the presence of oviposition holes or any other symptoms on floral parts that indicates infestation. Record such buds and flowers.
3. If the infestation symptoms are not clear, cut open the bud/flower and look for the presence of larvae or brown degenerated tissue which generally indicates infestation. If larvae are present, collect infested buds/flowers, and maintain them in a container under room conditions until the adult insect emerges. Identify the insect. Also record whether the infestation of the flower buds and flowers in the studied species is by one insect species or several species.
4. Calculate the percentage of flower buds and flowers predated by generalized and specialized insects.

12.7.2 Estimation of Pre-dispersal Seed Predation

Pre-dispersal seed predation occurs by the consumption of fruits on the plant by frugivorous vertebrates such as birds, monkeys, rodents and squirrels and infestation by insects. The consumption of fruits by vertebrates can be easily observed and recorded. Often they remove the fruits from the plant and carry them for hoarding; this has been referred to as seed removal rather than predation. Insect infestation is generally initiated by oviposition at the flower bud and/or flower stage. When their larvae emerge during fruit development, they consume parts of fruits and/or seeds.

12.7.2.1 Special Requirements

Hand lens
Sharp blade/razor

12.7.2.2 Procedure

1. Collect mature/ripened fruits randomly (when they are still on the plant) from different parts of the plant and from different plants.
2. Observe each fruit carefully for any symptoms indicating insect infestation. Often it is in the form of an oviposition site with a dark mark or a hole on the fruit or decoloration of the fruit wall. Use a hand lens, if necessary, for smaller fruits. The hole may also be formed by the exiting insects that completed their larval phase inside the fruits. If infestation of the fruits cannot be recognized externally, fruits have to be cut open to score for predation. Infested parts generally appear black/brown and may show the presence of larvae. Separate healthy fruits from infected fruits and count them.
3. Observe the extent of predation in fruits that were cut (under step 2). Cut open even those fruits that show external symptoms of infestation to observe the extent of predation. Give particular attention to record whether the damage is confined only to the fruit part or has extended to the embryo also. Separate the seeds in which infestation is confined only to the fruit wall and those in which embryo part is also damaged (Fig. 12.1).
4. Calculate the percentage of fruits in which the infestation is confined to the fruit wall and those in which embryo part is also damaged. Many of the infested fruits, in which the infestation is confined to the fruit part, may be able to germinate and produce normal seedlings. The fruits in which infestation extends to the embryo generally fail to germinate.

12.7.2.3 Modifications

1. Freshly fallen fruits can also be collected and used to estimate pre-dispersal predation. As fruits continue to fall below the ground over a period of time particularly in tree species, fruit samples collected from the ground would contain older as well as fresh fruits; it becomes difficult to separate fresh fruits from older fruits. In such studies, collection of fresh fruits is facilitated by laying quadrats of suitable size below the canopy. Old fruits are cleared from the quadrats, and fresh fruits fallen inside the quadrats are collected every day and studied for predation. Some investigators have spread a net or a cloth at about 1 m above the ground level to collect freshly fallen fruits on the net/cloth each day to study seed predation.
2. Predation of seeds by small vertebrates can also be studied by setting up camera traps.

12.7.3 Estimation of Pre-dispersal Insect Predation by Using an Insecticide

Some investigators (Louda 1982; Louda and Potvin 1995; Tsvuura et al. 2011) have used an insecticide to study pre-dispersal fruit and seed predation by insects. This requires some preliminary investigations to identify suitable insecticide, its concentration and frequency of application.

12.7.3.1 Special Requirements

Suitable insecticide available in the market
Hand sprayer
Tags

12.7.3.2 Procedure

1. Select a suitable number of plants (herbs) or inflorescences or branches (in large shrubs and tree species) from different plants and label them. Record the number of flower buds in each selected plant/inflorescence. Remove already opened flowers and young fruits, if any, in the selected plants/inflorescences/branches. Divide the samples into three sets of equal numbers and label them.
2. Spray one set with the insecticide and the second set with water, and leave the third set without any spray. If the insecticide used is effective, it should not permit any insect infestation in insecticide-treated flowers.
3. Repeat respective sprays at suitable intervals, depending on the insecticide, to prevent any later insect infestation in insecticide-sprayed set.
4. After the fruits have matured but before their dispersal, count the number of fruits developed from each treatment.
5. Collect mature fruits from each set separately and count the number fruits infested in each set. Cut open the infested fruits and record the extent of infestation (confined only to fruit wall and/or extended to the embryo).
6. Identify the insect by maintaining some infested fruits in the laboratory until the emergence of the adult. If there are more than one insect infecting the fruits, record the number of fruits infested with each insect.
7. Calculate per cent fruits infested from each set. There should be no significant differences between the control and water-treated sets. The difference between insecticide-treated set and the controls would indicate the efficacy of insecticide used in preventing infestation.

12.7.4 Estimation of Post-dispersal Seed Predation

Fruits and seeds are predated by vertebrates and a range of insects even after they fall on the ground beneath the plant or after they are dispersed away from the plant. Many of the seeds are also destroyed by pathogens particularly in humid habitats. The seeds to be studied for predation need to be marked to follow their fate. Investigators have used several methods to mark the seeds inside the sampling stations to keep track of seed removal/predation/germination:

1. Marking a small area of each seed with a permanent ink or paint
2. Gluing of each seed on to a plastic mesh and placing it on the ground
3. Tying one end of a thread to each seed and the other end of the thread to a small peg placed inconspicuously a little distance away

In the above methods, the fate of removed seeds cannot be studied; such seeds are generally considered as removed. By using the following methods, the fate of seeds can be studied (predated or secondarily dispersed):

1. A part of the seed is covered with aluminium foil before the onset of the study; the seeds removed are subsequently located with the use of a metal detector (Sork 1987).
2. Seeds are tagged by drilling a tiny hole in the seed (in such a way that it does not damage the embryo) through which a thin thread is inserted and tied; the other end of the thread is tied to a small, light, tin tag. Each tag can be numbered. When the dispersal agents carry the seed, the tag also moves along the seed and generally remains on the ground; the removed seed can be located by the tag (Xiao et al. 2005).
3. A bobbin intact with rolled nylon thread is used. The terminal part of a nylon thread is glued to the seed with superglue and the bobbin pegged to the forest floor with a toothpick. When the seed is removed, the bobbin rolls and allows the thread to be extended as the seed is carried away. Each seed can be labelled with a specific number marked on the bobbin (Ruiz 2008).

12.7.4.1 Special Requirements

Hand lens
Materials to lay quadrats
Required items to mark seeds by using any of the above methods

12.7.4.2 Procedure

1. Lay down quadrats of suitable size randomly. Generally, 10–12 % of the total area of the canopy is covered by quadrats. The pattern for laying quadrats depends on the objectives. For example, if the objective is to study the density- and distance-dependent predation, isolated trees have to be selected and the quadrats have to be laid starting near the trunk of the parent and extending at specific distances away from the trunk.
2. Cover the quadrats by erecting a cloth cover about 0.5 m above each quadrat to prevent new seeds falling into the quadrats.
3. Count all the fruits/seeds inside the quadrats and mark them using any one of the methods described above.
4. Keep all the fruits/seeds under observation at regular intervals. Observation frequency depends on the severity of predation. During each observation, record the state of each seed for predation or its removal. Depending on the method used to mark the seeds, those removed are generally scored as predated or their fate (predated or secondarily dispersed) is followed and recorded. If possible identify predating animals. If vertebrates are the predators, often the dental marks left on fruit wall/seed coat of the predated seeds are characteristic of specific vertebrate species (Fig. 12.2) and thus help in identifying the predator. The use of rodent traps baited with fruits/seeds of the focal species is another effective method to identify vertebrate predators. If insects are the major causes of seed destruction, follow the procedure described in Protocol 12.7.2, to record the infested seeds and the extent of infestation.
5. Calculate per cent of seeds predated.

12.7.4.3 Modifications

1. Relative importance of vertebrates vs. non-vertebrates in seed predation can be studied by enclosing half the number of seed quadrats with enclosures of suitable mesh size (depending on the prevailing predators) to exclude vertebrates. The other half is left without enclosures for recording both vertebrate and non-vertebrate predation.
2. Observations on seeds in the quadrats may be continued to study seed germination. At the end of the observation period, the percentage of predated, ungerminated (loss of viability) and germinated seeds is calculated. These studies can further be extended to study seedling predation (*see* Protocol 12.7.6) in these quadrats.

12.7.5 Estimation of Predation of Experimentally Placed Seeds

Many investigators have studied predation, germination and seedling establishment in seeds placed at seed stations. These studies are largely aimed to check the applicability of Janzen–Connell density- and distance-dependent model on seed and seedling mortality. Seed stations are placed in various designs. The most common method is to place seed stations at various distances from the tree trunk in concentric circles from the parent tree. Before the distance gradient for the seed station is determined, it would be useful to study the natural dispersal distances for the focal plant species. Suppose if seeds of a given plant are naturally dispersed only up to about 10 m from the parent tree/population, the seed stations are generally set up within that distance. Since density of dispersed seeds is an important factor determining seed loss to post-dispersal predators, it may be better to establish seed stations with different seed numbers along the distance. Ideally, the number of seeds used at seed stations should be based on the numbers normally found dispersed at each distance.

12.7.5.1 Special Requirements

Materials to lay quadrats

12.7.5.2 Procedure

1. Lay down a suitable number of quadrats of equal size at various distances from the tree trunk. The number and size of the quadrats

depend on the dispersal features of the seeds of the focal species.
2. Place sufficient number of seeds in the quadrats. Some investigators have placed single seeds in circles or along transects, spaced 5 m apart for various distances from the parent. If necessary, each seed in seed stations may be labelled using any of the methods mentioned in Protocol 12.7.4.
3. Observe seeds in each seed station at regular intervals, depending on the species, for predation. During each observation, record the number of seeds removed, eaten, untouched or infected by pathogens.
4. Calculate the percentage of seeds predated by various insects.

12.7.5.3 Modifications

1. Relative importance of vertebrates vs. non-vertebrates in seed predation.
   In most of the investigations, half of the seed lots placed at comparable distances are covered with enclosures of suitable mesh size to exclude vertebrates. The other half is left exposed for recording total predation.
2. Observations of seeds may be extended to study seed germination and seedling predation in experimentally placed seeds. Refer to Protocol 12.7.6 for additional steps.

12.7.6 Estimation of Seedling Mortality

Seedling mortality may start from the first day of seed germination. Ants are important predators of cotyledons, shoots and roots in the initial days of seed germination. Terrestrial crabs also cut the growing shoots in young seedlings. Leaf miners (Agromyzidae) and aphids are important herbivores of tender leaves. Membracids and leaf hoppers are the herbivores of tender shoots. Some plants are susceptible to specialized insect herbivory at the later phases of seedling establishment.

12.7.6.1 Special Requirements

Materials for raising quadrats

12.7.6.2 Procedure

1. Lay down the required number of quadrats of suitable size (generally 1 m^2) in the pattern needed for specific objective of the study.
2. Record the number of seeds in each quadrat. Mark the seeds with a paint or some other means (*see* Protocol 12.7.4). This would enable the identification of new seeds that may fall in the quadrats after starting observations; remove such seeds during each observation.
3. Observe seed samples at suitable intervals. Record the number of seeds removed, predated and those in healthy condition. Seeds of some species may be dormant and may not germinate until they complete dormancy period. For such seeds, observations have to be continued for longer period until they germinate/die.
4. After the seeds have germinated, record the number of seedlings and label each of them. Keep track of their growth at suitable intervals in terms of their height, number of nodes or leaves and at later stages the diameter of the main stem at collar level (just above the ground) or at breast height (DBH). Initially, the observations have to be at more frequent intervals.
5. Simultaneously, keep track of predated/infested seedlings. Record the stage of seedling at predation. Vertebrate herbivores may defoliate the seedlings or remove them. When the seedlings recorded earlier are missing, record them as removed. If the seedlings are infested with insects, record the insect species, the part of the seedling infested and the intensity of infestation. If the predator cannot be identified in the field, consider them as morphospecies and preserve them for later identification.
6. Continue the observation of plantlets at less frequent intervals, and keep record of their mortality and growth record (height, number of leaves, DBH, etc.) of surviving seedlings. If new seedlings come up during observation period, record and label them and monitor their growth/mortality.

7. Follow the growth of surviving plants for 1–3 years depending on the objective of the study.
8. Calculate the percentage of seeds and seedlings predated and those that survived until the end of the observation period.

12.7.6.3 Modifications

1. Predation by vertebrates and non-vertebrates can be studied by covering half of the quadrats with enclosures of suitable mesh size to prevent vertebrate predation.
2. If the studies are aimed at estimating only seedling mortality, study quadrats may be laid down on sites on which the seedlings have started emerging from the seeds. They are numbered and their mortality and survival monitored, instead of starting at the seed stage.

12.7.7 Estimation of Predation of Experimentally Placed Seedlings

Fresh seeds/germinating seeds/seedlings of known age are placed in suitable design and kept under observation. This protocol is also used to analyse the distance-dependent mortality of seedlings in relation to the parent.

12.7.7.1 Special Requirements

Materials needed to lay quadrats or establish seedling stations in any desired pattern

12.7.7.2 Procedure

1. Raise sufficient number of young seedlings in the nursery beds or under any other suitable conditions (germination trays, sand beds, polybags, etc.). The number of seedlings required depends on the situation and other logistics.
2. Place the seedlings of specific age in predetermined places in suitable designs. Some investigators have placed them in circles marked with flags or in quadrats. The number of seedlings placed is also variable according to the objective of the study (about 30–50 seedlings in each circle/quadrat). Some investigators have transplanted the seedlings of specific age to the study sites and monitored their mortality/survival (Broncano et al. 1998). In such studies, the seedlings that died within a few days of transplantation as a result of transplantation injury were replaced with fresh seedlings of the same age.
3. Follow steps 4–8 of Protocol 12.7.6 on seedlings and record their predation/survival.

12.7.8 Analysis of Regeneration Pattern

The analysis of regeneration pattern helps to understand whether the recruitment of the species is satisfactory or shows any constraints. In tropical forests, regenerating plants are generally grouped under pioneer and climax species. The saplings of pioneer species are found only in gaps as they require light throughout their life. The saplings of climax species are generally found as suppressed stems in the understorey with very slow growth or as fast-growing stems in the gaps. Their seeds are able to germinate in the shade, and the seedlings are able to tolerate shade of the understorey for longer periods; when gaps become available, they grow fast and reach the canopy (*see* Ghazoul and Sheil 2010).

If the recruitment is active, recruitment graph/histogram (number against size) shows typical reverse 'J' configuration with the highest frequency in small-size groups (on the basis of length and/or DBH) and a gradual decrease in the number of individuals towards large-size groups. When regeneration of a species is weak, the graph shows a single peak in the intermediate- or large-size groups and fewer, if any, individuals in small-size classes. They generally do not show any saplings or small trees. When the regeneration is strong, the graph shows more than one peak in the size classes (Tang and Ohsawa 2002).

12.7.8.1 Special Requirements

Requirements for laying quadrats

12.7.8.2 Procedure

1. Lay down adequate number of permanent quadrats/plots of suitable size at random in the study area. Quadrats of various sizes ranging from 10×10 m to 30×30 m have been laid depending on the objective. Some investigators have used contiguous area of 0.25–1.0 h for studies.
2. Record the seedlings and young plants of each species within the quadrat and measure their height.
3. Amongst adults, measure the height of each adult and its DBH.
4. Group them into different categories: seedlings, saplings, juveniles and adults of different sizes (on the basis of DBH/height).
5. Draw a frequency graph/histogram for each species (x axis-size/DBH class, and y axis-frequency) and compare the proportions of different life forms and analyse the pattern of recruitment of each tree species.

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Appendices

A.1 Preparation of Some Common Fixatives and Stains

A.1.1 Fixatives (*see* O'Brien and McCully 1981; Ruzin 1999)

A.1.1.1 Acetic-Alcohol

3 parts of absolute ethanol: 1 part of glacial acetic acid

A.1.1.2 Carnoy's Solution

6 parts of 95 % ethanol: 3 parts of chloroform: 1 part of glacial acetic acid

A.1.1.3 Formalin Acetic-Alcohol (FAA)

5 ml formaldehyde: 5 ml glacial acetic acid: 90 ml 70 % ethanol

A.1.1.4 Lactic Acid-Alcohol (LA)

3 parts of 70 % ethanol: 1 part of lactic acid. This fixative also does clearing to some extent.

A.1.2 Stains

A.1.2.1 Acetocarmine

Heat the mixture containing 55 ml of distilled water and 45 ml of glacial acetic acid to boiling in a round bottom flask and add 1 g of carmine. Stir and allow it to cool. Filter and store in a refrigerator.

A.1.2.2 Safranin (1.0 %)

Dissolve 0.5 g safranin in 50 ml of 50 % ethanol.

A.1.2.3 Water Soluble Aniline Blue (0.005 %)

Prepare a stock of 0.5 % water-soluble aniline blue in 0.05 M Na_2HPO_4 buffer (0.5 g in 100 ml). Mix 1 ml of stock with 99 ml of buffer to prepare 0.005 % working solution. Adjust the pH to 11 with 1 N NaOH and filter. The stain can be stored in brown bottle under refrigeration for many months. Under the alkaline pH, the stain generally gets decolourized; some batches may not get decolourized, but still work.

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A.1.2.4 Auramine O (0.02 %)

Prepare a stock solution of 0.2 % by dissolving 0.2 g of Auramine O in 100 ml of 0.05 M Tris HCl Buffer (pH = 7.2). For working solution, mix 1 ml of stock and 9 ml of the buffer.

A.1.2.5 Toluidine Blue O' (0.05 %)

Prepare 0.05 % (0.05 g in 100 ml) of toluidine blue O' in 100 ml of 0.1 M benzoate buffer. Adjust the pH to 4.4 by using 1 N NaOH.

A.2 Preparation of Buffers

A.2.1 Tris HCl Buffer (0.05 M, pH 7.2, 100 ml)

Dissolve 0.6057 g of Tris (2-amino-2-hydroxymethyl-propane-1, 3-diol) in 70 ml of distilled water. Adjust the pH using 1 N HCl to 7.2. Make up the final volume to 100 ml using distilled water. The buffer can be stored in a refrigerator.

A.2.2 Phosphate Buffer (0.15 M, 1 L)

Prepare the following two stock solutions:

Solution A: NaH_2PO_4. $2H_2O$: 23.40 g/l of distilled water

Solution B: $NaH_2PO_4.2H_2O$: 26.69 g/l of distilled water

Gradually mix 49 ml of solution B and 51 ml of solution A

A.2.3 Acetate Buffer (Sodium, 0.2 M, pH 4.0)

Prepare the following two stock solutions:

Solution A: Acetic acid (0.2 M) – Mix 1.2 ml of acetic acid in 50 ml of distilled water and make the final volume to 100 ml with distilled water.

Solution B: Sodium acetate (0.2 M) – Dissolve 2.7 g of sodium acetate in 50 ml of distilled water and make the final volume to 100 ml with distilled water.

Prepare the working solution by mixing 80 ml of Solution A and 20 ml of Solution B.

A.2.4 Benzoate Buffer (0.05 %)

Mix 0.25 g of benzoic acid and 0.29 g of sodium benzoate in 200 ml distilled water. Adjust the pH to 4.4 by using 1 N NaOH or HCl.

A.3 Preparation of Agar Plates and Glycerin Jelly

A.3.1 Agar Plates (1 %)

Take 100 ml of distilled water in a heat-resistant beaker or flask (Borosil) and suspend 1 g of agar. Dissolve agar by boiling for a few minutes in a water bath or microwave oven. It should not be boiled directly over the flame as the agar will char. Dispense the molten agar into Petri plates of suitable size until it fills the depth of 4–5 mm. Allow the medium to set, cover and seal the Petri plates with adhesive tape. Keep agar plates in a refrigerator until use. If properly sealed, the plates will last for more than a week.

A.3.2 Glycerin Jelly

Add 50 g of gelatin to 175 ml of distilled water in a beaker and heat it until the gelatin is dissolved. Add 150 ml of glycerin and 5 g of crystalline phenol when the mixture is still warm. If necessary, a stain can also be added at this stage. Palynologists have used basic fuchsin or fast green or ruthenium red at desired concentration to give light colour to the mixture. In the presence of a stain, pollen grains take up colour and appear more clearly. Filter the mixture when it is still hot through glass wool into a sterile container preferably with wider mouth. As the glycerine jelly becomes solid at lab temperature, wide-mouth container would help in taking out some jelly through forceps/spatula for mounting. Once prepared, the jelly can be stored for months.

A.4 Composition of Commonly Used Pollen Germination Media

The concentration of sucrose mentioned here is arbitrary. As osmolarity of pollen grains of different species is variable, one has to try different concentrations of sucrose in the culture medium. The concentration of osmoticum (sucrose/PEG) should not allow bursting or plasmolysis of the pollen. Two-celled pollen grains generally require sucrose around 10 % while three-celled pollen systems require higher concentrations. As the concentration of boric acid and calcium nitrate given in the composition is optimal for most of the species, there is no need to vary their concentration.

The following is the composition of several pollen germination media commonly used:

A.4.1 Standard Medium

Sucrose 10 %
Boric acid 100 mg/l
Calcium nitrate 300 mg/l

For many species we have found that calcium nitrate is not necessary for satisfactory pollen germination and pollen tube growth. It is worth testing a simple medium, containing sucrose and boric acid before adding calcium.

A.4.2 Brewbaker and Kwack's (B&K) Medium (Brewbaker and Kwack 1963)

Sucrose 10 %
Boric acid 100 mg/l
Calcium nitrate 300 mg/l
Magnesium sulphate 200 mg/l
Potassium nitrate 100 mg/l

Although this medium is the most commonly used medium by pollen biologists, we found that for most of the species, magnesium sulphate and potassium nitrate are not necessary. For some species even calcium nitrate is also not needed in the B&K medium for satisfactory germination.

A.4.3 Polyethylene Glycol (PEG) Medium

Polyethylene glycol (PEG) 10 %
Sucrose 2–5 %
Boric acid 100 mg/l
Calcium nitrate 300 mg/l

Polyethylene glycol of different molecular weights has been used. PEG has been reported to improve pollen germination, and more dramatically pollen tube growth in a number of species such as *Nicotiana*, *Brassica* and oil palm. We have used PEG of molecular weight 4,000, 8,000 and 10,000 around 10 % (Shivanna and Sawhney 1995; Shivanna et al. 1998; Tandon et al. 1999) and all of them have given good results. Sucrose concentration has to be reduced to 2–5% in the PEG medium. PEG is non-toxic, highly soluble in water, biologically stable and does not denature biological macromolecules (Powell 1980). It is a non-penetrating osmotic agent and decreases the water potential of the culture medium. PEG has also been suggested to regulate the permeability of the plasma membrane and to give stability to the membrane (Read et al. 1993). Sucrose, on the other hand, enters the pollen and increases the osmotic potential of the pollen cytoplasm which may not be conducive for pollen tube growth.

A.5 Simple Humidity Chamber

A pair of large Petri plates of ca 15 cm diameter lined with moist filter paper or tissue paper on the lower lid can be used for most of the studies on reproductive ecology (Shivanna and Rangaswamy 1992). Two glass rods of suitable length can be placed parallel about 4 cm apart on the moist filter paper; they help in handling slides used for incubation (Fig. A.1).

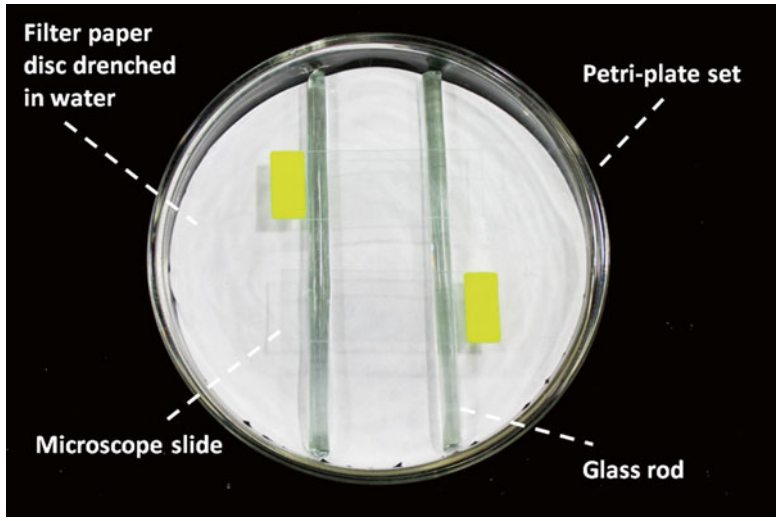

Fig. A.1 A set up of a simple humidity chamber made out of two large Petri plates with one or two layers of wet filter paper discs. Two glass rods of suitable length inside the plates help in handling the slides used for incubating pollen or pistils. The slides with labels on one side are kept across the glass rods

A.6 Field Kit

A fold-out plastic tool box of about $30 \times 15 \times 15$ cm with multiple trays (Fig. A.2) is very handy for field work. This may be used to carry portable instruments, collection/sampling vials, appropriate fixatives, pollination bags, tags, markers, paints, brushes, Petri plates, filter paper discs, slides and cover glasses which may be required to pursue the field based protocols.

A.7 Insect Traps

A.7.1 Sweeping Net

Use of insect nets, available in various sizes, is the most commonly used method to trap pollinating insects. The sweep should be swift enough to force the insect to the bottom of the collection bag; the rim of the net should be turned immediately after sweeping so that the bottom of the bag folds over the rim of the net to trap the insect inside.

A.7.2 Aspirator

Another method of collecting insects, particularly smaller insects, is to use an aspirator. A manually operated aspirator is available with any scientific instrument suppliers or can be easily prepared (Fig. A.3). It consists of a plastic or glass vial of suitable size, closed with a rubber stopper with two holes; two hoses are inserted through these holes in the stopper, one for sucking through user's mouth and the other as the inlet for the insect.

A.7.3 Wide Mouthed Vial of Suitable Size

We have been employing routinely a wide mouthed plastic vial (25–50 ml capacity) to collect insects of many species. Bees can be easily collected with this approach, particularly those which can enter the flower. After the insect has entered the flower, the vial is held over the opening of the flower. When the insect exits the flower, it enters the vial. The lid of the vial is closed immediately to prevent its escape. Keeping a piece of tissue or cotton dipped in ethyl acetate inside the bottle

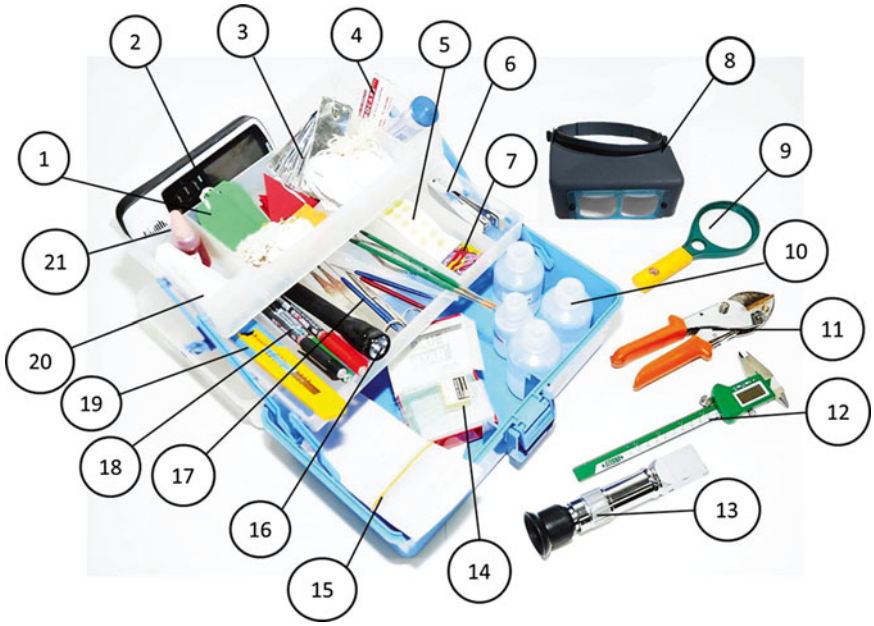

Fig. A.2 A field kit pulled out to show all the items that can be carried. Usually, a kit may have: *1*. Coloured paper tags, *2*. Portable geological positioning system (GPS), *3*. Metal tags, *4*. Microcapillaries, *5*. Sticky labels, *6*. Stapler, *7*. Non-tear-end clips, *8*. Optivisor, *9*. Magnifying glass, *10*. Suitable chemical fixatives, *11*. Secateurs, *12*. Digital vernier caliper, *13*. Hand refractometer, *14*. Glass slides and coverslips, *15*. Paper bags for pollination, *16*. Battery operated torch, *17*. Needles/forceps, *18*. Permanent marker, *19*. Box-cutter, *20*. Muslin cloth and *21*. Dyes. All these items may be carried in a locally available fold-out tool kit

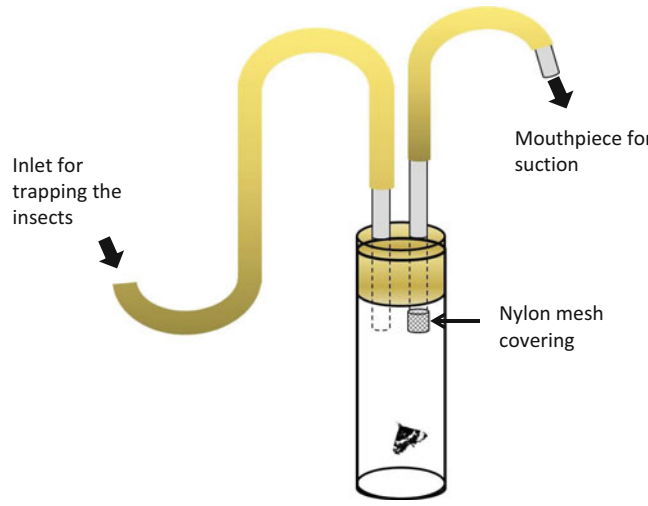

Fig. A.3 A diagrammatic representation of an assembly of a simple aspirator to capture small insects

Table A.1 Fluorochromes commonly used in studies on pollen biology, their specificity and fluorescence colour

Aspect of study	Flourochrome to be used	Specificity	Filter combination	Fluorescence colour
Pollen germination and pollen tube growth	Water soluble aniline blue	Callose	UV	Bright yellow
Pollen viability	Fluorescein diacetate	Fluorescein	Blue	Bright green/bright greenish yellow
Pollen cytology (nuclei)	DAPI (4′, 6-diamidino-2- phenyl indole)	DNA	UV	Blue
	Ethidium bromide	DNA	Blue	Yellow/orange
	Mithramycin	DNA	Blue	Yellow
	Hoechst 33258	DNA	UV	Bright bluish white
Pollen and stigmatic walls	Auramine O	Sporopollenin	UV	Greenish yellow
		Cuticle	UV	Brilliant orange yellow

before trapping would also help in immobilizing (by keeping for a shorter time) or killing (by keeping for longer time) the insect. Ethyl acetate should not be inhaled during capture of insects.

A.8 Commonly Used Fluorochromes and Their Excitation Filters

Fluorochromes are used in studies on various aspects of pollen biology. Their specificity, filter combination and fluorescence colour are given in Table A.1.

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