Pollination biology of *Echinacea angustifolia* and *Echinacea purpurea* (Asteraceae) in Saskatchewan

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ABSTRACT

The goals of this research project were to identify the various insects observed to visit inflorescences of *Echinacea angustifolia* DC, and to rank these visitors according to their importance as pollinators of *E. angustifolia* in Saskatchewan. Studying nectar and the nectary is essential to understanding the interaction of disc florets with pollinators. Nectar-sugar production by disc florets of *E. angustifolia* and *E. purpurea* (L. Moench) was quantified from anthesis to cessation with production per disc floret peaking in the afternoon of the staminate phase (191.7 µg) and at midday of the first day of the pistillate phase (156.6 µg), respectively.

Morphology of the disc-like floral nectaries of both *Echinacea* species was studied, as well as the ultrastructure of the nectary of *E. purpurea*. Modified stomata on the nectary rim are the most likely exits for nectar, but creases in the epidermis may also participate. The nectary of *E. purpurea* is vascularized by phloem alone, which occurred adjacent to the epidermis. Companion cells possessed wall ingrowths, and these cells may unload arriving sugar destined for either an apoplastic or symplastic pathway. Lobed nuclei were a key feature of secretory parenchyma cells, as was a predominance of mitochondria, suggesting that energy-requiring eccrine secretion predominates in *E. purpurea*.

E. angustifolia exhibited a generalist pollination system, with pollinating insects belonging to the orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera. The pollination efficiency of visitors was determined by single insect visits to bagged, virgin inflorescences followed by quantifying pollen tubes at the bases of receptive styles and/or calculating the percentage of shrivelled styles. It was determined that bumble bees (*Bombus* spp.) were efficient pollinators, indicating that they would likely contribute much to the pollination of *E. angustifolia*. Grasshopper bee flies (*Systoechus vulgaris* Loew) were plentiful but individually were not efficient pollinators, but taken together, they provided much pollination. Golden blister beetles (*Epicauta ferruginea* Say) were efficient pollinators but where yellow-petalled flowers occurred, their numbers on *E. angustifolia* decreased. Honey bees (*Apis mellifera* L.) were efficient pollinators and were present in low numbers without managed introduction. Pierid (2003) butterflies were regular visitors and efficient pollinators, and likely contributed

significantly to *E. angustifolia* pollination. When introduced, the alfalfa leafcutter bee (*Megachile rotundata* Fabr.) preferred not to forage on *E. angustifolia* and as such, these solitary bees were not suitable as managed pollinators. In large agricultural plantings of *E. angustifolia*, however, native insects may not be capable of providing sufficient pollination for seed production when floral competition occurs.

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Chapter 1: INTRODUCTION

1.1 Specialty crops and *Echinacea*

Saskatchewan farmers looking to diversify their crops to stay competitive have often turned to specialty crops like spices or herbs. The products of a spice crop are used to enhance the flavour of food. Herbs are often cultivated for use in herbal medicine. Some herbs fall into the category of nutraceuticals, "products that are isolated or purified from foods and sold as medical forms not associated with food" (Saskatchewan Agriculture, Food and Rural Revitalization, 2002). One example of these nutraceutical crops is feverfew (*Tanacetum parthenium*), whose leaf extracts have been approved by Health Canada as a migraine preventative medication. Another crop in great demand is *Echinacea*, the purple coneflower. Three species of *Echinacea* (Asteraceae: Heliantheae) are grown commercially for their medicinal qualities: *E. angustifolia* (DC), narrow-leaved purple coneflower; *E. purpurea* (L. Moench), purple coneflower; and *E. pallida* (Nutt.), pale-purple coneflower. McGregor (1968) identified nine species and two varieties of *Echinacea* and emphasized that all species will hybridize when grown in close proximity. Recent taxonomic revisions reclassify *E. angustifolia* as *E. pallida* var. *angustifolia*, to indicate that *E. angustifolia* is a variety of *E. pallida* (Binns *et al*., 2002) and not its own species, but the classical use of the name *E. angustifolia* will be employed for the purposes of this study. Of these species, *E. angustifolia* and *E. purpurea* are used most extensively as medicinal preparations to treat ailments ranging from the common cold to rattlesnake bites (Kindscher, 1989; Wagner, 1999). Studies have found that the roots of all three cultivated species contain a number of bioactive compounds and the flowers and green material of *E. purpurea* contain commercially-important quantities of them as well. The bioactive constituents of *Echinacea* plants are cichoric acid and echinacosides, which are derivatives of caffeic acid, and also alkamides, polyacetylenes, and glycoproteins/polysaccharides (Bauer, 1998). There is debate about which compound has the most potent pharmacological activity, but all of the bioactive compounds should be used together because they have a synergistic effect (Bauer, 1998).

Increasingly, Saskatchewan farmers are planting pharmaceutical crops to supply consumers' growing demand for organically produced "natural medicines". Saskatchewan has become a major exporter of these plants to the United States of America (U.S.A.) where, in 1996 and 1997, *Echinacea* products were the best selling herbs with 9.1% of the market share (Harbage, 2001). The popularity of *Echinacea* as an herbal medicine has led to increased harvesting of wild stands. This practice is termed "wildcrafting" and has led to such a reduction of wild plants that it has prompted concern and, in two cases, legislation to protect it. In the U.S.A., *E. tennesseensis* and *E. laevigata* are listed as endangered by the U.S. Fish and Wildlife Service (Apsit and Dixon, 2001; Walck *et al.,* 2001). The endangered rating means that wildcrafting these plants is now illegal. Farming of *E. angustifolia* and *E. purpurea* should keep them from being harvested into extinction. Selling wild stands of *Echinacea* creates other problems besides over-harvesting. The growing conditions of wild *Echinacea* species cannot be controlled and as a result, the levels of bioactive ingredients are inconsistent. Controlled farming of *Echinacea* will also result in the development of market worthy plants that contain standard levels of bioactive ingredients. The identity of the wild species is questionable, as well, if someone unfamiliar with species taxonomy is harvesting plants. A buyer cannot be assured that the *Echinacea* root they are buying is genuinely *E. angustifolia,* for example. Farming *Echinacea* ensures that the species identity is accurate so that growers and buyers can be certain that their product is authentic. Commercialized plantings of these economically viable species will reduce wildcrafting, increase the standardization of the medicinal ingredients, and create assurance of the harvested species.

1.1.1 *Echinacea* species grown in Saskatchewan

E. angustifolia is native to the south-eastern region of Saskatchewan (McGregor, 1968) and is drought tolerant, making it a promising crop choice for the prairie climate. It is also the only species of *Echinacea* that occurs naturally in Canada. Eighty percent of the cultivated *Echinacea* in Saskatchewan is *E. angustifolia*, with *E. purpurea* making up the remainder (Harbage, 2001). The other *Echinacea* species are endemic to the midwestern U.S.A. (Kindscher, 1989; McGregor, 1968). *E. angustifolia* is a popular crop choice because it contains more of the bioactive ingredients than the other

commercially grown species. *E. purpurea* is popular because the entire plant is harvested and the leaves and flowers are either used fresh and made into tinctures, gel capsules or juice, or dried and used for tea.

Much is known about the pharmacological value of *Echinacea* plants, whereas relatively little is know about their breeding system. This study seeks to elucidate the pollination ecology of the two economically valuable species of *Echinacea* (*E. angustifolia* and *E. purpurea*) in Saskatchewan. This pollination study begins by focusing on the floral inflorescences of *E. angustifolia* and *E. purpurea* in pollinator attraction, by performing a detailed analysis of the fertile disc florets and their pollinator rewards of nectar and pollen, with particular emphasis on the floral nectary as a poorly studied gland involved in attraction. Owing to our lack of knowledge in *Echinacea* spp., a study of the phenology of flowering and associated nectar secretion is of particular interest, to understand this extremely important insect attractant. The study also seeks to answer the following questions about insect pollination of the most economically valuable species of *Echinacea* in Saskatchewan, *E. angustifolia*. (i) Which insects are visiting inflorescences of *E. angustifolia* and of these insects, which ones are the most efficient pollinators? (ii) Are the levels of native pollinators sufficient to effectively cross-pollinate a field of *E. angustifolia*? (iii) Is *E. angustifolia* suitable for managed pollination by a readily available, non-*Apis* managed pollinator, the alfalfa leafcutter bee, *Megachile rotundata*? In answering these questions pertaining to pollination, other basic questions involving reproductive biology arose that needed to be answered in order to properly address the central question of *E. angustifolia* pollination. Controlled hand-pollinations needed to be performed to satisfactorily determine whether *E. angustifolia* and *E. purpurea* are self-compatible or self-incompatible, and to determine the intervals required following pollen-grain germination on the stigma before pollen tubes had reached the style base.

1.1.2 General morphology of *Echinacea angustifolia* and *E. purpurea*

Echinacea is a perennial flowering herb that, when mature, can be anywhere from one to three feet tall, depending on the species (McGregor, 1968). *E. angustifolia* is the shortest species and has a shrubbier habit than most other *Echinacea* species (Fig 1.1A). Its leaves are strap-shaped with almost parallel venation and defensive trichomes

cover the upper and lower leaf surfaces and the inflorescence stalk (peduncle). The plant begins in the spring as a basal rosette of leaves (Fig. 1.1A) that bolts in late spring or early summer to produce a capitulum-type inflorescence. Often, *E. angustifolia* does not produce inflorescences in its first year of growth; however, when it does, each plant typically produces only one inflorescence. In the third or fourth year of growth, however, the plant often takes on a bushy, shrub-like appearance and can produce multiple inflorescences (Fig 1.1A). The root of all *Echinacea* species is perennial and sends out new shoots and leaves each spring. *Echinacea* species typically possess thick taproots, with the exception of *E. purpurea*, which has a fibrous root system. *E. purpurea* is the taller of the two species, with ovate leaves having reticulate venation and always producing multiple inflorescences per plant (Fig 1.1B).

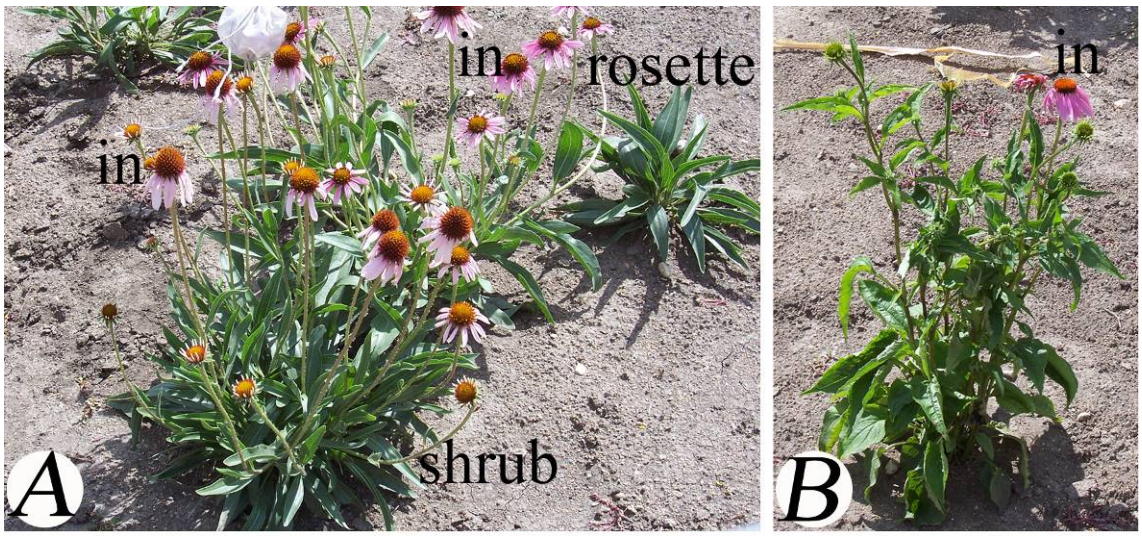


FIG. 1.1: (A) Mature plant of *Echinacea angustifolia* with multiple inflorescences (in) illustrating a shrubby (shrub) habit and younger plant with only a basal rosette (rosette) of leaves. (B) Second year plant of *E. purpurea* with multiple inflorescences (in) illustrating its tall, branching growth habit. Biology garden plot, 2005.

1.1.3 Nectar and asteracean nectaries

The ultimate goals of this research were to develop an understanding of the pollination ecology of *E. angustifolia* and *E. purpurea* so that pollination would never become a limiting factor in seed production and to add to the literature on asteracean nectar and nectaries. Nectar, as one of the major rewards offered to pollinators and the nectary, as the floral organ responsible for nectar production, are also important areas of

study to fully understand the interaction of the disc florets of *Echinacea* with their insect pollinators.

Knowledge of the process and phenology of nectar secretion is essential to understanding the attractiveness of a flower to nectar-foraging insects. Many studies link the surface and internal structure of floral nectaries with the function of nectar secretion, but relatively few have focused on the family Asteraceae. In spite of their widespread attractiveness to many generalist pollinators leading to reproductive success and an impressive species diversity in the Asteraceae (Proctor *et al*., 1996), plus the family's global importance in terms of honey production (Crane, 1975; Shuel, 1989), there are only two previous studies (Tacina, 1979; Sammataro *et al.* 1985) in which floral-nectary ultrastructure was investigated within the Asteraceae. Both involved the same species, sunflower (*Helianthus annuus*). Thus, this study represents only the second asteracean species in which nectary fine structure has been reported. Neither are there data on floral nectar production and dynamics in *E. angustifolia* or *E. purpurea*. Accordingly, goals of this research project were to examine the anatomy and ultrastructure of the floral nectary at three stages during flowering of the disc florets of *E. purpurea,* in relation to nectar secretion from its commencement to cessation, throughout floret phenology. Determining nectar dynamics of disc florets of *E. angustifolia*, as well, will assist in understanding the interaction between insect visitors and the inflorescence in the field.

1.2 Insect pollination of crop plants

Pollination is important to crop plants because it ensures that viable seeds will be formed to produce the next generation. Kevan (1997) has reviewed the basic breeding systems of plants. In angiosperms, flowers of some species are able to self-pollinate (autogamy), while others require that pollen be introduced from other flowers of the same plant (geitonogamy). Xenogamy occurs when pollen is introduced from flowers of another plant of the same species. This term is also known as cross-pollination or pollen out-crossing. Flowers are self-compatible if self-pollination can result in viable seed. Flowers of self-incompatible species can only set and produce viable seed if they are cross-pollinated. It is the flowers of these self-incompatible plants that are of primary

concern to this study, however, self-compatible species can benefit from the influx of new genetic material brought about by cross-pollination that often results in more or better quality seed or fruit set than self-pollination.

1.2.1 The value of honey bee pollination and current challenges

The usual practice to remedy inefficient crop pollination has been to import colonies of European honey bees (*Apis mellifera*) into the field to enhance cross-pollination of the flowers. This practice is efficient because honey bees are regarded as generalist foragers, being attracted to flowers of many species, so they have been used to pollinate many different crops (Free, 1993; McGregor, 1976). Not all scientists have complete confidence in the ability of honey bees to pollinate all crops. Westerkamp (1991) disagrees with the popular notion that honey bees are ideal for pollination of all crops because their long-lived societies prevent any adaptations to specific crops and force bees to continually forage on different plant species to meet the nutritional needs of the colonies.

Honey bees are used extensively as pollinators in agriculture for several reasons. Honey bees are domesticated and fairly easy to work with and can be moved long distances when necessary. When one crop has finished flowering, the hives of honey bees can be moved to another crop in flower. Honey bees also produce honey and wax that can be harvested and sold as valuable commodities so that the bees are beneficial as pollinators and producers. Early settlers in the17th century introduced honey bees from Europe to North America to produce honey. Honey bees adapted well to this climate and soon spread all over North America as domestic and feral colonies. The common practice of commercial beekeepers and pollinators is to hire their bees out to growers to pollinate crops. This practice is essential for the viability of insect pollinated crops in modern agricultural practices. Numerous studies have attempted to calculate the economic value of honey bee pollination to agriculture. Levin (1983) estimated that honey bee pollinated crops reap $18.9 US billion per year in the U.S.A., whereas in Canada, Winston and Scott (1984) estimated honey bee pollination resulted in profits of $1.2 US billion. These figures likely have grown significantly since the 1980's. Clearly, insect pollination of crops is essential for modern agriculture.

In the last few decades, honey bees in North America have encountered many new parasites and their ability to pollinate our crops is in jeopardy (Watanabe, 1994). These troubles are impacting on Saskatchewan beekeepers and farmers by reducing supplies of honey bees, and thus, have detrimental effects on crop pollination. Infestations of mites have decimated honey bee populations, and quarantines instituted in 1987 have limited the traditional supplies of replacement honey bee stocks to Saskatchewan from the U.S.A. Quarantines to prevent the northward spread of Africanized honey bees from the southern U.S.A. have also decreased the supply of honey bees to Saskatchewan. Today, honey bees can only be imported from countries free of mites like Australia and non-quarantined parts of New Zealand, which makes stock importation costly. These infestations of introduced parasites, the varroa mite (*Varroa destructor*) and tracheal mite (*Acarapis woodi*), have also decimated feral colonies of honey bees (Watanabe, 1994; Doebler, 2000). Mites reduce the longevity of honey bees and an infestation can swiftly spread through a colony, resulting in its fatality, without intervention. Apiculturists can treat mite infestations to save their bees from destruction but feral colonies are completely vulnerable. The added cost of treating colonies can strain a bee farmer's profit margin. The high price of imported bees, the Africanization of bees, and destruction by imported mites are producing an uncertain future for North American beekeepers. Agriculture's continued reliance on honey bees for pollination needs to change. The implementation of different pollination systems with pollinators other than *Apis mellifera* needs to be put in place to supplement the constrained honey bee system.

1.2.2 Native pollinators and current challenges

Insects are the primary vectors of pollen transfer for the majority of angiosperm species, whereas some species of birds (often hummingbirds), bats and the wind contribute as pollen vectors in other instances. There has been some recent concern that populations of native pollinators (wild insects) are not sufficient to effectively cross-pollinate crops (Buchmann and Nabhan, 1996; Richards, 2001). Kearns *et al*. (1998) warn of a "pollination crisis" where the declining numbers of native and managed pollinators, like *Apis mellifera*, are not able to properly pollinate the increasing acreages of crops. Modern agricultural practices may be responsible for the low number of native

pollinators. The planting of monocultures of crops and the destruction of natural habitats to produce more cropland reduces the insect biodiversity in agricultural areas (Cane and Tepedino, 2001; Richards, 2001). Monoculture of angiosperms results in large acreages of plants flowering for a short period of time. Native pollinating bees often require a continual supply of flowers for forage during their lifespan, to provide nectar and pollen for their brood. In a natural field there are many plants that flower at different times, so normally there are always flowers available for foraging. Most pollinators cannot survive with only one short-lived flowering species for forage and cropping systems that promote monocultures of flowering crops reduce pollinator diversity (Scott-Dupree and Winston, 1987). Modern agriculture has changed the landscape and forced pollinators to inhabit the few small plots of natural land that remain along roadways and railway tracks (Cane, 2001). Those low levels of pollinating insects that remain are not able to visit every flower in a monoculture and so pollination of the crop is sporadic and inefficient. In agriculture, the symptoms of less-than-effective cross pollination are manifest as a low number of seeds produced, unacceptably low seed germination rates and small seeds with less oil or nutritive value (Free, 1993). If the grower is producing a crop to harvest its seed, then proper pollination is of utmost importance. Seed may be gathered as the end product (many oilseed crops like sunflower, for example) or as the means to propagate the next generation. In either case, the seed is sold and growers will benefit from high quality seeds with high levels of germination produced by cross-pollination.

In a paper commissioned by the journal "Conservation Biology", leading pollination biologists suggested that future pollination research should address several key areas (Allen-Wardell *et al*., 1998). Efforts should be directed towards conserving native pollinators, developing new, managed insects for pollination and towards identifying the pollination systems of poorly studied specialty crops. The overall objective of this thesis was to address the key areas of pollination research specified by Allen-Wardell *et al.* (1998). *Echinacea*, as a specialty crop, has been poorly studied in regards to its pollination system and native insect pollinators. Through this study, a survey of the populations and identities of native pollinators around Saskatoon was undertaken and the interaction between insects and the inflorescence of two

economically relevant *Echinacea* species, *E. angustifolia* and *E. purpurea*, were studied. Introduction of thousands of individuals of a domesticated solitary bee, the alfalfa leafcutter bee (*Megachile rotundata* Fabr.; Hymenoptera: Megachilidae), was also undertaken to ascertain whether these insects would be suitable for pollinating an agricultural field of *E. angustifolia*. Developing *M. rotundata* as a managed pollinator of *E. angustifolia* would add to the usefulness of this domesticated bee species in Saskatchewan and possibly increase demand for this unique insect commodity that is supplied by alfalfa seed growers in Saskatchewan. Identifying new crops suitable for managed pollination by *M. rotundata* reduces the need for reliance on the European honey bee as a supplementary pollinator, if levels of native pollinators are insufficient to fulfill the pollination needs of *E. angustifolia* crops or other specialty crop requiring pollination.

1.2.3 Breeding system in *Echinacea*

Echinacea angustifolia and *E. purpurea* are supposedly self-sterile and unable to produce seed without cross-pollination (McGregor, 1968). In that study, self-sterility was determined by observing that bagged inflorescences did not produce viable seed. A wild population of *E. angustifolia* was determined to operate with a mixed breeding system (Leuszler *et al.*, 1996), where geitonogamy occurred at 9% and autogamy at 7% in hand pollination treatments, in contrast to the absolute self sterility determined by McGregor (1968). McGregor (1968) did not discuss the possible methods employed by *Echinacea* species to effect cross-pollination, however, but insects are overwhelmingly the most likely candidates for pollen vectoring. The typical asteracean inflorescence is a highly evolved aggregation of florets that seems to be designed for the sole purpose of attracting insects. In all other taxa within the asteracean family, insects are cited as the primary mode of pollen dispersal (Mani and Saravanan, 1999). It is hypothesized that the only animals identified as pollinators of *E. angustifolia* and *E. purpurea* in Saskatchewan will be insects. The reproductive biology of *E. angustifolia* was studied in a wild population in North Dakota (Leuszler *et al.*, 1996), where insect visitors to inflorescences of *E. angustifolia* belonged to 12 species in four families of bees (Colletidae, Andrenidae, Halictidae, and Megachilidae) and four families of butterflies (Nymphalidae, Pieridae, Hesperidae and Papilionidae).

Widely available literature directed at producers of *Echinacea* consistently fails to mention insect cross-pollination as a growing consideration (Greenfield and Davis, 2004; Porter, 2003). In this same production literature, it is often mentioned that germination of achenes of *E. angustifolia* is poor if proper pre-treatment methods are not employed (Feghahati and Reese, 1994). It is possible that in some instances the problem of poor germination may be the result of insufficient cross-pollination, rather than the result of a failure to break seed dormancy. Little mention of insect pollination in this literature may indicate that pollination of *E. angustifolia* and *E. purpurea* is not a serious issue. Native insects may have been sufficient to produce seeds when seed harvests of *E. angustifolia* were mostly through "wildcrafting" of natural stands of plants but when large scale production occurs, a subsequent decrease in the ability of naturally available (native) insect pollinators to effectively pollinate the large number of available flowers often occurs (Allen-Wardell *et al.*, 1998; Richards, 2001). Pollination and subsequent fertilization may also hasten maturity and senescence of the plant, which is desirable for growers that need to harvest and dry a crop before winter. A well-pollinated field then may have the potential to mature more quickly then an insufficiently pollinated field.

A need for pollination research on *Echinacea angustifolia* and *E. purpurea* exists even though the primary economic value of these plants is in the root and shoot. Insect pollinators may be essential to seed production and need to be a research focus to identify the specific insects responsible for pollination. If a pollinator shortage occurs then a failure of seed production may result that might otherwise be blamed on other environmental factors. *Echinacea* seeds are often sold at a higher price per kilogram than the root, indicating that a good seed yield can reap large profits for growers. The seeds of each species have different values on the market. The seeds of *E. angustifolia* are sold for three times as much as the seeds of *E. pallida* (Dr. B. Barl, personal communication) and so it is imperative that buyers know what seeds they are acquiring. Currently, *E. angustifolia* seed sells in Saskatchewan for $150 to $250 per pound (0.45 kg), and *E. angustifolia* root sells for about $35 per pound (0.45 kg) (Lone Wolfe Herb Resource, 2005).

Several of the phytochemicals possessing immunostimulatory effects like the alkamides, typically found in *Echinacea* roots, are also found in the achenes of *E.*

angustifolia and *E. purpurea* in significant amounts (Schulthess *et al.*, 1991). The phytochemical content of the achenes could increase the value of *Echinacea* seeds if they are suitable for use in herbal formulations. It is a biological fact that a seed is initially required to grow a plant. Any agricultural plantings of *Echinacea* must initially come from seed except in cases where plants are propagated through vegetative techniques. An *E. angustifolia* crop is not harvested until at least its third year of growth and often not until its fourth or fifth year, and thus does not produce any profits for the grower for the first three years. *E. angustifolia* plants typically flower in their third year, and the seed produced may be sold to produce income before the main root crop is harvested. Guidelines produced for growers by provincial governmental agricultural branches recommend methods to harvest and market seed, so the production of seed is an established commercial practice (Porter, 2003; Manitoba AFRI, 2001). After harvest, growers require additional seed to reseed or expand their acreage.

1.3 Project objectives

To increase our current state of knowledge of the reproductive biology and pollination ecology of *Echinacea angustifolia* (narrow-leaved purple coneflower) and *E. purpurea* (purple coneflower) in Saskatchewan, this study had four main objectives:

1) to record the flowering phenology and flower structure of both species, particularly in relation to the dynamics of nectar and pollen production (Chapter 2), two attractive resources utilized by flower-visiting insects for nutrition;
2) to examine microscopically the morphology, anatomy and ultrastructure of the floral nectary of *E. purpurea* in relation to flower development and nectar production (Chapter 2), especially in light of the paucity of similar studies within the highly-successful family, Asteraceae;
3) to document the native arthropod fauna that visited the inflorescences of *E. angustifolia* at two or three field sites over two consecutive seasons, and to develop and test different techniques for evaluating and ranking the various flower visitors as pollinators (Chapter 3), to ensure that cross-pollination can be maximized for seed production in this species; and
4) to conduct preliminary trials with a domesticated solitary bee species, *Megachile rotundata* (the alfalfa leafcutter bee), as a potential pollinator of *E. angustifolia*

in the field (Chapter 4), to potentially broaden the scope of dependable pollinating species for this specialty crop.

Chapter 2: FLORAL MORPHOLOGY, NECTARY ANATOMY AND ULTRASTRUCTURE AND NECTAR SECRETION BY DISC FLORETS

2.1 Introduction

The asteracean inflorescence (capitulum) is a highly modified aggregation of small florets that creates the illusion of one large flower, and can thus be termed a highly modified pseudanthium (Weberling, 1989). Commonly in the capitulum of the subfamily Tubuliflorae of the Asteraceae, ray florets surround a central disc in the same manner that the corolla of a single flower surrounds the central floral organs of the androecium and gynoecium. In this way, the asteracean capitulum mimics the appearance and attractiveness of a single, large flower while gaining the genetic advantage of dozens to hundreds of flowers. With disc florets maturing and becoming receptive at different times on the central disc, the potential for many pollen donors is enhanced, which increases genetic variability of the offspring. Seeds of an asteracean inflorescence thus will not all be genetically similar and the chance for development of novel genes is enhanced.

The inflorescences of species within the genus *Echinacea* were studied in detail (McGregor, 1968) and included measurements of floral organs, so this numerical information was not duplicated by this study of *E. angustifolia* and *E. purpurea* inflorescences and disc florets. The inflorescence and disc florets of *E. angustifolia* and *E. purpurea* are similar enough to warrant discussion in one section devoted to a study of their morphology and anatomy with differences identified where necessary. Moreover the ultrastructure of the nectary of *Echinacea purpurea* was also studied using transmission electron microscopy.

Seed is required for this commercial industry, yet the inflorescence's disc florets are almost exclusively self-infertile and must be cross-pollinated by insects (McGregor, 1968). Insect visitors are attracted by pollen presented during the staminate phase and by nectar secreted throughout floret phenology. Nectar is the only reward

produced during the pistillate phase of disc floret phenology and is thus an important addition to the study of insect pollination of *E. purpurea* and *E. angustifolia*. To fully understand the attractiveness of the capitulum to insect visitors, nectary structure and nectar secretion by the disc florets must be identified.

In the Asteraceae (Compositae), floral nectaries are annular, multicellular outgrowths that form atop the inferior ovary and surround the style base (Frei, 1955; Mani and Saravanan, 1999). Nectary morphology is highly variable, ranging from flat, to cup-shaped, and even multi-lobed rings, as depicted in line drawings from 48 species (Gopinathan and Varatharajan, 1982; Mani and Saravanan, 1999). Within a capitulum, some florets may lack nectary tissue. Also, disc florets tend to produce more nectar than ray florets, which may lack or possess only a very small nectary (Mani and Saravanan, 1999), although a notable exception occurs in *Heterothalamus alienus* (Vogel, 1998).

Similarly, members of the Asteraceae provide a wide diversity in the type of vascular supply to their floral nectaries (Fahn, 1979), which originates from vascular bundles of the style and ovary (Gulyás and Pesti, 1966). Of 30 species investigated to date, 17% have their floral nectaries innervated by both phloem and xylem, 30% by phloem alone, and 53% completely lack vascular tissue (Frei, 1955; Kartashova, 1965; Gulyás and Pesti, 1966; Ma *et al.,* 2002). Various types even exist among species within the same genus (e.g., *Centaurea* – Frei, 1955; Gulyás and Pesti, 1966). Despite this internal variation in structure, the nectary surface in over 70 asteracean species (see Davis, 1992) possesses modified stomata that can serve as exits for nectar escape from the gland (Caspary, 1848; Bonnier, 1879; Gopinathan and Varatharajan, 1982; Vogel, 1998; Mani and Saravanan, 1999; Warakomska and Kolasa, 2003).

2.2 Materials and Methods

2.2.1 Plant material and field sites

Mature plants of *E. angustifolia* were sampled at three field sites; Meewasin Valley Authority, Valley Road and the garden plots outside the Biology (W.P. Thompson) Building in the summers of 2004 and 2005. Mature plants of *E. purpurea* were sampled in 2004 from plants started in the Biology garden plots, before some were transferred to a growth chamber later that year. In 2005, nectar from plants of *E.*

purpurea growing under field conditions in the Biology garden plots was sampled for its carbohydrate content.

2.2.1.1 Meewasin Valley Authority's Chief Whitecap Park

The Meewasin Valley Authority (MVA) field site is located near the South Saskatchewan River on the northeastern side of Saskatoon. Entrance to the field is gained from a gate off Saskatchewan Road in the Rural Municipality of Corman Park. *E. angustifolia* plants were initially hidden by a dense covering of alfalfa (*Medicago sativa*) that had been seeded by a farmer renting the land from the MVA. This field site was an old cultivated *E. angustifolia* plot that had been harvested in 2001, two years prior to the beginning of this study. This field was mostly free of alfalfa and had thousands of *E. angustifolia* plants in flower. These plants must have sprung up from un-harvested roots and dispersed seeds and today is an approximation of an organic stand of poorly maintained *E. angustifolia* transitioning to a wild condition. This field was approximately 3100 square feet and had a diversity of weedy plant species amongst the *E. angustifolia* plants dominated by Canadian fleabane, *Erigeron canadensis*. It bordered a roadway on the eastern side and an area of native grassland on all other sides that transitioned to prairie forest on the western and northern sides. With permission of the Meewasin Valley Authority, this field became the primary field site in 2003 and one of three main sites in 2004.

2.2.1.2 Valley Road

The designation Valley Road (VR) is given to an *E. angustifolia* field site added in 2004. VR is an organic farm located approximately 20 kilometres west of Saskatoon on Valley Road, on the west bank of the South Saskatchewan River near Poplar Bluffs. The property is the second on the right side after turning left onto Chenille Road from Valley Road. The agricultural site, belonging to Doug Pchajek, is referred to as Valley Road (VR) as it is located near Valley Road. This site had 1/3 of an acre planted to *E. angustifolia* with approximately 22,000 plants. The plants were in their fifth year of growth and many plants had attained a bushy habit with numerous inflorescences per plant. Several other organic crops, such as wheat (*Triticum aestivum*) occupied the remainder of the acreage with a shelterbelt border of trees on the south side, a caragana hedge border on the north, and pastureland on the west and east border.

2.2.1.3 W.P. Thompson (Biology) garden plot and growth chamber

Seedlings of *E. purpurea* L. (Moench), purchased from Helga's Herbs (Saskatoon, SK) were transplanted into a plot near the W.P. Thompson (Biology) building at the University of Saskatchewan in May, 2004. Five plants that initiated flowering by mid-September were transplanted into individual pots of Sunshine Sphagnum Mix (Sun Gro Horticulture, Seba Beach, AB, Canada) placed in a growth chamber at 22°C day and 16°C night temperatures with 400 foot candles illumination on a 16:8 hour light: dark cycle. Plants were watered daily and fertilized with 20:20:20 fertilizer (Plant-prod, Plant Products Co. Ltd., Brampton, ON). Nectar was sampled from 17 inflorescences from these five plants.

Eight plants of *E. angustifolia* were transplanted from MVA to the garden plots in 2003. In 2004 and 2005, the number of *E. angustifolia* plants at this site increased and they were used for field analysis of nectar production.

2.2.2 Phenology and morphology of ray and disc florets of the capitulum

Various phenological stages were identified according to the development and relative location of disc florets within a capitulum. In-depth observations of inflorescences of *E. purpurea* were used to deduce the phenology of flowering, but *E. angustifolia* disc florets follow the same phenology.

Macro images of floral features were captured using a Nikon Coolpix 950 and modified where necessary using Adobe Photoshop® to enhance picture quality. Pictures were printed on Epson Glossy Photo Paper.

2.2.2.1 Pollen grain counts

The average number of pollen grains per disc floret was determined by counting the total grains in one indehiscent, mature anther tube from five plants of *E. purpurea*. Four extra anther tubes from the same capitulum of one plant (5 total) were randomly selected from the staminate whorl and also counted. The anther tube was first removed from the disc floret before dehiscence, to ensure that all pollen grains produced in the anthers were accounted for. The anther tube was then split open with a razor blade and the pollen released into a drop of water on a microscope slide. The opened anther tube was transferred through five subsequent water droplets until all of the pollen was removed from the anthers and suspended in water. Coverslips were placed over the

droplets and sealed with nail polish to prevent evaporation. All pollen grains from the anther tube of a disc floret were counted at 100x using a compound microscope (Olympus).

2.2.2.2 Pollen grain cytology

Fresh pollen grains were harvested from 2 florets each of *E. angustifolia* and *E. purpurea*, just after anther dehiscence. Nuclear material was identified by placing pollen grains onto a microscope slide and staining them with iron acetocarmine (Belling, 1921; Kearns and Inouye, 1993). Pollen grains were examined with a Zeiss Universal microscope and photographed with Fujifilm Superia Iso 100 Daytime Colour film.

2.2.2.3 Pollen viability

The fluorochromatic reaction (FCR) protocol (Kearns and Inouye, 1993) was employed to identify the number of viable pollen grains released by disc florets of the staminate phase and the continuing viability of pollen grains remaining on the underside of the stigmas of pistillate florets in their first and third days of receptivity of *E. purpurea* in a growth chamber (see 2.2.1.3). A 1:50 mixture of FDA: sucrose (FDA: 2 mg FDA/mL acetone; 0.5 M sucrose solution) was placed onto a glass microscope slide and pollen grains from one floret each in the staminate (SP) phase and the first day of the pistillate (PP1) of one inflorescence from each of seven plants were sampled. Viability of pollen grains from three additional PP1 florets was compared against that of three florets in their third day of stigmatic receptivity (PP3) from three plants (three plants, one phase from each plant). SP pollen grains were removed from the pollen mass on the anther one h after presentation and at the same time point pollen was removed from the underside of stigmas from PP1 and PP3 florets where it was held by the sweeping trichomes. The pollen suspension on the slide was incubated at room temperature for 10 minutes in a humidity chamber (60x15 mm Petri dish sealed with moistened filter paper), then covered with a coverslip and observed under fluorescence at 160x magnification (Zeiss Universal microscope). Total grains were counted in 10 randomly selected fields of view, averaging 295 ± 41 pollen grains per anther tube. Viable grains fluoresced green/yellow while unviable grains were not visible.

2.2.3 Disc floret structure and nectary ultrastructure

2.2.3.1 Scanning electron microscopy

For morphological study of overall structure, disc florets of the mature bud, indehiscent staminate (SPi), dehiscent staminate (SPd) and pistillate (PP1 and PP2) stages (see 2.3.1.2 for description) were harvested from *Echinacea purpurea* plants in the growth chamber and *E. angustifolia* plants from MVA and the Biology garden plots, and processed for scanning electron microscopy (SEM) as follows. Florets were removed from inflorescences before fixation in 2% glutaraldehyde (GA) in 25 mM Na phosphate buffer, pH 6.8, for a minimum of 0.5 h. After rinsing three times with buffer, tissues were post-fixed for 2 h in 1% OsO_4 in buffer. Three rinses each of buffer and distilled water were followed by dehydration in a graded series of acetone. Tissues were critical-point dried with liquid CO_2 (Polaron Instruments, Watford, UK), mounted on aluminum stubs with two-sided tape, coated with gold (Edwards S150B Sputter Coater), observed with a Philips SEM 505 at 30 kV, and photographed with Polaroid 665 positive/negative film. Negatives and positives were scanned (Epson 3200 Photo) and images edited using Adobe Photoshop® 7.0.

For detailed studies of nectary morphology, nectaries from disc florets in three distinct phenological stages (mature bud, SPd, and PP1) were removed from mature inflorescences of two *E. purpurea* plants and processed for SEM. Floral organs were removed except for the nectary, which remained upon the inferior ovary. Nectary dimensions and number and developmental stage of the modified stomata on the nectary surface, were compared between stages.

2.2.3.2 Light and transmission electron microscopy

Eleven nectaries of *E. angustifolia* were fixed in 1:3 glacial acetic acid: 70% ethanol for a minimum of 24 h, then softened overnight at room temperature in a 2.5M solution of NaOH, rinsed with distilled water and stained with either I_2KI (starch specific) or 1% decolourized aniline blue (callose specific) and observed using a Zeiss Universal microscope under bright field and fluorescent light.

Disc florets of three distinct developmental stages (SPi - within 1 h following anthesis; late SPd – 24 h after anthesis, as pollen accumulated on the pre-receptive stigma lobes; early PP2 - 48 h post-anthesis, with stigma lobes fully bifurcated and

receptive) were harvested from the same inflorescence and dissected to the nectary atop a cross-sectioned ovary. Tissues were fixed in 1.5% GA in 25 mM Na phosphate buffer, pH 6.8, for 0.5 h at room temperature, before transferring to 3% GA in buffer for 2 h. On ice, samples were rinsed with buffer over 1-12 h, post-fixed overnight with 1% OsO_4, then rinsed with distilled water before dehydration in an ethanol series. A gradual substitution of ethanol by propylene oxide preceded sample infiltration and embedding in Araldite 502 resin at 60°C for 24 h.

For light microscopy, semi-thin (0.5-1 μm) sections of floral nectaries, cut with glass knives on a Reichert OMU3 ultramicrotome, were heat-fixed to microscope slides, stained with toluidine blue, and then mounted in immersion oil under coverslips. Sections were examined with a Zeiss Universal microscope and photographed with Fujifilm Superia Iso 100 film.

For transmission electron microscopy (TEM), ultrathin (70-90 nm) sections were floated onto copper grids and air-dried before staining in 2% uranyl acetate and lead citrate (Reynolds, 1963). Sections were examined using a Philips TEM 4101 LS and photographed with Kodak film. Micrographs were processed electronically as for SEM (section 2.2.3.1).

2.2.4 Nectar dynamics during floret phenology

To estimate the quantity of nectar sugar per stage of floret phenology encountered by potential pollinators on the capitulum of *Echinacea purpurea*, sampling of nectar was attempted from disc florets of 17 inflorescences from five plants, during three intervals (morning: 0900-1100 h; midday: 1101-1400 h; afternoon: 1401-1900h) for each phenological stage (SP, PP1-PP4). *E. purpurea* plants were sampled in a growth chamber (see section 2.2.1.3) in 2004 and 2005 and *E. angustifolia* plants were sampled under field conditions in 2004 and 2005. Nectar was sampled from four inflorescences of *E. angustifolia* plants at MVA and four inflorescences at VR in 2004. The majority of nectar sampling of disc florets of *E. angustifolia* was conducted on 12 plants and 14 inflorescences in the Biology garden plot in the summers of 2004 and 2005. *E. angustifolia* inflorescences were bagged to exclude insects and no insects were present in the chamber that housed plants of *E. purpurea*.

2.2.4.1 Nectar volumes

A 0.2 µL microcapillary tube (Drummond Microcaps®) of common bore was inserted to the corolla base, and volume of withdrawn nectar calculated from the height of the nectar column. From 5-15 disc florets per phenological stage per inflorescence were sampled.

2.2.4.2 Nectar-sugar concentrations

Following volume measurements, nectar was expelled onto the prismatic surface of a hand-held refractometer (0-50%, 40-85%; Bellingham and Stanley, Tunbridge Wells, Kent, UK) modified by the manufacturer for small quantities of nectar. Approximately 90% of nectar samples yielded a reading on the refractometer. These nectar-solute concentrations based on weight (NCW) were corrected to 20.0°C (manufacturer's reference manual).

2.2.4.3 Nectar-sugar quantities per disc floret

Corrected nectar-solute concentrations (%, NCW) were converted to µg sugar per µL nectar (NCV) using the quadratic equation of Búrquez and Corbet (1991). The nectar-sugar quantity per disc floret was calculated as the product of the nectar volume and NCV.

2.2.4.4 Nectar-carbohydrate composition

Nectar samples (1 µL) were pooled from approximately five florets of the SP, PP1 and PP2 phases, and together with 1 µL reference solutions of sucrose, glucose and fructose were applied to a baseline on Whatman No. 1 paper and then overnight placed in a sealed glass vessel containing a solvent mixture of n-propanol: ethyl acetate: H_2O (7:1:2, by volume). The paper chromatogram was air-dried before spraying with p-anisidine-HCL reagent (Block *et al*., 1958) and heated at 70°C for 0.5 h to reveal the colour spots from which R_f values were determined.

For 3-5 plants per species, nectar was collected separately for each of three phenological stages (SP, PP1, PP2) by pooling nectar from several florets per whorl into a 1 µL microcapillary before expulsion onto a filter-paper wick (McKenna and Thomson, 1988). Sampling from these three stages of florets was achievable from consecutive whorls within the same inflorescence, thereby allowing comparison of nectar-carbohydrate composition within a genotype. Stored wicks were later eluted

individually in 2 µL Eppendorf tubes containing 150-1000 µL of pure distilled water, depending on the dilution rate required to allow carbohydrate peaks to fall within the range of standard curves (r = 0.99 for each carbohydrate) created for each major nectar sugar (glucose, fructose, sucrose; 5-200 ppm). After filtering, 50 µL of each sample was analysed in duplicate using a Waters HPLC system, as described in Davis *et al*. (1998).

2.3 Results

2.3.1 Phenology and morphology of ray and disc florets

The phenology and morphology of *E. purpurea* and *E. angustifolia* inflorescences and florets differs very little so they were discussed as a whole. In both species, the first inflorescence to reach anthesis is borne at the terminal end of the central stalk. Other inflorescences may develop lower down on the shoot at the nodes depending on the age of the plant.

2.3.1.1 Ray florets

The cone-shaped inflorescence (capitulum) of *E. purpurea* and *E. angustifolia* enters anthesis with maturation of the outer, single whorl of sterile, ligulate (ray) florets, which surround multiple whorls of disc florets (Fig. 2.1A). Ray florets are the first whorl of florets to reach anthesis. They range in colour from a purplish-pink to a deeper purple and in most cases they droop when mature. The ray florets appear as a "false corolla" surrounding the receptacle that bears the disc florets and must function as pollinator attractants. Ray florets are ligulate, strap-shaped flowers and have three points at their end indicating that the ligulate corolla is the result of fusion of three petals. The corolla is mono-coloured and does not appear to have "nectar guides" visible in the ultraviolet spectrum. Ray florets are sterile, lacking androecium and gynoecium, except for the presence of an ovary. The ovary wall of this achene hardens but does not produce a viable seed within due to the absence of a complete gynoecium.

2.3.1.2 Phenology of disc floret development

After expansion and pigmentation of the corolla of the outer row of ray florets (Fig. 2.1A) that coincided with lateral expansion of the inflorescence, the protandrous

disc florets developed distally and sequentially, in whorls. Disc florets matured from the periphery of the capitulum to the centre. One whorl of mature buds reached anthesis per day, as the indehiscent staminate phase (SPi) characterized by stamen elongation beyond the tubular corolla passed to the SPd stage upon anther dehiscence (Fig 2.1A, C). On the second day of flowering, the elongating style extended through the anther tube and pollen mass before the two stigmas unfolded; this initial pistillate phase was designated PP1 (Fig 2.1A). Stages PP2, PP3 (Fig 2.1C) and PP4 marked the second, third and fourth days of stigmatic receptivity, and the third, fourth and fifth days of flowering, respectively. Disc florets remained in the pistillate stage for several days (eg. PP3 in Fig. 2.1C) until anthesis was complete, or until cross-pollination occurred, wherein the stigmas and style shrivelled and withdrew into the corolla tube as it senesced.

2.3.1.3 Disc floret morphology

Disc florets are born on the "dome shaped" receptacle in the centre of the capitulum (Fig. 2.1A). Each disc floret is subtended by a bract (palea) (Fig. 2.1C left) that gives the capitulum's centre an echinate appearance (Fig. 2.1A). Paleas of *E. purpurea* are predominantly green at the base with a transitional colour zone from yellow in the centre to a conspicuously red summit (Fig. 2.1A, C left). Paleas of *E. angustifolia* are quite similar with green at their base, but the yellow in the centre is more pronounced and red makes up approximately the upper one third. Each disc floret is perfect, having both an androecium and gynoecium. Florets are epigynous with the inferior ovary embedded in the cone shaped receptacle. Mean number of disc florets per capitulum of *E. angustifolia* was 234 ± 7 (n = 76) and 276 ± 19 (n = 10) for *E. purpurea*.

2.3.1.3.1 Calyx

Sepals of the calyx are highly reduced and exist only as a fringe of green tissue on top of the ovary surrounding the base of the corolla (Figs 2.1B left, 2.2D) and are often referred to as a pappus. The pappus is "crown like" with its unevenly toothed margins (Fig. 2.2D).

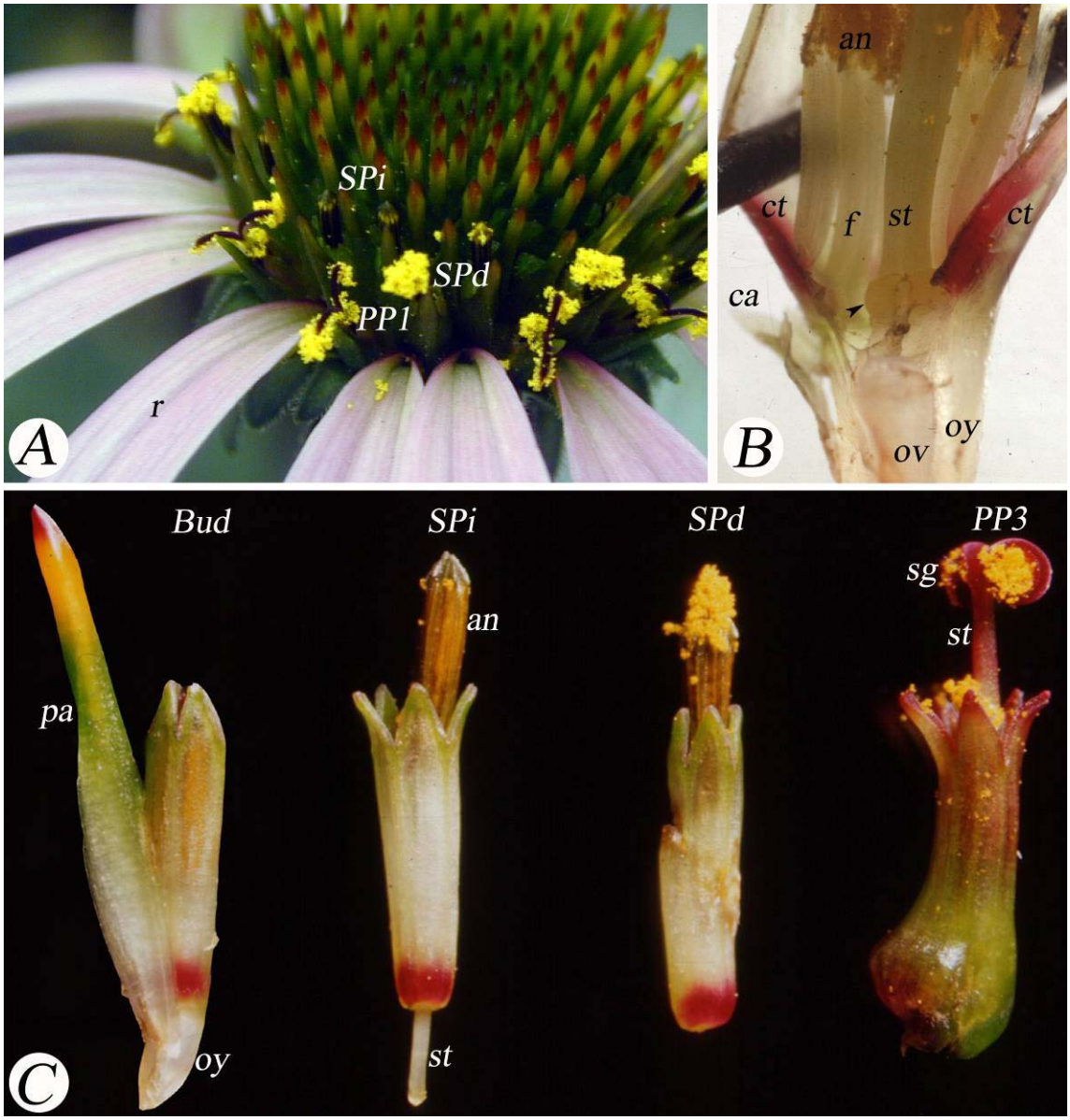
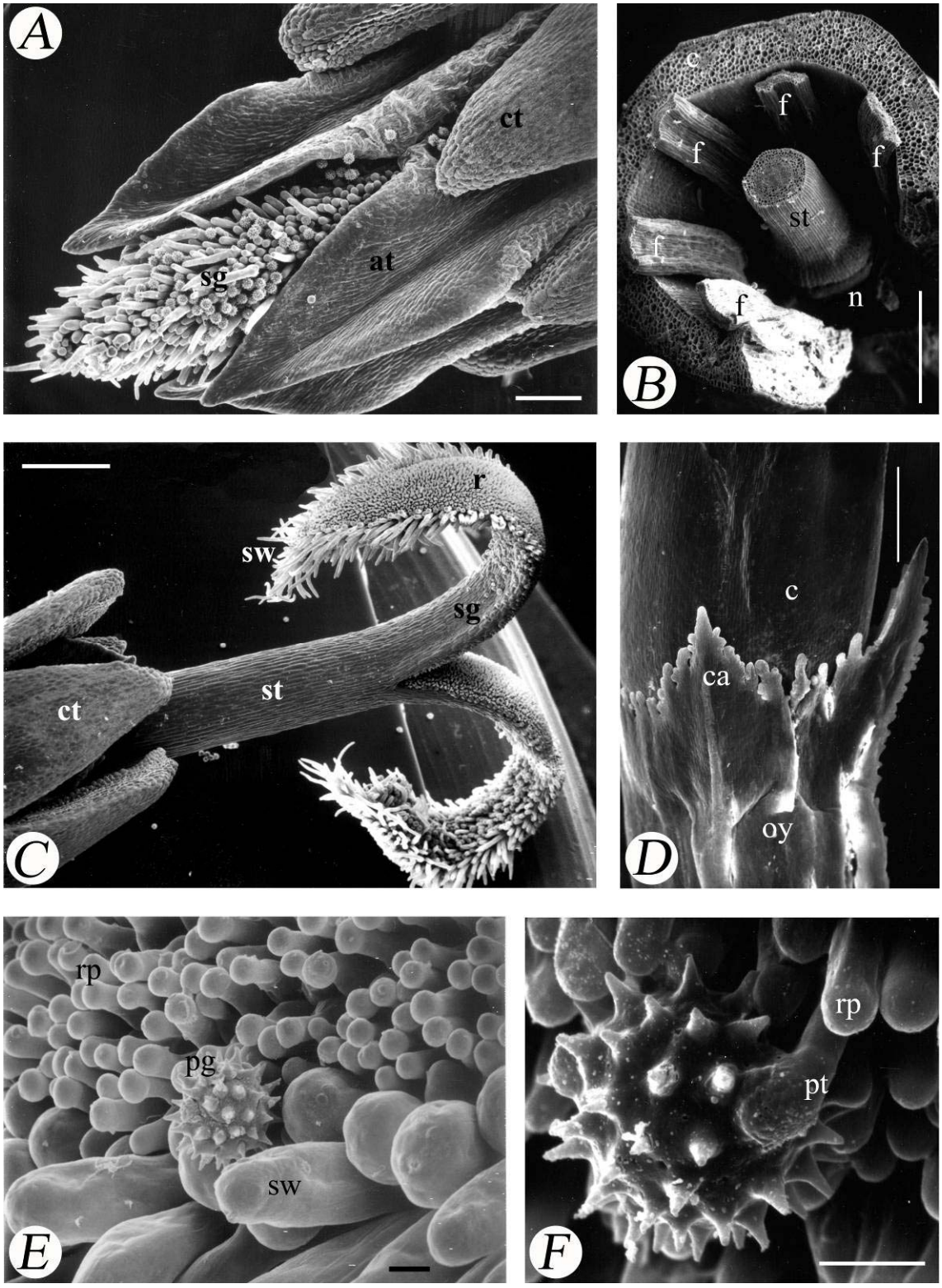


FIG. 2.1: (A) Inflorescence of *E. purpurea* showing disc florets of the indehiscent staminate phase (SPi), dehiscent staminate phase (SPd), first day of the pistillate phase (PP1), and ray florets (r). (B) Disc floret of *E. purpurea* dissected to show the tubular corolla (ct), anthers (an) containing pollen, style (st), ovary (oy), single ovule (ov), calyx (ca) adnate filament (f) and nectary (arrow). (C) Disc florets from four floral phases: mature bud with palea (pa), indehiscent staminate phase (SPi) with style base (st), dehiscent staminate phase (SPd), and third day of the pistillate phase (PP3) with recurved stigmas (sg). Ovary present on bud and absent elsewhere.

FIG. 2.2: SEMs of disc florets. (A) Staminate phase floret of *Echinacea purpurea*. Corolla tube (ct), anther tube (at), stigma (sg) acting as pollen presenter. Scale bar = 0.1 mm. (B) Transverse section through the base of the corolla (c) of a disc floret of *E. angustifolia* indicating the location of staminal filaments (f), style (st) and nectary (n). Scale bar = 0.5 mm. (C) Pistillate phase floret of *E. purpurea*. Stigma (sg) fully reflexed to reveal the receptive surface (r) with the sweeping trichomes (sw) of the pollen presenter curled underneath. Style (st). Corolla tube (ct). Scale bar = 0.5 mm. (D) Disc floret of *E. angustifolia*. Corolla (c), calyx (ca), ovary (oy). Scale bar = 0.5 mm. (E) Self pollen grain (pg) on the receptive surface of an *E. purpurea* stigma at the zone where receptive papillae (rp) meet sweeping trichomes (sw). Scale bar = 10 μm. (F) Pollen grain of *E. angustifolia* with pollen tube (pt) penetrating the receptive surface of a stigma. Receptive papilla (rp). Scale bar = 10 μm.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2.3.1.3.2 Corolla

The five petals were fused into a campanulate corolla (Fig. 2.1C) whose base gradually enlarged (Fig. 2.1C right) and functioned as a nectar reservoir (Fig. 2.2B). At floret maturity, the base of the corolla bulged (Fig. 2.1C). The corolla colour varied from green at anthesis (Fig. 2.1C left) to a reddish-purple in the pistillate phase (Fig. 2.1C right). The tips of the five petals can be distinguished at the upper margin of the corolla tube (Fig. 2.1C, 2.2C).

2.3.1.3.3 Androecium

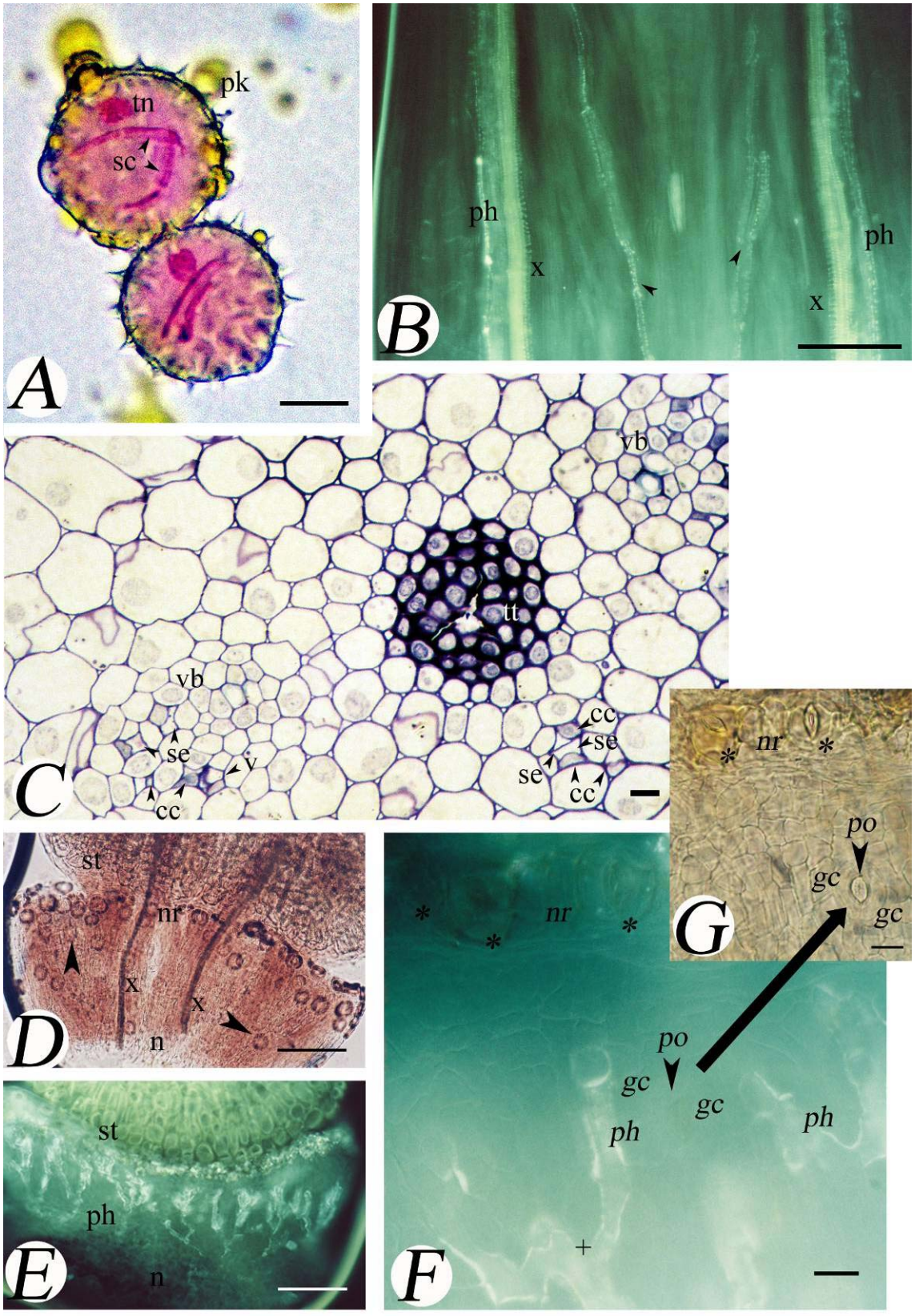
The androecium was composed of five stamens. The filament bases of the five stamens were epipetalous, being adnate to the bases of the five petals (Figs 2.1B, 2.2B, 2.4A left) of each disc floret. Anthers were coalescent and fused into a tube (Fig. 2.2A) while the filaments remained free (Fig. 2.2B). Pollen was released within the anther tube, which split at dehiscence along the apical margins where the anthers met (Figs 2.1C, 2.2A). Pollen grains were released inside the anther tube and were "swept" out and presented upon the top of the anther by the extrusion of the stigma and contraction of the filaments, which was evident when gentle pressure was applied to filaments with a glass micropipette.

2.3.1.3.3.1. Pollen grain structure

Pollen grains of *E. purpurea* and *E. angustifolia* were yellow at dehiscence (Fig. 2.1 A, C) and had a tendency to clump together due to the presence of yellow droplets of oily pollenkitt which were evident adhering to the exterior of pollen grains during light microscopy (Fig. 2.3A). Grains of both species were echinate with microperforated spines, and tricolporate with three typically prominent pores.

Pollen grains of *E. angustifolia* and *E. purpurea* were trinucleate with three distinct nuclei visible (Fig. 2.3A) at the time of anther dehiscence. One round tube cell nucleus (vegetative nucleus), and two laminar sperm cells (each with their own nucleus) were visible within the cytoplasm.

FIG. 2.3: Light and fluorescence micrographs. (A) Trinucleate pollen grains of *E. purpurea* stained with iron acetocarmine to show the round tube nucleus (tn) and the two laminar sperm cells (sc). Pollenkitt (pk). Scale bar = 10µm. (B) Central portion of a longitudinally-oriented style of *E. angustifolia* stained with aniline blue to show isolated sieve tubes (arrows), and the position of phloem (ph) and xylem (x) within the style's two vascular bundles. Scale bar = 50 µm. (C) Transverse section through the style base of a disk floret of *E. purpurea* in the bud phase stained with toluidine blue to show the thick walled cells of the transmitting tissue (tt), isolated phloem sieve elements (ph) with companion cell/sieve element complexes, and the sieve elements (se), companion cells (cc) and xylem vessels (v) of the two vascular bundles (vb). Scale bar = 0.1 µm. (D) Nectary of *E. angustifolia* stained with I_2KI to show guard cells (arrows) containing starch grains along the rim (nr) and side walls of the nectary (n). Note the two vertically-oriented vascular bundles entering the style base (st). Xylem (x). Scale bar = 50 µm. (E, F) Nectaries of *E. angustifolia* stained with aniline blue. (E) Note the position of phloem (ph) sieve tubes of nectary (n) viewed with fluorescence microscopy after staining with aniline blue. Style (st). Scale bar = 50 µm. (F) Fluorescence micrograph of aniline-blue stained nectary showing phloem (ph) sieve tubes branching horizontally (+) before terminating vertically near pores (po) of modified stomata. Guard cells (gc). Modified stomata (*) on nectary rim (nr). Scale bar = 10 µm. (G) Bright field image of nectary from F indicating position of stomatal pore (po) and guard cells (gc) of modified stomate on the nectary wall. Modified stomata (*) on nectary rim (nr) correspond to those in F. Scale bar = 10 µm.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2.3.1.3.3.2 Pollen grain counts

The total number of pollen grains from nine anther tubes representing five inflorescences of *E. purpurea* was counted. Pollen grains counts were as follows: Plant 1: 7464, Plant 2: 10916, 10021, 7722, 10127, 7695, Plant 3: 14231, Plant 4: 11046, Plant 5: 10034. The average number of pollen grains per anther tube was 9917 ± 714 (S.E., n = 9). From plant two, the average number of pollen grains from five indehiscent anther tubes randomly selected from one inflorescence was 9296 ± 666 (S.E., n = 5).

2.3.1.3.3.3 Pollen viability

Viability of grains from pollen masses on SP florets after one hour of presentation was 31.6 ± 8.3% (n = 7) and the viability of pollen on the underside of stigmas of PP1 and PP3 florets was 21.5 ± 17.5% (n = 10), and 2 ± 2% (n = 3), respectively.

2.3.1.3.4 Gynoecium

The gynoecium consisted of one bi-lobed stigma, one style and one ovary. Within the ovary was one ovule (Fig. 2.1B). The presence of the bi-lobed stigma indicated that this was a compound pistil resulting from fusion of two carpels, even though only one ovule was present within the ovary. The ovary was inferior, with attachment of floral parts occurring at its summit. The bi-lobed stigma had long "sweeping" trichomes (Figs 2.2A, C) on the non-receptive surface that collect and trap pollen grains from the interior of the anther tube and present them. The receptive surfaces of the stigma were covered at this time which prevents the majority of self-pollen from contacting it. Reflexing of the stigmatic lobes signified the beginning of the pistillate phase. The receptive surface of the stigma is covered with stigmatic (receptive) papillae (Figs 2.2C, E). The stigma lobes were equal in length and were shorter than the length of the style. If the stigma was not cross-pollinated after several days of receptivity, the stigma lobes reflexed further to bring the receptive surface into contact with self-pollen grains (Fig. 2.1C right) that may remain on the anthers.

One vascular bundle containing both xylem and phloem entered each stigmatic lobe after traveling up either side of the style (Fig. 2.3B) from the style base (Fig. 2.3D), often with several isolated phloem sieve tubes (Figs 2.3B, C) found several cell layers

away from the main sieve tubes of the vascular bundle. In the centre of the style was the transmitting tract for conducting pollen tubes to the ovule (Fig. 2.3C).

2.3.1.3.5 Nectary morphology, anatomy and ultrastructure

Although absent in the ray florets, internal to the base of the corolla tube of the disc floret was a yellowish nectary which formed around the style base (Figs 2.2B, 2.4A) atop the inferior ovary (Fig. 2.1B). Outwardly, the floral nectary had a pentagonal shape (Fig. 2.4B) with a collar-like profile (Fig. 2.4A) and cup-like structure from above (Fig. 2.4B). The nectary of *E. purpurea* was about 360 μm in its widest dimension and 122-154 μm high (Table 2.1). The five-sided nature of the nectary's external form was attributed to its formation following establishment of the pentamerous corolla base that surrounded it. The interior surface of the nectary, however, had a more regular, circular circumference molded around the cylindrical style (Fig. 2.1B), whose inserted, tapered apex (Fig. 2.1C, bottom of SPi stage) below a dilated portion of the style slightly higher up (Fig. 2.4A) may also reflect growth pressure on this organ, exerted by the expanding nectary.

The multicellular floral nectary of *E. purpurea* consisted of three distinct tissues: a direct supply of phloem situated between a large region of secretory parenchyma, located within the interior, and the epidermis, outermost.

2.3.1.3.5.1 Epidermis

This single cell layer was composed of modified stomata dispersed among non-specialized epidermal cells. Trichomes, and subsidiary cells around the paired guard cells per stomate, were absent.

Pistillate phase nectaries of *E. angustifolia* had an average of 36.8 ± 5.1 (S.E., range = 25-59, n = 11) modified stomata with the majority of stomata found on the upper rim but with several found on the lower nectary walls (Fig. 2.3D). Guard cells contained abundant amyloplasts (Fig. 2.3D, F). On average, the nectary surface of *E. purpurea* possessed 29.2 modified stomata per gland, and the data in Table 2.1 suggests that very few of these structures initiate after the mature bud phase is attained. The modified stomata were densest along the uneven upper rim of the nectary (Figs 2.4A, B, 2.5A, B), although they were oriented in different planes and were rarely adjacent to one another (Figs 2.4B, 2.5A, D). Less frequently, stomata were located below the rim and

could be found on both the exterior and interior nectary surfaces (Figs 2.4A, B, 2.5E). Along the rim, the two kidney-shaped guard cells of a modified stomate were raised slightly (Figs 2.4B, D-F), but were flush with adjacent epidermal cells on the external and internal walls of the nectary (Figs 2.5D, E). Each guard cell possessed a centrally-located nucleus (Figs 2.5C top left, 2.5D top right) and abundant amyloplasts (Figs 2.5A-F).

Closest to their anticlinal-wall junctions with non-specialized epidermal cells, circumferential ridges occurred along the external guard-cell walls (Figs 2.4C-E) that contrasted the relatively smooth cuticle originally covering the pore developing between guard-cell pairs. Sometimes the overlying cuticle intervening the two guard cells was folded (Fig. 2.4C), rather than taut, possibly an indication of a reduced guard-cell turgor during fixation and dehydration. As guard cells expanded, this outer cuticle was stretched and eventually could be torn to reveal the pre-formed pore below (Fig. 2.4D).

Each guard cell possessed an outer ledge on its relatively thick ventral wall lining the stomatal pore (Fig. 2.5D top right), which demarcated the future (Fig. 2.4C) and actual (Figs 2.4D, E) site of cuticle rupture.

Various stages of stomatal development were encountered on nectaries examined from mature bud to pistillate-phase florets (Table 2.1): immature stomata in which the pore is not yet revealed externally (Fig. 2.4C), stomata with visibly open pores (Figs 2.4D, 2.5A, B, D top right), and stomata with the pore occluded and not visible (Figs 2.4E, F). From 7.1 to 9.3 % of stomata on average remained immature even in actively-secreting glands (Table 2.1). The majority (87.7%) of the nectary's modified stomata are already open even in the mature bud phase (Table 2.1) prior to anthesis, before nectar secretion begins. Nectar presumably exudes through some or all of the unblocked stomatal pores.

Stomatal pores do not appear to close by guard-cell movements, and pores can still remain open after nectar secretion has ceased. However, pores may become occluded, in up to 16 % of stomata in the pistillate phase (Table 2.1), in different ways that evidently block nectar flow. Figure 2.4E demonstrates the occlusion of a stomatal pore with unknown material completely filling the area below the bases of the cuticular ledges. Occlusion may also occur from below, by a plug of material that protrudes

TABLE 2.1: Dimensions (Mean ± S.E.) of the floral nectaries of *Echinacea angustifolia* and *E. purpurea* and developmental stages and dimensions of the modified stomata of *E. purpurea* at three phenological phases of disc florets. Four florets are represented per phase. Stomatal measurements were taken from 7 open stomata per nectary. *E. angustifolia* measurements were from 7 nectaries (1 mature bud, 3 staminate, 3 pistillate).

			Modified stomata						
	Nectary dimensions (µm)		Developmental stage (%)				Dimensions (µm)		
Floret phase	Exterior width	Height	Total	Immature	Open	Occluded	Width	Length	Pore width
Mature bud	357.5 ± 17.5	137.0 ± 35.0	32.5 ± 4.2	2.8 ± 2.1 (8.3)	28.5 ± 4.7 (87.7)	1.3 ± 0.5 (4.0)	19.8 ± 0.6	21.1 ± 0.7	2.79 ± 0.30
Staminate	367.5 ± 14.4	154.0 ± 22.5	26.8 ± 0.5	2.5 ± 0.9 (9.3)	21.8 ± 0.8 (81.3)	2.5 ± 0.3 (9.3)	19.8 ± 0.5	21.6 ± 0.4	1.71 ± 0.11
Pistillate	362.3 ± 24.5	121.5 ± 12.1	28.3 ± 1.7	2.0 ± 1.2 (7.1)	21.8 ± 2.5 (77.0)	4.5 ± 1.4 (15.9)	22.3 ± 0.5	22.2 ± 0.8	2.43 ± 0.29
E. angustifolia	381.4 ± 22.6	124.3 ± 8.4							

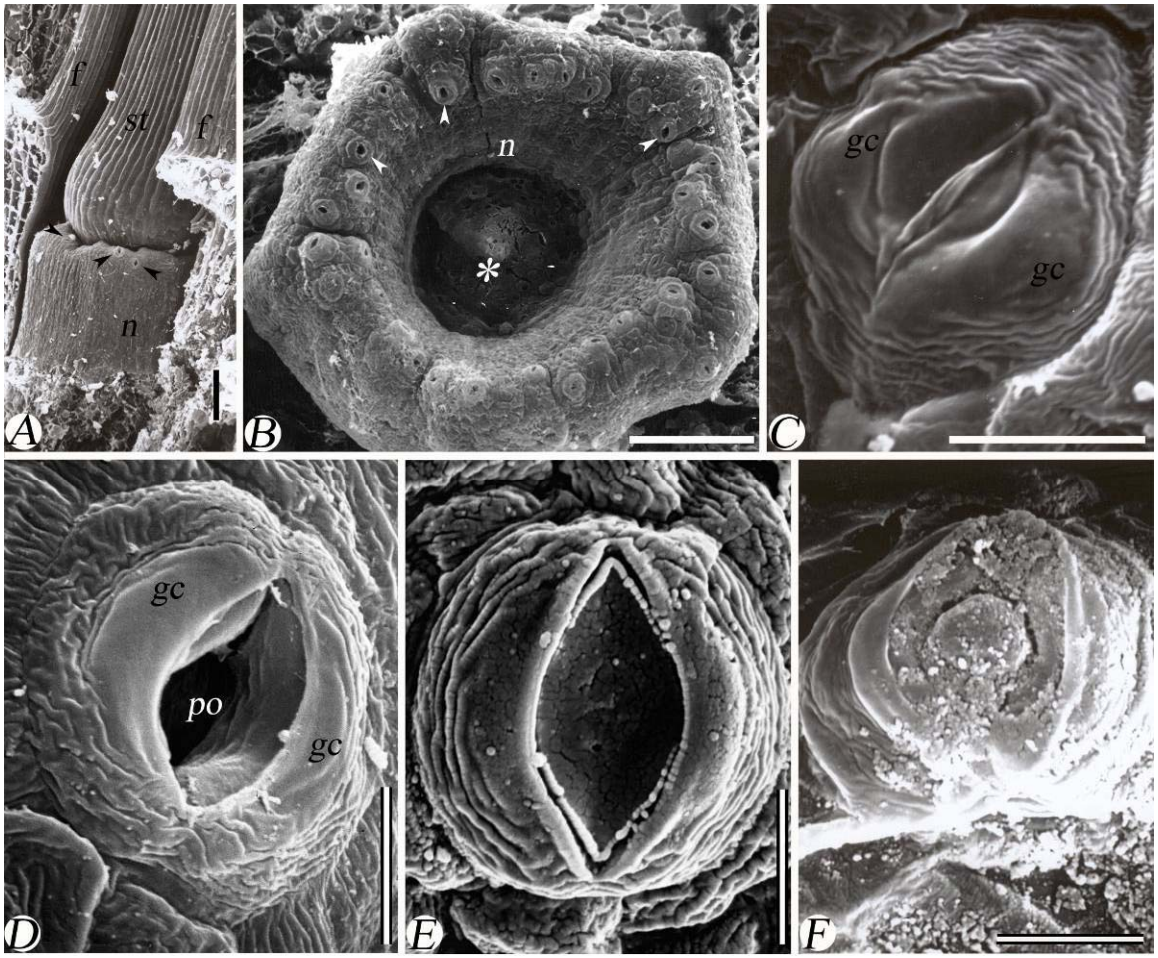


FIG. 2.4: SEM micrographs of the floral nectary and modified stomata of disc florets of *E. purpurea*. (A) Dissected disc floret showing a lateral view of the floral nectary (n) below the swollen base of the style (st). Modified stomata (arrows) along the nectary rim. Filaments (f). (B) Top view of a floral nectary (n) from the bud phase. Modified stomata (arrows) clustered along the rim; note depression (asterisk) where the style base resided. Scale bars = 0.1 mm. (C-F) Developmental stages of modified stomata. (C) Immature stoma with intact cuticle between guard cells (gc). (D) Mature stoma with open stomatal pore (po). Guard cells (gc). (E, F) Mature stomata demonstrating two mechanisms of pore occlusion. Scale bars = 10 μm.

beyond the ledges (Fig. 2.4F); it is possible that a portion of a parenchyma cell normally underlying the guard cells (Figs 2.5C, D, F) may contribute to stomatal pore occlusion in such instances.

Serial sections (Figs 2.5A-C) demonstrated that the substomatal space below a modified stomate was typically small, owing to contact maintained between the guard cells and parenchyma cells immediately below (Figs 2.5D, E). Below an obliquely-sectioned stomate, however, a continuous passage connecting the diminutive substomatal space with a larger intercellular space among the nectary's parenchyma cells, was evident (Fig. 2.5F). The majority of the epidermis comprised less differentiated cells which were compact and tight-fitting (Figs 2.5D, G-J). Recessions representing a limited number of creases particularly along the nectary's interior wall and rim (Fig. 2.4B) permit contact of subepidermal cells to the exterior, between slightly separated epidermal cells (Fig. 2.5E bottom left).

Externally, epidermal cells were ornamented by ridges (Figs 2.4C-E) usually oriented along the cell's length, and similar in pattern to the circumferential ridges on the outer walls of adjacent guard cells (Figs 2.4C-E). Part of a ridge is evident in Fig. 2.6B (top right) and consists of a thickening of the external primary wall, covered throughout by an osmiophilic cuticle (Figs 2.6A-C) of 185 ± 10 nm thickness. Above a junction of anticlinal walls of non-guard epidermal cells, the deposition of osmiophilic globules (possibly pre-cuticle components) beneath an established layer of cuticle, was evident (Fig. 2.6D). The cuticle had a bipartite nature, consisting of a very fine, intact epicuticular layer (42.6 ± 4.0 nm) subtended by a thicker (142 ± 11 μm) layer regularly interrupted by channels reaching the epicuticle (Fig. 2.6D). These thicker regions of wall above the anticlinal wall junctions stained lightly (Figs 2.6C, D) or densely osmiophilic (Fig. 2.6B).

Each epidermal cell typically possessed a single, large vacuole (Figs 2.5C-E) traversed by cytoplasmic strands (Fig. 2.6A left). Occasionally, various vacuolar inclusions such as multitubular and multilamellar bodies, as well as a flocculant material, occurred (Fig. 2.6A). In the cytoplasm, a prominent nucleus of spherical (Figs 2.5G, H) or more irregular (Figs 2.5D, E) outline, resided.

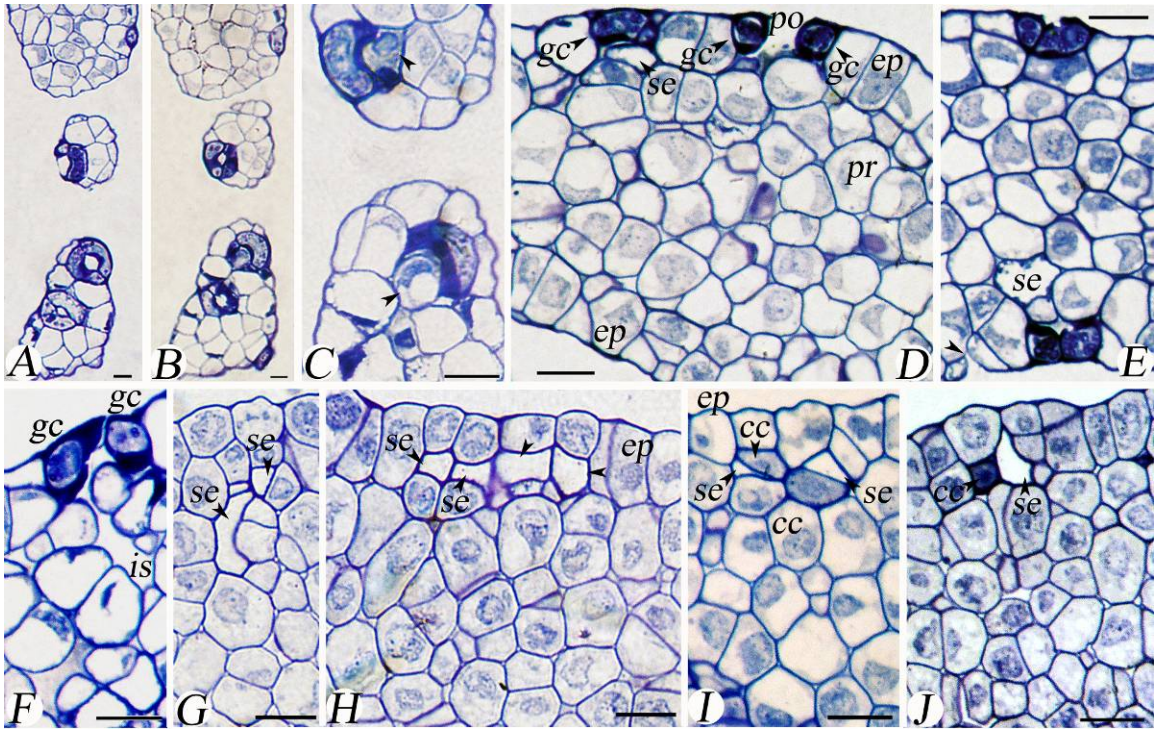


FIG. 2.5: Light micrographs of transverse sections through the floral nectary of a pistillate phase floret of *E. purpurea* stained with toluidine blue. (A-C) Serial sections through the rim of the floral nectary showing stomata and upper parenchyma. (C) Parenchyma cells (arrows) evident directly beneath the stomatal apertures. (D-F) Sections taken immediately below the apical rim, where stomata are cut in various planes. (D) Two stomata on the outer epidermis of the nectary. Outer (uppermost), inner (lowermost) epidermal layers (ep), and intervening parenchyma cells. Guard cells (gc), stomatal pore (po). Possible sieve element (se) below guard cells at top left. (E) Stomata on the outer and inner nectary surfaces. Sieve element (se) near stomate on inner epidermis. Note the non-stomatal opening (arrow) in the inner epidermis. (F) Substomatal chamber is continuous with a large intercellular space (is). (G-J) Mid-region of the floral nectary. (G) Sieve tube (arrows) branching toward the epidermis. (H) Four sieve tube elements (se) next to the outer epidermis (ep). (I) Two sieve tube elements (se) of a sieve tube and their darkly staining companion cells (cc) adjacent to the outer epidermis (ep). (J) A companion cell (cc) and adjacent sieve element (se) intervening the bases of cells of the nectary's outer epidermis. Scale bars = 10 µm.

An abundance of ribosomes in the ground substance gave a dense and granular appearance to the cytoplasm (Figs 2.6A-D). Mitochondria with prominent cristae (Figs 2.6A-D) and plastids with plastoglobuli but very few thylakoid membranes (Fig. 2.6B) were abundant organelles. Microbodies (Fig. 2.6B), rough endoplasmic reticulum (Figs 2.6A, B) and dictyosomes (Figs 2.6B, C) were also evident. The latter were common in the vicinity of the cell membrane (Figs 2.6B, C) and the irregular internal surface of the cell membrane and primary wall beneath (Figs 2.6A-D) may reflect cell-wall deposition involving dictyosome vesicles. Plasmodesmata traversed the walls between epidermal cells and between epidermal cells and the nectariferous parenchyma cells below them (Fig. 2.6A).

2.3.1.3.5.2 Nectary vasculature

Xylem was not present in the nectary vasculature of *E. angustifolia* but many phloem sieve tubes were observed (Fig. 2.3E, F). Certain developmental features of the nectary phloem are noteworthy. Depending on the particular phloem trace, contact of a sieve tube with the epidermis may occur predominantly via the sieve tube elements (Figs 2.5H, 2.5I right), the companion cells (Figs 2.5G, 2.5I left, 2.6A), or be shared almost equally (Fig. 2.5J). Sieve tube branching was often observed where a vertically oriented sieve tube turned, travelled horizontally, and branched to form two or three sieve tubes (Fig. 2.3F, left). Also, in a horizontally-oriented sieve tube (Fig. 2.5H) believed to yield a vertically-oriented trace leading higher up within the nectary as branches at each of its left and right ends (Fig. 2.3F bottom left), the origin of the sieve element-companion cell precursor cells, from epidermal cells on the outer nectary surface, can be inferred. Based on their common widths between anticlinal walls, the epidermal cells above these four sieve elements had divided periclinally, evidently to yield the sieve element-companion cell precursors which themselves subsequently have divided periclinal to the outer surface, to form companion cells below the sieve elements. That multiple companion cells may eventually arise per sieve element is suggested from the two right-most sieve elements, which are superior to at least two smaller companion cells each (Fig. 2.5H). Meanwhile, epidermal cells to the left and right of the mature sieve elements remain columnar (Fig. 2.5H), possibly delineating the original size of the four epidermal cells between them, which have participated in the formation of phloem.

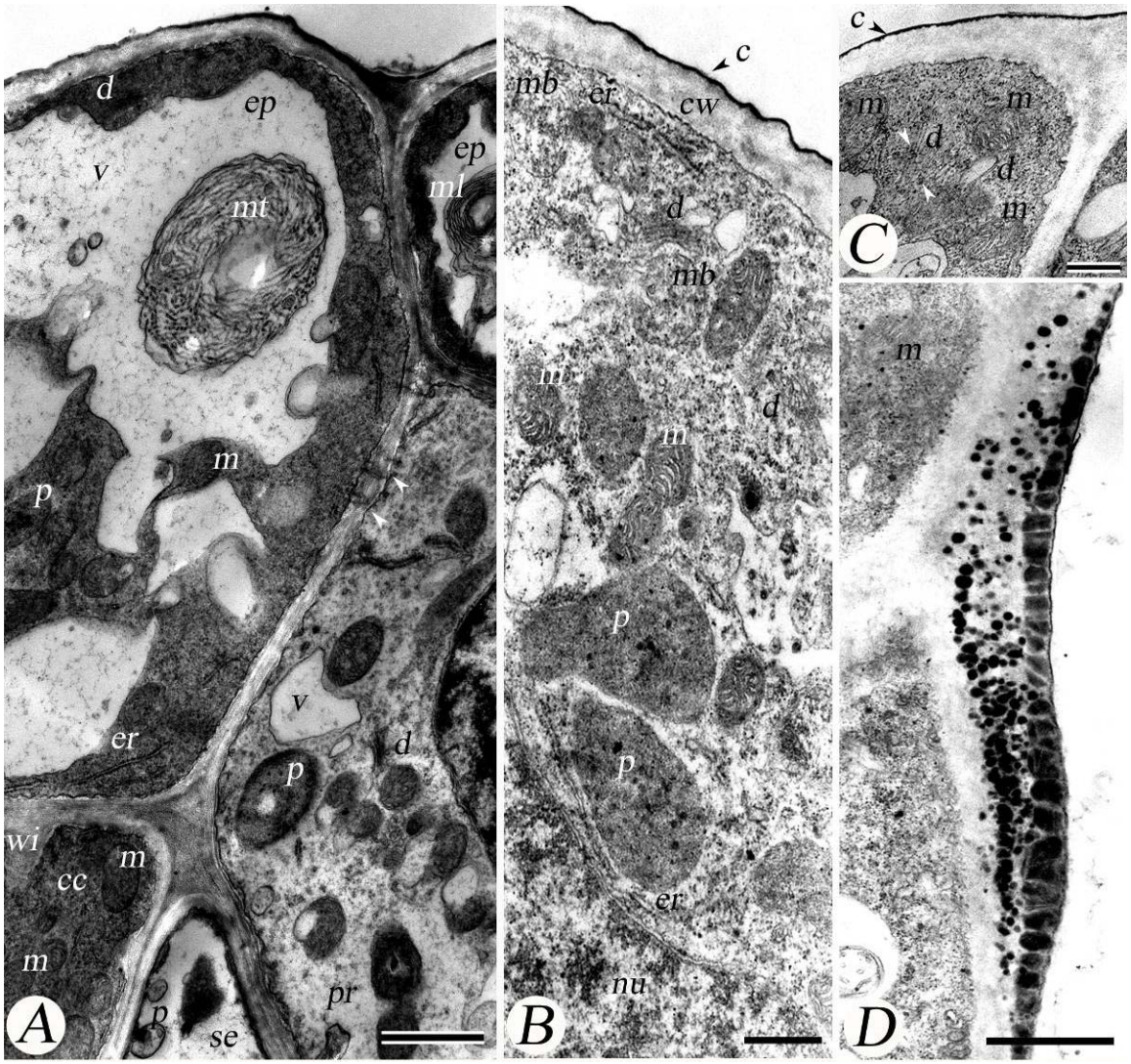


FIG. 2.6: Transmission electron micrographs of the epidermis of floral nectaries of *E. purpurea.* (A) Epidermal cells (ep) showing mitochondria (m), plastids (p), and rough endoplasmic reticulum (er) in association with ribosomes and membranous multilamellar (ml) and multitubular (mt) bodies within large vacuoles (v). Plasmodesmata (arrows) evident in the wall between epidermal and phloem parenchyma cell (pr). Sieve element (se) with plastids (p) and companion cell (cc) with mitochondria (m) and wall ingrowth (wi) next to epidermis. PP1 phase. Scale bar = 1 µm. (B) Epidermal cell with thin cuticle (c) covering the outer wall (cw), plastids (p) containing electron-dense plastoglobuli, rough endoplasmic reticulum (er), dictyosomes (d), mitochondria (m), microbodies (mb), and nucleus (nu). SPi phase. Scale bar = 1 µm. (C) Note thin cuticle (c) and two dictyosomes (d) surrounded by vesicles (arrows) and the unevenness of the anticlinal wall between the two epidermal cells. SPd phase. (D) Outer junction of two epidermal cells showing deposition of osmiophilic globules along the outer walls. Microchannels evident in cuticle. SPi phase. Scale bars = 0.2 µm.

Phloem sieve tubes in nectaries of *E. angustifolia* did not reach modified stomata on the nectary rim but extended into the upper third of the nectary, several micrometres below the rim (Fig. 2.3E). Sieve tubes sometimes terminated near pores of modified stomata midway up the nectary wall (Fig. 2.3F).

Mature sieve elements of the nectary interior were anucleate (Fig. 2.7A) and possessed a peripheral cytoplasm (Figs 2.5E, 2.7A) that included organelles such as plastids (Figs 2.6A, 2.7A top), mitochondria (Figs 2.7A centre, 2.7D) and endoplasmic reticulum (Figs 2.7D, E). The lumen contained p-protein (Figs 2.6A, 2.7A, B, D). The sieve plate between sieve elements contained pores lined by unstained (white) callose (Fig. 2.7A). Sieve elements were connected by plasmodesmata to phloem parenchyma (Figs 2.7A bottom left, 2.7D) and companion cells (Fig. 2.7E). Intercellular spaces occurred next to sieve elements (Figs 2.5E, 2.7A top, 2.7B).

Companion cells were larger than (Fig. 2.5I), similar in size (Fig. 2.7A), or dwarfed by (Fig. 2.5H right) their adjacent sieve elements. The dense-staining cytoplasm contained plentiful ribosomes in its ground substance (Figs 2.7C, E), as well as a prominent nucleus (Figs 2.5H, I, 2.7A, C) and dictyosomes (Fig. 2.7E). Their small vacuome (Figs 2.7A, E) contained a flocculent material, and inclusions ranging from multiple vesicles (Fig. 2.7E left) to multilamellar bodies (Fig. 2.7E right).

Characteristics of the companion-cell walls were also noteworthy. Companion cells were the only nectary cells to be modified as transfer cells (Type A; Gunning and Pate, 1969), their wall ingrowths consisting of secondary wall material atop the primary wall (Figs 2.6A, 2.7E). The wall ingrowths were directed toward epidermal cells (Fig. 2.6A), phloem parenchyma (Figs 2.7A, E), other companion cells (Fig. 2.7A top), but never toward sieve elements (Figs 2.6A, 2.7A, E). Putative plasmalemmasomes (Fig. 2.7C) and vesicles external to the cell membrane (Fig. 2.7C top right) also occurred at the primary wall. Plasmodesmata connected companion cells to sieve elements (Fig. 2.7E) and phloem parenchyma (Fig. 2.7C). Intercellular spaces were also located next to companion cells (Figs 2.7A, C).

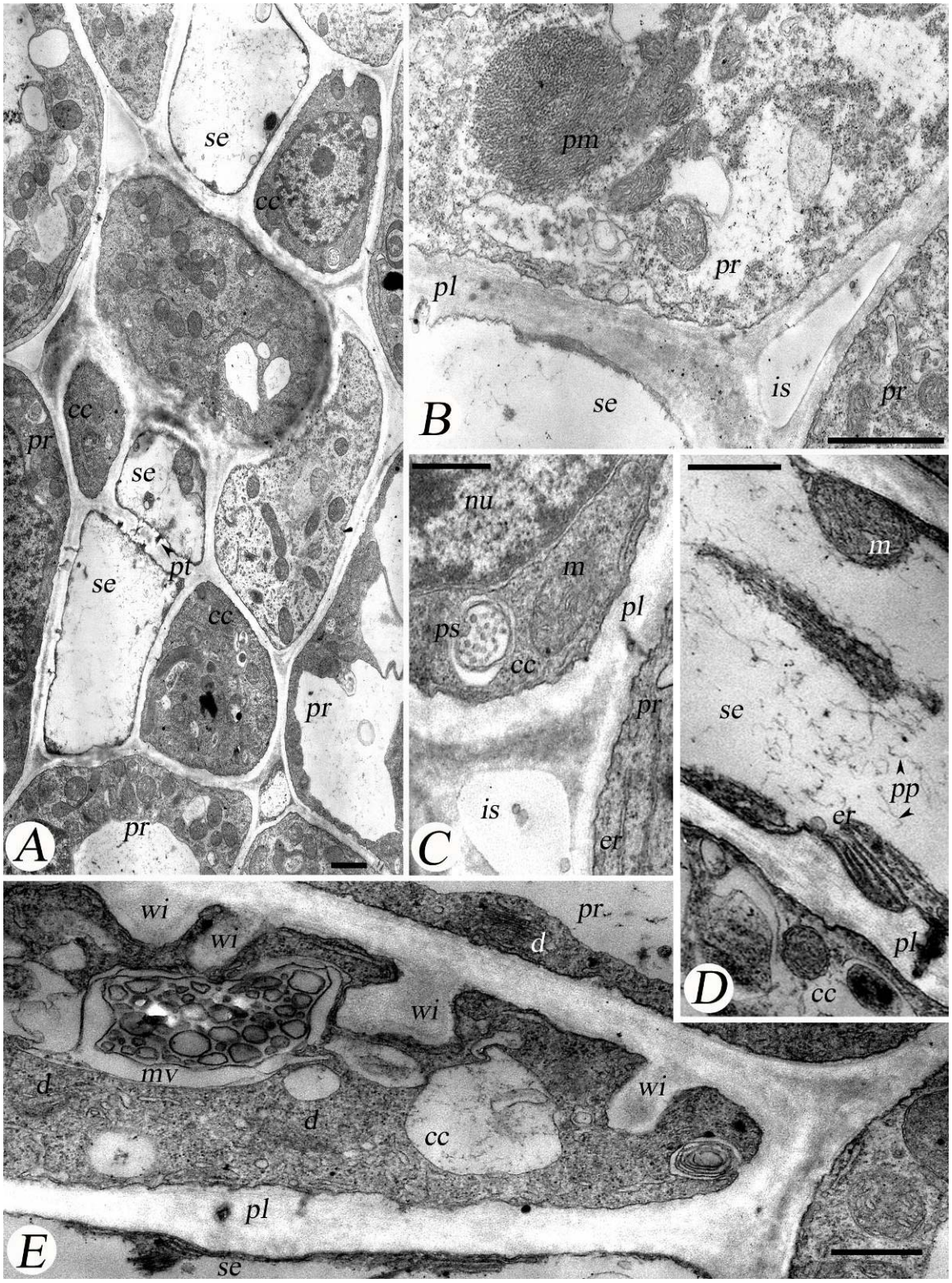
Parenchyma cells adjacent to sieve elements and companion cells were typically larger and less densely stained (Figs 2.6A, 2.7A), and were designated phloem parenchyma. These cells possessed very similar features to the parenchyma cells (see

below) that formed the remainder of the nectary. Phloem parenchyma cells often contained a multilobed nucleus (Fig. 2.8B), fibrillar proteinaceous material (Fig. 2.7B), dictyosomes (Figs 2.6A, 2.7E, 2.8B), mitochondria (Fig. 2.8B), endoplasmic reticulum (Fig. 2.7C), and plastids (Figs 2.6A, 2.7A) that can contain starch (Fig. 2.8B). Phloem parenchyma cells were connected by plasmodesmata to sieve elements (Fig. 2.7A), companion cells (Fig. 2.7C) and epidermal cells (Fig. 2.6A), and they also opposed intercellular spaces (Figs 2.7A-C).

2.3.1.3.5.3 Parenchyma cells

Thin-walled parenchyma cells comprised the bulk of the nectary interior (Figs 2.5D-J). Each cell contained a dense-staining nucleus, often spherical (Fig. 2.8A) or lobed (Figs 2.5H, J, 2.8B), with multiple dense nucleoli (Figs 2.5E, H, J). Plastids were homogenous with osmiophilic plastoglobuli (Figs 2.8A, 2.9A-C), very few thylakoids (Figs 2.9A-C), and lacked starch (Fig. 2.8A) or possessed a single starch grain per plastid profile (Figs 2.8B, 2.9B). Plastids commonly were lobed (Fig. 2.9A) and often surrounded a mitochondrion (Figs 2.7A top right, 2.9C top right). Mitochondria were very abundant and had well-developed cristae (Figs 2.8A, 2.9A, B). Dictyosomes (Figs 2.8A, B, 2.9A, B), rough endoplasmic reticulum (Fig. 2.8A) and microbodies (Fig. 2.9A) were also common. Each cell contained a large vacuole (Figs 2.5D-F, I, 2.9C) or multiple small vacuoles (Figs 2.5H, J, 2.8A, 2.9A). Many vacuoles contained a flocculent material (Figs 2.9A-C). Inclusions of variable form were occasionally present. A multilamellar body was continuous with a multitubular body within a vacuole (Fig. 2.9B), indicating that the two inclusions may intergrade. In other examples, cells with the nucleus confined to the periphery and containing several membrane-bound inclusions (Fig. 2.9C) may indicate a stage of degradation. Plasmodesmata occurred between parenchyma cells (Figs 2.8A, 2.9B, C). Intercellular spaces were located between parenchyma cells (Fig. 2.5F), sometimes with apparently cellular debris (Fig. 2.8A) and darkened regions where the middle lamella ended (Figs 2.9B, C).

FIG. 2.7: Transmission electron micrographs highlighting the vascular tissue of floral nectaries of *E. purpurea*. (A) Nectary interior showing portion of a sieve tube with pores of a sieve plate (pt) between adjacent sieve elements (se). Companion (cc) and parenchyma (pr) cells. PP1 phase. (B) Sieve element (se) next to an intercellular space (is). Parenchyma cells (pr). Part of a plasmodesma (pl). Proteinaceous material (pm). SPi phase. Scale bars = 1 μm. (C) Companion cell (cc) with nucleus (nu), mitochondrion (m), and possible portion of a plasmalemmasome (ps). Plasmodesma (pl) connecting companion cell with parenchyma cell (pr). Rough endoplasmic reticulum (er). PP1 phase. Scale bar = 0.5 μm. (D) Peripheral cytoplasm of a sieve element (se) containing endoplasmic reticulum (er) and a mitochondrion (m) adjacent to a companion cell (cc). P-protein (pp) in the lumen. Plasmodesma (pl). PP1 phase. Scale bar = 1 μm. (E) Companion cell (cc) with wall ingrowths (wi) opposing a parenchyma cell (pr). Dictyosomes (d), and a multivesicular inclusion (mv) in vacuole (v). Portions of plasmodesmata (pl) between sieve element and companion cell. Peripheral cytoplasm visible in sieve element (se). Parenchyma cell (pr) with dictyosome (d). PP1 phase. Scale bar = 0.5 μm.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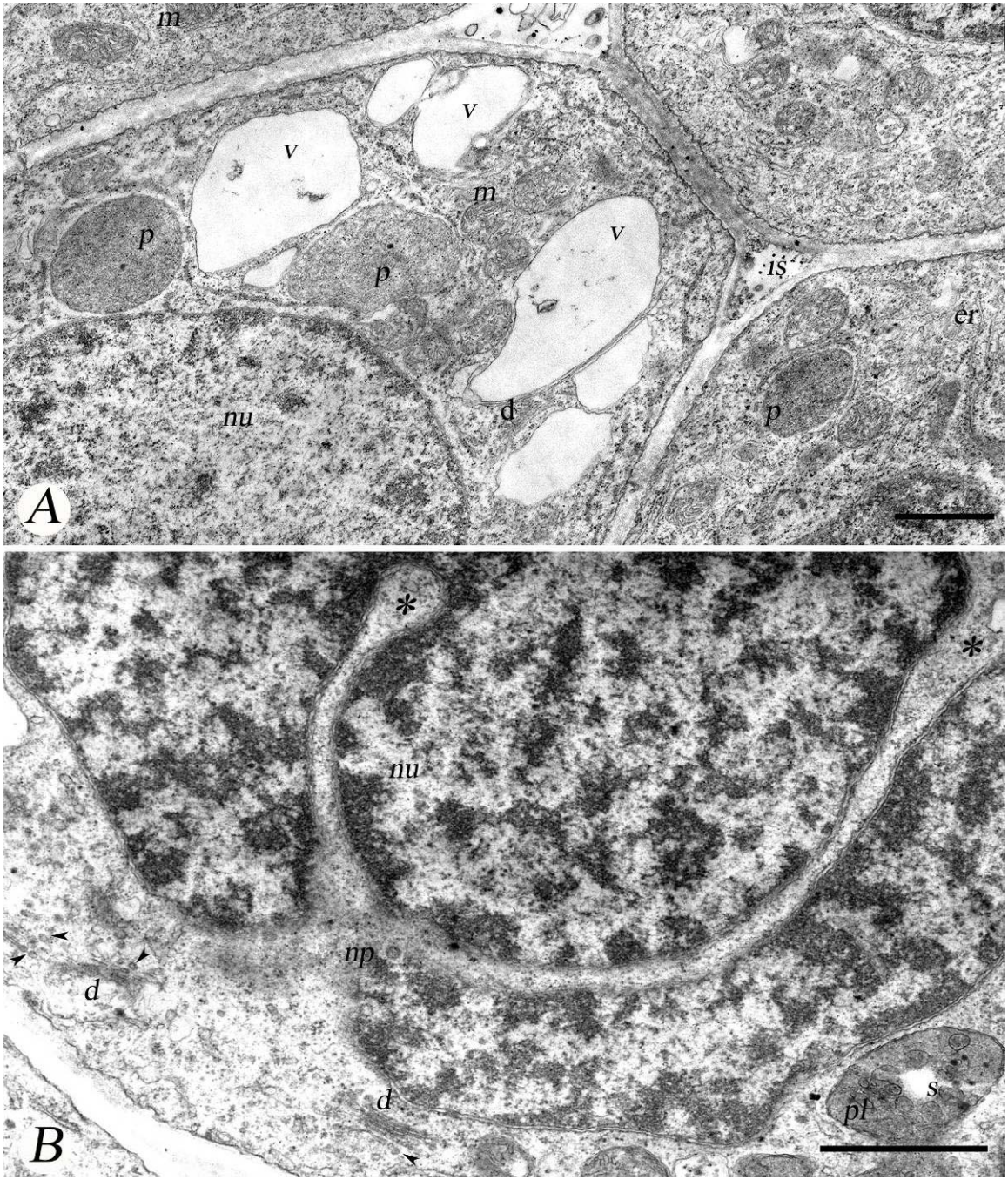
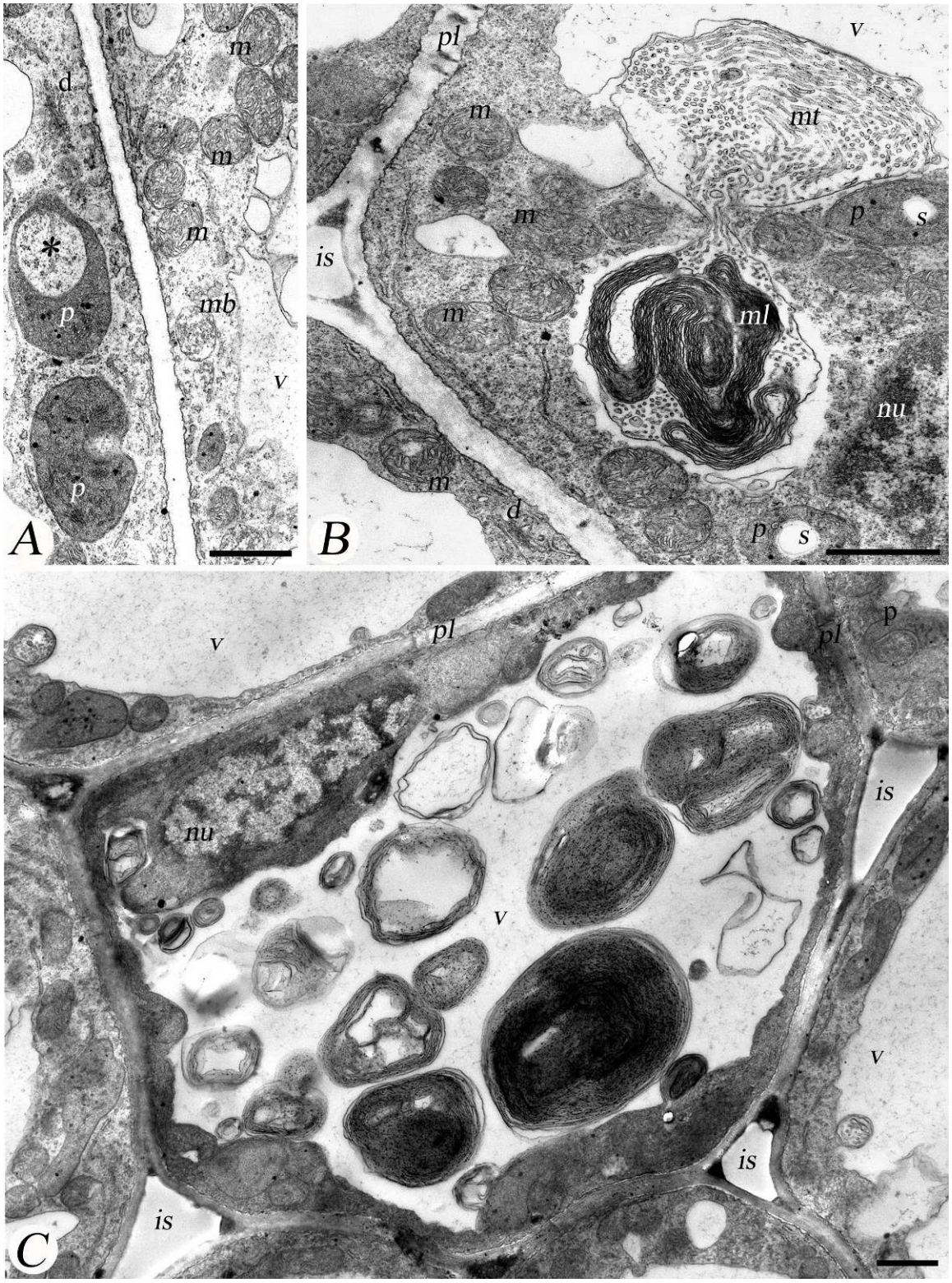


FIG. 2.8: Transmission electron micrographs of the parenchyma of floral nectaries of *E. purpurea.* (A) Nuclei (nu), vacuoles (v), plastids (p), mitochondria (m), dictyosome (d) and rough endoplasmic reticulum (er). Intercellular spaces (is) containing amorphous material. SPi phase. (B) Nucleus (nu) with three prominent lobes encompassing cytoplasm (*). Nuclear pores (np) in nuclear membrane. Dictyosomes (d) and vesicles (arrows). Plastid (p) with starch grain (s). PP1 phase. Scale bars = 1 μm.

FIG. 2.9: Transmission electron micrographs of parenchyma cells of floral nectaries from disc florets of *E. purpurea* in the pistillate phase (PP1). (A) Lobed plastids (p) enveloping cytoplasm (*). Aggregation of mitochondria (m). Dictyosome (d). Microbody (mb). (B) Multitubular (mt) and multilamellar (ml) bodies apparently enclosed by a continuous membrane that abuts the tonoplast of a vacuole (v). Plastids (p) containing starch grains (s), mitochondria (m), dictyosome (d), nucleus (nu) and plasmodesmata (pl) between parenchyma cells. Intercellular space (is). (C) Parenchyma cell with vacuole (v) containing many membrane-bound inclusions possibly undergoing degradation. Nucleus (nu). Plasmodesmata (pl). Scale bars = 1 μm.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2.3.2 Dynamics of nectar secretion: *Echinacea purpurea*

Plants of *E. purpurea* grown in a growth chamber under a regular regime of lighting and temperature (see 2.2.1.3) were not subjected to environmental fluctuations typical of field conditions. Thus, the major variable in this study was phenological stage of the disc florets. Fig. 2.10A illustrates mean nectar volume per disc floret spanning the commencement of nectar accumulation (morning of the staminate phase SP) to the cessation of nectar production by the end of the third day of the pistillate phase (PP3), four days post anthesis.

2.3.2.1 Nectar volume

At anthesis, disc florets of *E. purpurea* in the staminate phase held an average of 0.07 μL of nectar that increased to 0.15 μL later that first day (Fig. 2.10A). Nectar volume declined overnight, reaching 0.1 μL in the morning before rising significantly to 0.19 μL (midday) and 0.18 μL (afternoon) - the peak values for disc florets – on the second day post anthesis (PP1). On the second day of the pistillate phase (PP2), nectar volume remained significantly lower and fairly constant at about 0.1 μL per disc floret, before declining to 0.05 μL during the third day of the pistillate stage (Fig. 2.10A), when only 24% of sampled florets yielded nectar. All florets lacked nectar by the fourth day of stigma receptivity (PP4) (Fig. 2.10A). On a daily basis, nectar volumes tended to be highest at midday (Fig. 2.10A).

2.3.2.2 Nectar-solute concentrations

Average nectar-solute concentrations (refractometer measurements) rose throughout the staminate phase of disc florets from 33.4% (morning) to 43.2% (midday) and 53% (afternoon). However, from the first day of the pistillate phase (PP1) to the end of nectar secretion, nectar solute concentrations remained constant ($P = 0.204$, $\alpha = 0.05$), averaging 61.1 ± 1.1%.

2.3.2.3 Nectar-sugar quantity

Nectar-sugar quantity per disc floret rose from 26.9 μg in the first hours after anthesis to 94.3 μg by the afternoon of the staminate phase (SP) (Fig. 2.10B). Owing to the consistency in nectar-solute concentration throughout stigma receptivity, thereafter nectar-sugar quantity per disc floret (Fig. 2.10B) closely mirrored the pattern of nectar volume (Fig. 2.10A). Thus, peak quantities of nectar sugar per floret occurred at midday

(156.6 µg) and afternoon (150.7 µg) of PP1 (Fig. 2.10B). The following day, 80 µg of nectar sugar per floret had fallen to 65.9 µg in the afternoon of PP2, and decreased to 42.5 µg on the third day of stigma receptivity (PP3) before cessation of nectar production at PP4 (Fig 2.10B).

In 2005, several bagged inflorescences of *E. purpurea* under field conditions were sampled for nectar to be analysed for its carbohydrate composition, and it was noted that nectar was plentiful in SP and PP1 disc florets but available only sporadically in PP2 disc florets. This finding indicates that nectar secretion diminishes after PP1 and reabsorption of nectar begins as florets enter the second day of receptivity (PP2).

2.3.2.4 Nectar-carbohydrate composition

Nectar of all three disc-floret stages (SP, PP1, PP2) contained sucrose, glucose and fructose according to paper chromatography. Fructose (R_f = 0.446) and sucrose (R_f = 0.393) standards produced yellow spots, whereas glucose (R_f = 0.436) was brown. Nectar of the three disc-floret stages contained all three carbohydrates, but relative quantities differed per stage. Glucose appeared most abundantly in the PP1 and PP2 stages, sucrose was highest in the SP and PP1 stages, and fructose remained approximately the same, throughout.

In accordance with this preliminary study using paper chromatography, the floral nectar of *E. purpurea* was confirmed by HPLC to be dominated by the hexose sugars throughout flowering phenology, with a reduction in sucrose content as florets aged. For example, from one inflorescence of plant 5 in the growth chamber, the G/F/S ratio of floral nectar sampled separately from disc florets of the three adjacent whorls (SP, PP1, PP2) was 1.8/2.0/1, 3.3/3.4/1, and 3.9/4.1/1, respectively. The increase in the hexose sugars with concurrent reduction in sucrose content was even more striking in three field-grown plants, by the PP2 stage. For example, in Plant 1 the G/F/S ratio at SP (1.3/1.2/1) and PP1 (1.2/1.3/1) changed dramatically by PP2 (27.2/27.8/1). In Plant 3, the G/F/S ratio at SP (2.2/2.4/1) and PP1 (2.3/2.5/1) had become modified at PP2 (6.0/7.8/1). In Plant 2, hexose dominance occurred sooner and was actually highest at PP1; G/F/S ratio was 2.4/2.0/1 at SP, 25.7/21.8/1 at PP1, and 16.0/18.1/1 at PP2.

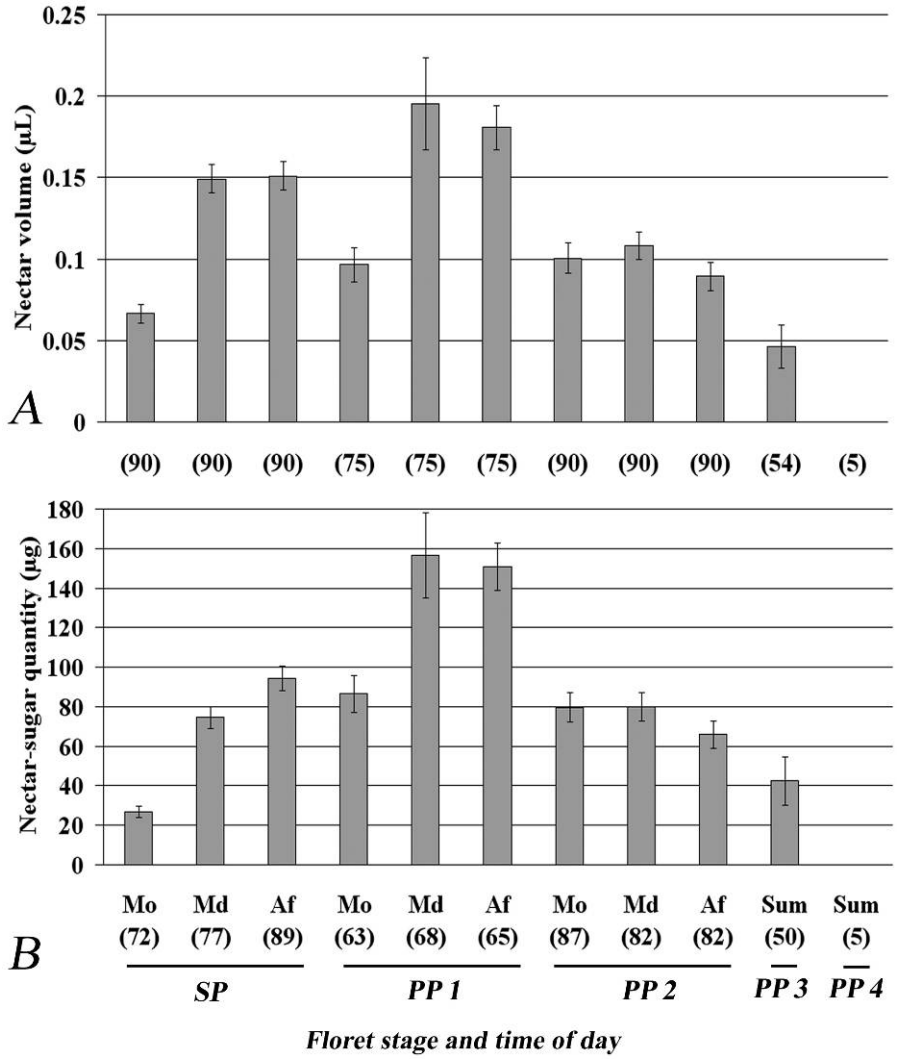


FIG. 2.10: Nectar characteristics of disc florets of *E. purpurea*. (A) Nectar volume (Mean ± S.E.). (B) Nectar-sugar quantity (Mean ± S.E.). Morning (Mo), midday (Md), afternoon (Af). Staminate phase (SP), first pistillate phase (PP1), second pistillate phase (PP2), third pistillate phase (PP3) and fourth day of the pistillate phase (PP4). The numbers in brackets represent, per floret stage and time of day, the total number of disc florets sampled (A) and all sampled florets except those that failed to yield sufficient nectar for refractometry (B). Many of the SP (Mo) florets were indehiscent; all other staminate phase florets were dehiscent.

Although the floral nectars of both *Echinacea* species were hexose-rich or –dominant, they differed in which hexose sugar predominated. In *E. angustifolia*, the quantity of glucose exceeded the fructose in 6 of 9 samples, whereas in *E. purpurea*, fructose exceeded glucose in 12 of 15 samples. The reduction in sucrose content as florets aged was less striking in *E. angustifolia*, but for the most part still followed the same general trend as *E. purpurea*. For example, from plant 1, the G/F/S ratio of floral nectar sampled separately from disc florets of the three adjacent whorls (SP, PP1, PP2) was 5.3/5.8/1, 3.8/3.8/1, and 6.8/6.8/1, respectively, whereas plant 3 had more sucrose in PP2 (4.4/4.3/1) than in SP (12.8/9.9/1) or PP1 (15.8/16.5/1) florets.

2.3.3 Dynamics of nectar secretion: *Echinacea angustifolia*

Plants of *E. angustifolia* that grew at three field sites (see 2.2.1) were also sampled for nectar similarly to *E. purpurea*, and these data were combined. Fig. 2.11 illustrates the changes in mean nectar volume and nectar-sugar quantity per disc floret spanning the commencement of nectar accumulation (morning of the staminate phase SP) to the cessation of nectar production by the end of the third day of the pistillate phase (PP3), four days post anthesis.

2.3.3.1 Nectar volume

At anthesis, disc florets of *E. angustifolia* in the staminate phase (SP Mo) held an average of 0.06 µL that increased to 0.18 µL at midday and peaked at 0.24 µL in the afternoon (Fig. 2.11A). Nectar volume did not decrease significantly overnight and remained high for the morning of the first pistillate day (PP1 Mo) at 0.23 µL. Nectar volume began to decrease during the day of the first pistillate phase to 0.17 µL at midday and 0.19 µL in afternoon (Fig. 2.11A). Mean nectar volume decreased significantly on the second day of the pistillate phase (PP2) and remained fairly constant during the day at just below 0.1 µL per disc floret (Fig. 2.11A). By the morning of the second pistillate day (PP2), only 60% of sampled disc florets contained nectar and at midday and afternoon samplings only 42% of florets yielded nectar. Nectar volume declined to 0.01 µL during the third day of the pistillate phase where most florets (84%) did not yield nectar. By the fourth day of the pistillate phase (PP4), nectar production had all but ceased with only one of 62 florets yielding nectar.

2.3.3.2 Nectar-solute concentrations

Average nectar-solute concentrations (refractometer measurements) rose throughout the staminate phase of disc florets from 40.4% (morning) to 54.4% (midday) and 63.0% (afternoon). However, during the first day of the pistillate phase (PP1) nectar solute concentrations remained relatively constant, averaging 63.6 ± 0.76%. Nectar solute concentration averaged 62.6 ± 1.36% during the second pistillate day (PP2) for florets containing nectar.

2.3.3.3 Nectar-sugar quantity

Nectar-sugar quantity per disc floret rose from 40.6 µg in the first hours after anthesis (SP Mo) to a peak of 191.7 µg by the afternoon of the staminate phase (SP Af) (Fig. 2.11B). Owing to the consistency in nectar-solute concentration throughout floret phenology, nectar-sugar quantity per disc floret (Fig. 2.11B) closely reflected the pattern of nectar volume (Fig. 2.11A). The nectar sugar quantity per floret remained high in the morning (187.5 µg) of PP1 (Fig. 2.11B). Nectar-sugar quantity decreased and remained relatively constant for the rest of the first day of the pistillate phase. The following day, 80.1 µg of nectar sugar per floret had fallen to 73.0 µg in the afternoon of PP2, and decreased to 7.4 µg on the third day of stigma receptivity (PP3) before cessation of nectar production at PP4 (Fig 2.11B), with only a single floret yielding 0.63 µg from the total of 62 disc florets sampled.

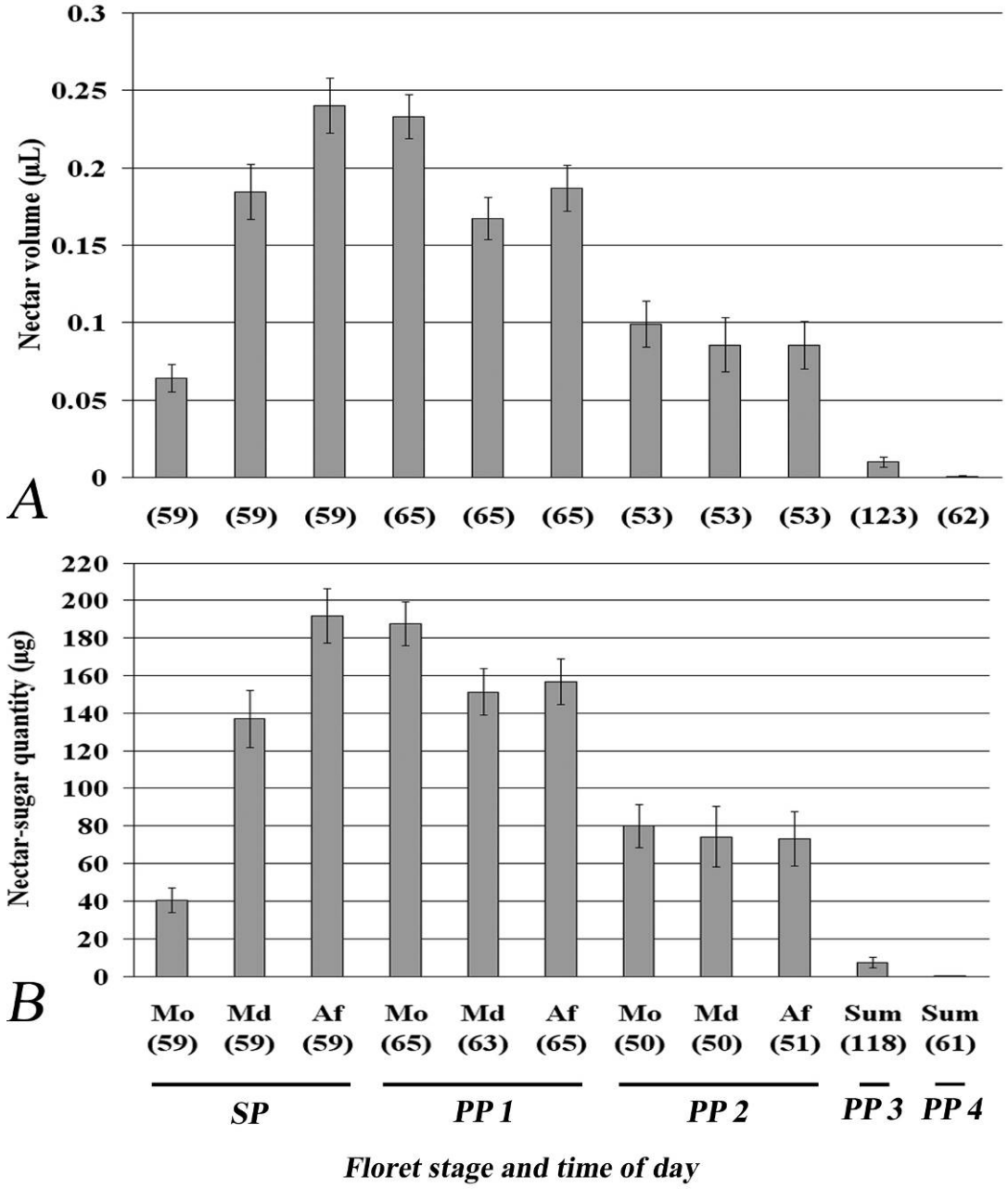


FIG. 2.11: Nectar characteristics of disc florets of *E. angustifolia*. (A) Nectar volume (Mean ± S.E.). (B) Nectar-sugar quantity (Mean ± S.E.). Morning (Mo), midday (Md), afternoon (Af). Staminate phase (SP), first pistillate phase (PP1), second pistillate phase (PP2), third pistillate phase (PP3) and fourth day of the pistillate phase (PP4). The numbers in brackets represent per floret stage and time of day, the total number of disc florets sampled (A) and all sampled florets except those that failed to yield sufficient nectar for refractometry (B).

2.4 Discussion

2.4.1 Characteristics of reproductive structures of *E. angustifolia* and *E. purpurea*

2.4.1.1 Inflorescence morphology and flowering phenology

The capitulum of *E. angustifolia* and *E. purpurea* is a highly modified aggregation of small florets, thus creating an illusion of one large flower that attracts pollinators. Neither *Echinacea* inflorescence differs much from the other in any significant way, except that on average it seems that the inflorescence of *E. purpurea* is larger and therefore contains more disc florets. The common name, "purple coneflower", is derived from the appearance of the flower heads or inflorescences. The term "cone" refers to the raised receptacle bearing the disc florets. The outer, colourful, "petal like" ray florets attract insects visually but typically are sterile, lacking an androecium, gynoecium or nectary and are the first florets to reach anthesis, followed soon by the outermost whorl of disc florets. Each disc floret is subtended by a modified bract (palea) that protrudes well beyond the five petals of the corolla. Each palea is stiff and "spiny", and this condition is the source of the generic name from the Greek *Echinos*, a hedgehog (Kindscher, 1989). Pollinators are rewarded by these inner disc florets in the staminate phase (pollen and nectar) preceding the pistillate phase (nectar, and collection of any residual pollen available as secondary presentation on the style below the stigmas - Inouye *et al*., 1994; Pacini, 1996). Nectar is produced by individual disc florets in small volumes, which entices insects to visit more than one floret, and often more than one inflorescence per foraging trip in order to become satiated. In this way, one insect may transfer pollen grains to many disc florets on other plants of the same species.

Each morning between sunrise and 9:00 AM, a new whorl of disc florets opens into the indehiscent staminate stage (SPi). The florets dehisce their pollen from the top of the fused anther tube between 9:00 and 11:00 AM, weather permitting, as they enter the dehiscent staminate phase (SPd). By 9:30 PM, florets have passed through the staminate phase and have entered the receptive pistillate phase (PP1) characterized by reflexed stigmas, which in *E. angustifolia* lasts until cross-pollination of the floret or floret senescence after 8-10 days (Wagenius, 2004). The disc florets are protandrous,

wherein the anthers mature first in the staminate phase and present pollen before the stigma lobes become receptive during the pistillate phase of the floret. This developmental sequence is a mechanism employed to prevent autogamy (Kevan, 1997) in *Echinacea* and in general, throughout the Asteraceae (Pacini, 1996). Sammataro *et al.* (1985) describe the transition in floret phase between the staminate and pistillate phases in sunflower as the pre-pistillate phase. This phase is characterized by the full extrusion of the stigma from the anther tube prior to the reflexing of the stigmatic lobes, and is identical to the dehiscent staminate stage identified in *Echinacea* for the following reason. If the pollen bundle is removed from the top of the anther in the dehiscent staminate phase, the stigma can be seen extruding beyond the anther tube, which is the defining characteristic of the pre-pistillate stage. These phases are one and the same and the terminology can be used interchangeably.

2.4.1.2 Disc floret morphology

The fused corolla tubes of *E. angustifolia* and *E. purpurea* did not differ from the descriptions of McGregor (1968) who reported their lengths as 6-8 mm and 4.5-5.5 mm, respectively. The bell shape of the corolla serves to protect the androecium and gynoecium before anthesis and the corolla's basal expansion serves as a nectar reservoir after anthesis. The base of the corolla also serves as attachment points for the filaments of the stamens. This epipetalous nature of the stamens with their coalescent anthers and free filaments attached to the base of the petals had not previously been reported for the genus *Echinacea*.

2.4.1.3 Pollen morphology

McGregor (1968) measured *E. angustifolia* and *E. purpurea* pollen grains and found them to be in the range of 19-26 μm and 19-21 μm, respectively. Erdtman (1945) classified pollen grains with diameters of 10-25 μm as small and grains with diameters of 25-50 μm as medium. Therefore the majority of pollen grains of these two species of *Echinacea* can be classified as small. Pollen grains were echinate with protruding spines, a defining feature of asteracean pollen grains, and the spines assist in pollen dispersal by helping pollen grains adhere to body hairs of visiting insects.

This study was the first report of trinucleate pollen in *E. angustifolia* and *E. purpurea*. In addition to the round vegetative nucleus, two laminar sperm cells are

visible, indicating that the generative cell has already divided into two sperm cells by anther dehiscence. Trinucleate pollen differs from the more common bi-nucleate pollen in that the generative cell divides into the two sperm cells before the pollen is released from the anther and before pollen tube germination has occurred. The generative cell of binucleate grains does not divide into sperm until it has reached the base of the pollen-tube, just before the sperm cells effect double fertilization. The Asteraceae typically have trinucleate pollen grains (Brewbaker, 1967), so this feature is not surprising but is the first such evidence for the genus *Echinacea*. Following anther dehiscence, trinucleate pollen typically looses its viability quickly compared to binucleate pollen (Brewbaker, 1967) which may explain the seemingly low viability of *E. purpurea* pollen grains one h and 24 h after dehiscence.

2.4.1.4 Calyx

The thin, membranous, scale-like pappus is the reduced form of the upper free limbs of the sepals, and apparently serves no functional purpose to disc florets of *E. angustifolia* and *E. purpurea* other than the base of the calyx fusing around the ovary as the outer wall, and hence becoming a part of the mature fruit (Mani and Saravanan, 1999). This reduced, membranous pappus is also present on disc florets of other asteracean genera such as *Helianthus*, *Parthenium*, *Senecio*, *Coreopsis*, *Galinsoga*, and *Gaillardia* (Mani and Saravanan, 1999). Other asteracean disc florets have a hair-like pappus that remains attached to the achene, such as in dandelion (*Taraxacum officinalis*), where it aids in wind dispersal of the fruits. Without a pappus specialized for wind dispersal, the achenes of *E. angustifolia* and *E. purpurea* do not disperse far from the inflorescence when shed.

2.4.1.5 Gynoecium

Stigmas of 17 asteracean species were described as dry and this feature was thus assumed to be a general feature of asteracean stigmas; dry stigmas occur in species that employ a sporophytic self incompatibility system to prevent self pollination (Heslop-Harrison and Shivanna, 1977). The stigmas of *E. purpurea* and *E. angustifolia* bifurcated into two lobes that reflex when receptive and the stigma of *E. angustifolia* may become moist, according to Leuszler *et al*. (1996). No evidence of a wet stigma was found in any observations of *E. angustifolia* or *E. purpurea*, but the stigma of

Senecio squalidus (Asteraceae) combines characteristics of both wet and dry stigmas into a "semidry" stigma which may actually be the general condition of the Asteraceae (Hiscock *et al*., 2002). In this semidry stigma of *S. squalidus*, a cuticle and pellicle are present as in dry stigmas but a small amount of surface exudate was observed. Stigmas of *Echinacea* then may also share this semidry condition.

Stigmas of both *E. angustifolia* and *E. purpurea* were nearly identical with receptive surfaces composed of papillae that were exposed by the reflexing of the stigmatic lobes in the receptive pistillate phase. Bilobed stigmas are a typical feature of the asteracean gynoeciums (Mani and Saravanan, 1999) and gynoecia of *Echinacea* do not differ from this asteracean convention. *Echinacea* stigmas serve a dual purpose; in the staminate phase the stigma is utilized as a secondary pollen presenter (see Pacini, 1996) and in the pistillate phase the stigma serves its more conventional purpose of a receptive surface for pollen. The non-receptive surface of the stigma consists of long trichomes modified for pollen presentation in the staminate phase, wherein trichomes sweep pollen grains out of the anther tube as the style lengthens and the staminal filaments contract. This type of active pollen presentation is typical of asteracean disc florets and occurs also in the Lobeliaceae, Goodeniaceae, Brunoniaceae and Calyceraceae (Ladd, 1994). The stigma lobes reflex in the pistillate phase to expose their receptive papillae. Xenogamous pollen grains transferred by pollinators adhere to the surface of papillae where germination of pollen tubes initiates from pores on the surface of pollen grains. Compatible pollen tubes penetrate the stigma surface between receptive papillae, presumably where the cuticle is absent or thin, and grow down into the style in the same manner as do pollen tubes of *Senecio squalidus* (Hiscock *et al*., 2002). Stigmas that remain un-crosspollinated for several days will curl down to collect any self pollen that may yet cling to the style and anther tips (Fig. 2.1C) in order to attempt autogamy, although the effectiveness of this self-pollination technique is unknown.

The style serves as the conduit for the passage of pollen tubes from the stigma surface to the ovule. The interior of the style is interesting with its clearly defined transmitting tract for the passage of pollen tubes and with isolated phloem sieve tubes

occurring extraneous to the vascular bundles. These isolated sieve tubes will be considered later during discussion of pollen tubes (see 3.3.3.2.3).

The ovary of each disc floret of *E. angustifolia* and *E. purpurea* is inferior which occurs in all asteraceans. The single ovule within the ovary suggests that only one compatible pollen grain is required to pollinate a disc floret. At maturity, the outer ovary wall and the base of the calyx harden into an indehiscent fruit coat that surrounds the seed coat and developing embryo. The mature fruit of *E. purpurea* and *E. angustifolia* is called an achene, but it is functionally analogous to a seed.

2.4.1.6 Nectary

2.4.1.6.1 Nectary morphology

The floral nectary of *E. purpurea* and *E. angustifolia* is similar in morphology to that of many asteracean species, where the gland sits atop the inferior ovary and surrounds the style base (Mani and Saravanan, 1999). By anthesis, spatial restrictions imposed by the adjacent floral organs of the disc floret had contributed to the final nectary shape and size, as in other species (Davis *et al*., 1996). On its internal face, the circular outline of the nectary surface evidently reflected the nectary's expansion around a cylindrical style base, which was dilated above the floral nectary disk. The swollen style base immediately above the nectary in *Gochnatia polymorpha* (Sancho and Otegui, 2000), *Helianthus annuus* (Tacina, 1974), *Heterothalamus alienus* (Vogel, 1998) and *Tridax procumbens* (Gopinathan and Varatharajan, 1982), like that of *E. purpurea*, may result from constriction of the surrounding, expanding nectary around the narrowed, extreme base of the style.

Externally, the pentagonal outline suggests that the late-maturing floral nectary is partially molded, during its expansion, against the five-preformed, established petals of the corolla tube adjacent to it. Sammataro *et al*. (1985) reported that sunflower nectaries from many cultivars ranged from 4-8 sided, and even circular, although it is unknown whether nectary sidedness corresponded to the number of floral parts in whorls adjacent to the gland. Evidence of opposing external pressure applied by the surrounding corolla (Fig. 1F) and even the filament bases (Figs 2A, 3F), resulting in the undulating,

pentagonal nature of the tall floral nectary of *G. polymorpha* (Sancho and Otegui, 2000) is also clear.

Floral nectaries of *E. purpurea* were slightly more than half the width and approximately 100 µm shorter than those of sunflower (Tacina, 1974; Sammataro *et al*., 1985). The internal diameter of sunflower nectaries varied from 470 to 800 µm and their heights varied from 200-360 µm (Sammataro *et al*., 1985). Diameters of the floral nectaries of *Wedelia chinensis* (440 µm) and *Tridax procumbens* (400 µm) (Gopinathan and Varatharajan, 1982) are also slightly larger than those of *E. purpurea*.

2.4.1.6.2 Modified stomata

Modified stomata on the floral nectary of *E. purpurea* are anomocytic and are borne between non-specialized epidermal cells. Stomata occur predominantly on the apical rim of the nectary and occur only sporadically on the outside lateral walls. Stomata that do occur on these walls are more often than not immature and are not raised above the epidermal surface. Modified stomata on the nectary rim are the most likely route for the exit of nectar from the nectary (Davis and Gunning 1992; Gaffal *et al.* 1998). These modified stomata are termed "modified" because they do not open and close to regulate the pore like the stomatal guard cells of leaf stomata (Fahn, 1979). Rather, these stomata remain permanently open and do not serve to regulate the passage of nectar but simply act as a conduit. The small size of the substomatal chamber indicates that guard cells are in contact with subepidermal cells which hinder guard-cell movement. Modified stomata are termed "open" when the cuticle covering the stomatal pore ruptures (Davis and Gunning, 1992). Open stomata are present on nectaries of *E. purpurea* from all phases of disc floret development, as are immature and occluded stomata. The presence of immature stomata on nectar producing nectaries (staminate and pistillate) suggests that some stomata may never reach maturity. Occluded stomata occurred predominantly in pistillate phase florets where nectar secretion is decreasing as the floret ages, but were found to a lesser extent on nectaries of bud phase and staminate phase florets. Occlusion of modified stomata in *Vicia faba* occurs with the deposition of a second cuticle over the stomatal pore that is distinct from the original cuticle (Davis and Gunning, 1992). Occlusion of modified stomata in *E. purpurea* occurred either from below or from another mechanism where an unknown material covered the

stomatal pore and could prevent the release of nectar through that pore. Stomata of *E. purpurea,* when found on the nectary rim, are raised slightly above the surrounding non-specialized cells as are the stomata of *Helianthus annuus* (Sammataro *et al*., 1985). In floral nectaries of *Digitalis purpurea*, stomatal guard cells are level with the epidermis before nectar secretion and become raised during nectar secretion but sink below the surface of the epidermis following nectar secretion (Gaffal *et al*., 1998).

Nectar is secreted by secretory parenchyma cells into intercellular spaces that are contiguous with the stomatal pores of modified stomata and in this manner passes to the exterior of the nectary (Gaffal *et al*. 1998). Nectar secretion through modified stomata occurs with the rupturing of the thin cuticular membrane covering the stomatal pore (Razem and Davis, 1999). Modified stomata are well documented on floral nectaries of *Pisum sativum* L. (Razem and Davis, 1999), *Vicia faba* L. (Davis and Gunning, 1992, 1993), *Cucurbita pepo* (Nepi *et al*., 1996) and *Linaria* (Pacini *et al*., 2003). Stomata on the surface of floral nectaries are reported for 72 species of asteraceans and are listed by Davis (1992). Modified stomata are implicated in the secretion of nectar in disc florets of seven asteracean taxa: *Helianthus annuus* (Sammataro *et al*., 1985), *Cosmos, Wedelia, Tridax*, *Synedrella* (Gopinathan and Varatharajan, 1982), *Tussilago farfara* (Warakomska and Kolasa, 2003) and *Taraxacum officinale* (Frey-Wyssling, 1955). Modified stomata are the most likely route of exit for nectar in nectaries of *E. purpurea* as they are continuous with intercellular spaces. Other openings in the epidermis may also serve as a route of nectar escape from the intercellular spaces. Several openings in the epidermal surface were observed with light (Fig. 2.5E) and scanning electron microscopy. These non-stomatal openings were not associated with stomata but occurred as creases in the epidermal layer that allowed contact between subepidermal cells and the exterior of the nectary. Breakages in the nectary epidermis were observed in *Vicia faba* (Davis and Gunning, 1992, 1993) but these openings were associated with clusters of modified stomata.

2.4.1.6.3 Anatomy of the floral nectary of *Echinacea purpurea*

2.4.1.6.3.1 Nectary phloem

The floral nectary of *E. purpurea* was innervated directly and solely by phloem consisting of sieve tubes that extended from the nectary base to about ¾ the height of the

gland. This occurrence of phloem alone within the gland interior is in accordance with 30% of Asteraceae studied previously (see 2.1). Although it is common for angiosperm nectaries to receive a direct vascular supply of phloem (Fahn, 1979), it is unusual to observe an intimate relationship between the phloem and epidermis. In the structureless nectaries on the outer perianth of *Paeonia albiflora*, Zimmermann (1932) drew phloem cells, beneath a guard cell, but these hypodermal elements of phloem did not match the length of the epidermal cells immediately above them. Interestingly, in the extranuptial nectaries of the involucral bracts of the capitulum of *Centaurea* spp., phloem also resides close to the epidermis and guard cells but is separated from them by a few cell layers of sclerenchyma (Zimmermann, 1932). This phenomenon of phloem in the hypodermis of floral nectaries in the Asteraceae has been demonstrated before in *Cirsium arvense*, *Helianthus tuberosus* (Frei, 1955) and *H. annuus* (Frei, 1955; Sammataro *et al*., 1985). Indeed, now in *E. purpurea*, there is anatomical evidence for involvement of at least some epidermal cells in the ontogeny of sieve elements adjacent to them. Periclinal divisions of epidermal cells can result in the subepidermal precursors that yield the sieve tube element-companion cell complex. Moreover, within a sieve tube, the length of adjacent sieve elements varies and appears to depend on the size of the precursor cell itself. Furthermore, it appears that multiple, though diminutive, companion cells may form per sieve element.

Whereas the propinquity of the sieve tubes to the nectary exterior strongly invokes phloem sap as a major contributor to nectar sugar in *E. purpurea*, and phloem exudates of the Asteraceae contain sucrose and some raffinose (Zimmermann and Ziegler, 1975), the demonstration of glucose and fructose in nectar of this species suggests that sucrose of phloem sap is not simply transported externally, but rather, sucrose inversion still occurs prior to ultimate secretion. If enzymes contributing to this modification of sucrose are only bound to cell walls rather than present in nectar itself, it could be fruitful to explore regions of the epidermis near the sieve elements, for localization and characterization of these catalysts.

The closeness of the sieve elements to the nectary epidermis of *E. purpurea* also probably allows an efficient retrieval system, for net reclamation of uncollected nectar carbohydrates. Recently, Masierowska and Stpiczynska (2005) detected radioactivity in

sieve tubes following reabsorption of 14C-labelled sucrose by the lateral nectaries of *Sinapis alba*, another species whose floral nectaries are supplied by phloem alone.

Cytologically, the sieve elements of the floral nectary of *E. purpurea*, share several features with those of other nectaries, including pore connections to adjacent elements at sieve plates (Durkee, 1983*a*), and various types of plastids, mitochondria and endoplasmic reticulum (Figier, 1971; Durkee, 1983 *a*, *b*; Davis *et al*., 1988; Davis, 1992; Belmonte *et al*., 1994; Razem and Davis, 1999). Like in *Echinacea*, sieve elements of nectary phloem can be connected by plasmodesmata to companion (Davis *et al*., 1988) and parenchyma (Sammataro *et al*., 1985; Davis *et al*., 1988) cells, and sieve elements may lie directly beside intercellular spaces (Sammataro *et al*., 1985; Davis, 1992; Razem and Davis, 1999).

The companion cells were particularly rich in mitochondria and ribosomes in the ground substance and stained densely, but with variability in intensity. In *E. purpurea*, companion cells were distinguished as the only units of the entire nectary modified as transfer cells (Gunning and Pate, 1969; Pate and Gunning, 1972), their wall ingrowths enhancing the opportunity for active transport of pre-nectar constituents across the enlarged surface area of their cell membrane. In the floral nectary of *Helianthus annuus*, wall ingrowths were not reported by Sammataro *et al*. (1985), but Tacina (1979) identified wall protuberances in what might be a companion cell, if the unlabelled cell in bottom right of Fig 5 is a sieve element. Interestingly, companion cells involved in phloem loading at minor veins in leaves of *H. annuus*, are modified as transfer cells (Turgeon *et al*., 2001), but evidently is unknown for *Echinacea*. Other floral nectaries as multicellular outgrowths that possess companion cells as their only transfer cells include *Eccremocarpus scaber* (Belmonte *et al*., 1994) and *Pisum sativum* (Razem and Davis, 1999), but they differ somewhat because those of *E. purpurea* had wall ingrowths along their walls opposite epidermal cells, but lacked ingrowths opposite the sieve elements. Otherwise, the companion cells of *E. purpurea* share with *Pisum sativum* (Razem and Davis, 1999) and *Vicia faba* (Davis *et al*., 1988) the orientation of wall ingrowths opposite other companion cells, phloem parenchyma and intercellular spaces. Accordingly, companion cells in *E. purpurea* may be instrumental in the unloading of

phloem sap, arriving within the adjacent sieve elements, and passing pre-nectar constituents to the phloem parenchyma and intercellular spaces next to them.

The fibrillar substance found in phloem parenchyma cells (Fig. 2.7B) may be proteinaceous, sharing affinities with material identified in parenchyma adjacent to vascular tissue of floral and extrafloral nectaries (Durkee, 1983*a*; Belmonte *et al*., 1994) and elsewhere within the glands (Eymé, 1963; Baker *et al*., 1978; Schnepf and Deichgräber, 1984; Horner *et al*., 2003).

2.4.1.6.3.2 Parenchyma cells of the nectary

The presence of lobed nuclei lined by nuclear pores along cytoplasmic invaginations occurs in parenchyma (Zandonella, 1970*b*; Perrin and Zandonella, 1971) and guard cells (Davis, 1992) in other floral nectaries and hydathodes, where it enables close contact between organelles with the nucleus and generally reflects a cell's heightened physiological activity (Perrin and Zandonella, 1971).

Plastids were common and possessed plastoglobuli but few thylakoids, and little chlorophyll, in their stroma. Using oil immersion and light microscopy, plastids from unstained, fresh nectaries appeared yellowish, but it was impossible to ascertain whether the gland's pale-yellow colour (Fig. 2.1B) was attributable only to chromoplasts, or included faintly-pigmented vacuolar contents. In sectional profile, each plastid had one starch grain, or none. This paucity of starch in combination with the relative longevity (up to four days) of nectar secretion, in small quantities per floret, is generally indicative of floral nectaries dependent on other plant organs for their photosynthate throughout secretion (Pacini *et al*., 2003). The rich and direct supply of phloem to the gland strongly favours phloem sap, supplemented to a lesser extent by plastid starch, as the principal source of nectar sugar. Some of the starch present may even originate from carbohydrate delivered by nectary phloem.

More starch per plastid was evident in pistillate-phase (Figs 2.8B, 2.9B) than staminate-phase (Fig. 2.8A) florets, and may serve as an intermediate reserve later in the secretory duration. However, it is also possible that some plastid starch is deposited during the net reabsorption process preceding cessation; plastid starch was highest in parenchyma of nectaries with fading petals than in the fully-opened flower of red clover (Eriksson, 1977) and in post-secretory glands of *Cucurbita pepo* (Nepi *et al*., 1996).

Plastids were often adjacent to mitochondria, sometimes wrapped around them closely, as in other nectaries (Zandonella, 1970*b*; Baker *et al*., 1978; Stpiczynska, 2003*a*).

Mitochondria were the most abundant macroorganelle in floral nectaries of *E. purpurea*, often existing in groups similar to other studies (Baker *et al*., 1978; Fahn and Benouaiche, 1979). Their omnipresence suggests that the mode of nectar secretion in *E. purpurea* is eccrine, dependent on energy for active transport of pre-nectar carbohydrates across cell membranes (Eriksson, 1977; Davis *et al*., 1986; Razem and Davis, 1999). However, a granulocrine secretory mechanism, dependent on reverse pinocytosis of vesicles to expel pre-nectar constituents from cell-to-cell and to the exterior, is less likely because endoplasmic reticulum remained a minor component of the cytoplasm compared to other nectaries (reviewed by Kronestedt-Robards and Robards, 1991). Dictyosomes, however, existed continually in both epidermal and subepidermal cells, and were also common in nectaries of sunflower (Tacina, 1979). Although their role may have been associated with cell-wall formation, involvement of dictyosomal vesicles in a granulocrine process cannot be totally excluded. Nectar formation in *E. purpurea* did not coincide with cellular disintegration of the nectary, and hence is not a holocrine process.

Complex vacuolar inclusions were detected in, or associated with, vacuoles of epidermal and parenchyma cells. In Fig 2.9B, a constricted connection between a myelin-like multilamellar body and a multitubular body was evident, corroborating the finding of Eymé (1967) in floral nectaries of *Diplotaxis erucoides*, that these bodies are confluent. Eymé proposed that these inclusions originated near plasmodesmata from internalized trabeculae that form vesicles of membranes eventually residing in vacuoles, where they are a reserve of tonoplast membranes for new, interlocking vacuoles. In the cyathial nectary of *Euphorbia candelabrum*, Schnepf and Deichgräber (1984) indicated an involvement of endoplasmic reticulum in such bodies which myelinize in the centre. Eriksson (1977) found myelin figures in floral nectaries of *Trifolium pratense* before, during, and after nectar secretion. Rather than serving as a source of new vacuoles (Eymé, 1967), in *E. purpurea* we propose that these types of inclusions have a lysosomal function, the bodies accumulating layers of membrane as they participate in a continual degradation of senescing organelles during nectary development and function. In certain

cells of the pistillate phase of secretion, over 25 inclusions of a related nature were detected in a single cell profile (Fig. 2.9C). An enlarged vacuole and peripheral nucleus (Fig. 2.9C) have been associated with cell degradation in senescing nectaries (Zandonella, 1970*a*; Davis *et al*., 1986; Horner *et al*., 2003); apoptosis might even result. These types of inclusions have been reported in floral (Eymé, 1996, 1967; Zandonella, 1970*b*; Eriksson, 1977; Durkee *et al*., 1981; Davis *et al*., 1986) and extrafloral (Figier, 1971; Schnepf and Deichgräber, 1984; Freitas and Paoli, 1999) nectaries with regularity, such that it is difficult to dismiss them simply as artefactual, although much remains to be verified about their origin and role.

2.4.1.6.3.3 Features of modified stomata on the nectary surface

Evidently, very few modified stomata originate after the mature bud phase is reached, the overall average number (*E. angustifolia* - 36.8, *E. purpurea* - 29.2) per gland being in close accord with *Cosmos bipinnatus* but exceeding three other species (Gopinathan and Varatharajan, 1982). Modified stomata occurred predominantly around the entire rim of the nectary of *E. purpurea* and were often raised as in *Helianthus annuus* (Sammataro *et al*., 1985), but only occurred sporadically on the sides of the gland. The latter commonly were immature and not raised above the epidermal cells, a pattern observed elsewhere (Webster *et al*., 1982; Davis and Gunning, 1992). The modified stomata were anomocytic, like those on other nectaries (Gopinathan and Varatharajan, 1982) and leaves (Sanchez, 1977) of the Asteraceae.

The stomata on the nectary surface of *E. purpurea* are considered "modified" (Fahn, 1979) because they typically lack the ability to close their pores by guard-cell movements (Zandonella, 1967; Davis and Gunning, 1992, 1993). Movement is hindered because of contact maintained between guard cells and the secretory parenchyma below, resulting in a small substomatal space (Davis and Gunning, 1992, 1993; Gaffal *et al*., 1998; Razem and Davis, 1999), unlike foliar stomata of sunflower (Sanchez, 1977). The stomatal pores on the surface of asteracean nectaries remained open throughout day and night (Gopinathan and Varatharajan, 1982). Greatest average pore widths in *E. purpurea*, comparable to those of *Cosmos bipinnatus* and *Tridax procumbens* (Gopinathan and Varatharajan, 1982), occurred in the mature bud phase similar to the floral nectary of *Vicia faba* (Davis and Gunning, 1992), wherein the pore-width decrease

associated with the onset of secretory activity may be related to wetting and turgor-pressure changes in guard cells (Davis and Gunning, 1992, 1993). Widths of the nectary stomata in *E. purpurea* also were similar to those of *Barnadesia odorata* (20.3 μm) and *Hyaloseris rubicunda* (19.5 μm), two species of Asteraceae studied by Galetto (1995).

Apart from mechanical constraints, the lack of synchrony between development of the modified stomata and nectar production also mitigates against a finely-controlled regulation of nectar passage by the stomatal pores. Apertures were fully open – even widest – before secretion began, and immature stomata still occurred by the pistillate phase of secretion, suggesting that some nectary stomata never reach maturity. However, it is known that not all open stomata participate as exits for nectar (Davis and Gunning, 1992; Beardsell *et al*., 1989; Gaffal *et al.*, 1998). Also, direct observations are still required to determine whether nectar may yet pass through the developed pore of immature stomata, despite the intact, overlying outer cuticle that conceals the pore from view externally, by SEM.

Despite the restrictions on guard-cell movements that cause several open pores to persist, pores of the modified stomata on the floral nectaries of *E. purpurea* could become occluded by different means. Not reported previously in the Asteraceae (Gopinathan and Varatharajan, 1982), pore occlusion occurred like that in *Vicia faba* (Davis and Gunning, 1992) and *Pisum sativum* (Razem and Davis, 1999), throughout floret phenology but was greatest after nectar secretion commenced, and as florets aged. The nature of the occluding materials, and whether pore occlusion necessarily results in complete blockage of nectar flow, also requires investigation.

2.4.1.6.3.4 Pathway of pre-nectar to the nectary exterior

Ultrastructural evidence exists to support two commonly-proposed routes of pre-nectar movement – the apoplastic and symplastic pathways – in the floral nectary of *E. purpurea*. The apoplastic route exists as a continuum of cell walls and intercellular spaces, the latter already formed prior to commencement of nectar secretion in the early staminate phase. Intercellular spaces abutting the sieve elements and companion cells of the gland interior suggest that arriving phloem sap already may enter the apoplast at this step. The established continuity of intercellular spaces extending from the phloem to the substomatal chambers and pores of the modified stomata would facilitate pre-nectar

transport and escape onto the nectary surface. Such an apoplastic route terminating at stomatal pores has been illustrated for several asteracean nectaries (Frei, 1955; Frey-Wyssling, 1955). An apoplastic pathway has been advocated for several non-trichomatous nectaries (Vassilyev, 1971; Gaffal *et al*., 1998; Durkee *et al*., 1999; Koteyava *et al*., 2005). In *E. purpurea*, an additional apoplastic pathway formed by gaps in the epidermis, exist at a limited number of creases (Figs 2.4B, 2.5E) that apparently circumvents the epidermal cells altogether. Epidermal gaps in nectaries are reported elsewhere (Vogel, 1998), but in *Vicia faba* are apical, non-stomatal openings adjacent to guard cells (Davis and Gunning, 1993), unlike *E. purpurea*.

A symplastic route dependent on intercellular plasmodesmatal connections is also available in the floral nectary of *E. purpurea*, from the phloem to the epidermis. Plasmodesmata connect sieve elements to companion and phloem-parenchyma cells, and these in turn have plasmodesmatal connections to parenchyma and epidermal cells. A continuous symplastic path to the epidermis requires an apoplastic step across the anticlinal or outer periclinal wall to the nectary surface. This transfer to the exterior may be facilitated by microchannels in the cuticle (Fig. 2.6D) lining the outer nectary surface, similar to other nectaries (Radice and Galati, 2003).

In many nectaries, ultrastructural evidence has demonstrated that both apoplastic and symplastic routes for pre-nectar transport and escape are feasible (Davis *et al*., 1986, 1988; Kronestedt and Robards, 1987; Nichol and Hall, 1988; Razem and Davis, 1999), and we conclude that carbohydrate arriving in the nectary phloem of *E. purpurea* may simultaneously be transferred to the nectary exterior by a combination of these routes.

2.4.2 Phenology and nectar-production dynamics

In *Echinacea purpurea* in the growth chamber, nectar was available continuously from disc florets for up to four days, commencing on the morning of anthesis during the indehiscent staminate phase (SPi) and ceasing production after the third day of stigma receptivity (PP3). The actual pattern of nectar production observed can be related to potential pollination by insects in the field. For example, a functional benefit might be low if the disc florets provided a higher quantity of nectar at SPi, because pollen is not yet presented for distribution. Mani and Saravanan (1999) instructed, however, that in

some Asteraceae the probing proboscis may stimulate the filaments of indehiscent stamens to contract, typically leading to dehiscence. The rapid rise in nectar volume and concentration throughout the first day, concurrent with anther dehiscence (SPd), could serve to enhance pollen dissemination by insect vectors.

In contrast to growth chamber studies of nectar production, *E. purpurea* inflorescences sampled under field conditions produced nectar consistently only in disc florets of the staminate (SP) and first-day pistillate (PP1) phases. Very little nectar was produced by florets of the second day of the pistillate phase (PP2). On three separate inflorescences, less or nearly the same amount of nectar was available in all of the florets of the PP2 whorl (19-24 florets) than was available in a single floret of the SP or PP1 phases. A similar pattern of nectar secretion was observed in bagged *E. angustifolia* plants under field conditions, where nectar production was greatest in staminate and first-day pistillate florets but decreased significantly in second-day pistillate florets (PP2) when approximately half of sampled disc florets did not yield nectar. It seems that these field grown, bagged florets of *E. angustifolia* and *E. purpurea* reveal a much sharper division between nectar production and cessation than did the plants in the growth chamber, suggesting that nectar production ceases after the first-day pistillate phase (PP1) and nectar reabsorption occurs during the night and day of the second-day pistillate phase (PP2) culminating in almost no available nectar in third-day pistillate phase (PP3) florets. This finding may have uncovered a difference in nectar production between inflorescences from the growth chamber and those cultivated under field conditions, but do not contradict any arguments about pollinator attraction based on growth chamber studies.

Throughout disc-floret phenology, the quantity of nectar sugar approximated a normal distribution, centred at midday/afternoon of the first day of the pistillate stage (PP1) in *E. purpurea* and the afternoon of the staminate phase and morning of the first day of pistillate phase in *E. angustifolia*. After pollen collection by insects during the staminate phase (SP), nectar becomes the major reward available thereafter. A relatively large nectar quantity in disc florets immediately internal to the whorl of staminate-phase (SP) florets could enhance microgametophyte transfer by pollen laden, inflorescence-jumping insects to stigmas on their first day or receptivity (PP1). At PP2, a reduction in

nectar-sugar quantity per floret to levels comparable to the late-SP and early-PP1 stages could prolong insect attention to this pistillate-phase whorl in events such as missed pollination or poor foraging conditions. The marked decline in nectar-sugar quantity at PP3 and almost complete cessation by PP4 may result in a loss of potential pollination in a minority of florets, but likely encourages insect interest in the richer, less-pollinated whorls situated more distantly from the visually-attractive ray florets. Accordingly, nectar-secretion dynamics in these *Echinacea* species appears to represent a classic example whereby events among adjacent whorls of disc florets of the capitulum mimic the succession of reproductive events occurring temporally within an individual flower (Proctor *et al*., 1996).

Various, established physiological and environmental phenomena may account for the pattern of floral nectar secretion in *E. purpurea* and *E. angustifolia*. When pollinators were absent, disc florets produced nectar at a high rate from late morning throughout the afternoon on each of the first two days (SP,PP1) of anthesis, perhaps in relation to plant photosynthesis (Pacini *et al.*, 2003). The concurrent increase in nectar-solute concentration throughout SP might be attributable to water evaporation from accumulating nectar. Moreover, the lower nectar volumes on the mornings of PP1 and PP2, compared to the previous afternoons, also may reflect some water loss from nectar, by evaporation. However, during these same intervals, a marked decrease in nectar-sugar quantity by morning, also evident during consecutive days in sunflower (Hadisoesilo and Furgala, 1986), suggests a net reabsorption of nectar sugar. Nectar sugar reabsorption is also suggested in *E. angustifolia* by a decrease in nectar-sugar in PP2 florets (as a result of many florets yielding no nectar) and an almost complete absence of nectar in PP3 florets. Nectar-sugar reabsorption occurs very frequently when contact between nectar and nectary is maintained (for examples and previous literature see Búrquez and Corbet, 1991; Nicolson, 1995; Langenberger and Davis, 2003; Stpiczynska, 2003*a*), apparently still promoted in *E. purpurea* and *E. angustifolia* by the nectar reservoir formed by the enlarged corolla base. In this study where insect pollinators were excluded and cross-pollinations not performed by hand, a net reclamation of uncollected nectar continued throughout the PP2 and PP3 stages until eventual cessation in PP4. In future, it would be interesting to determine the fate of

reabsorbed nectar-sugar molecules (Pedersen *et al*., 1958; Shuel, 1961; Stpiczynska, 2003*b*) in the Asteraceae, including whether any can re-appear in the nectar of florets of adjacent whorls within a capitulum.

In sunflower (*Helianthus annuus*), like *E. purpurea*, greatest nectar quantities occurred in pistillate-stage florets (Hadisoesilo and Furgala, 1986). However, maximum average volumes (0.19 and 0.24 µL), nectar-solute concentration (61 and 63%) and quantity (157 and 192 µg) per disc floret of *E. angustifolia* and *E. purpurea*, differed from that study, wherein nectar volumes and concentrations ranged from 0.71-1.13 µL and 36-57%, respectively, among 18 cultivars of *H. annuus*, with up to 569 µg per pistillate-stage floret (Hadisoesilo and Furgala, 1986). In other studies of sunflower, however, mean nectar volumes and concentrations of 0.02-0.16 µL and 32-39% (7 cultivars; Tepidino and Parker, 1982) and 0.04-0.32 µL and 26-70% (47 lines; Vear *et al.,* 1990) were comparable to *E. angustifolia* and *E. purpurea.*

Sucrose, fructose and glucose were detected in nectar of *E. purpurea* sampled from three floret phases (SP, PP1, PP2), although less of the former occurred at PP2. Hexoses are predominant over sucrose in most asteracean species (Percival, 1961; Galetto and Bernadello, 2003), although sucrose is occasionally even absent (Percival, 1961; Torres and Galetto, 2002). The high solute concentration and mixture of hexoses with sucrose in the nectar of *E. purpurea* are common in a generalist pollination syndrome involving bees, butterflies and moths (Baker and Baker, 1983; Cruden and Hermann, 1983; Galetto and Bernadello, 2003). With the lower proportion of sucrose compared to hexoses, the nectar of *E. angustifolia* and *E. purpurea* can be termed hexose dominant (Baker and Baker, 1983). The decrease in sucrose in PP2 stage florets may be due to the action of invertase cleaving sucrose (Heil *et al*., 2005) into hexoses but the possibility of selective sucrose reabsorption cannot be completely ruled out.

Nectar dynamics between the two species of *Echinacea* studied are similar but there are some notable differences. However, any comparison of nectar secretion between *E. angustifolia* and *E. purpurea* must take into account discrepancies that could be attributable to comparing data collected from plants in a growth chamber with data collected from plants in the field. Nectar volumes at anthesis are nearly identical between the species (0.064 and 0.07 µL), but nectar sugar per floret in *E. purpurea* was

lower than *E. angustifolia*. This difference may be due to a discrepancy in the length of time that plants received light in growth chamber vs. field sampling. Lights came on at 8:00 AM in the growth chamber and the sun came up before 6:00 AM in the field. This discrepancy afforded two more hours of light to field sampled plants in the mornings which may have enabled them to generate more sugar by photosynthesis before nectar sampling than plants in the growth chamber. An earlier start to photosynthesis may also explain the earlier peak of nectar sugar in *E. angustifolia* in the staminate phase (as opposed to the PP1 Af time-point in *E. purpurea*) where the extra hours of light gained during the SP and PP1 allow the nectar sugar maximum to peak sooner and provide approximately 30 µg more sugar. Nectar sugar was identical in morning PP2 florets (80 µg) followed by a similar pattern of decreasing nectar-sugar availability during PP2, until at PP3 and PP4 florets the amount of available nectar sugar is minimal to nonexistent.

In conclusion, the capitulum of both *E. angustifolia* and *E. purpurea* is ringed by colourful ray florets that most likely act as a visual attractant to potential insect visitors. Both capitula provide pollen and nectar rewards in their uppermost whorls (SP, PP1 and PP2) to entice insect visitors that may transfer out-cross pollen onto receptive stigmas and carry self-pollen from the capitulum to other plants. With the majority of nectar available within the two youngest whorls of open florets (SP and PP1), it seems that the pollination strategy of *Echinacea* is to concentrate insect visitation to these whorls. Each day, a new staminate whorl reaches anthesis and the staminate whorl from the previous day passes into its pistillate phase and new insect pollinators visit these two whorls of florets and cross pollinate the newly receptive stigmas of PP1 florets. In this way, attractiveness to insect visitors via a succession of flowering whorls, is maintained throughout capitulum phenology.

Chapter 3: Insect pollination of *Echinacea angustifolia*

3.1 Introduction

Asteracean flowers are typically pollinated by insects so it was likely that insects would be the dominant, if not the only, animal pollinators of *Echinacea angustifolia* in Saskatchewan. *E. angustifolia* is native to Saskatchewan (McGregor, 1968), so it should have a diversity of native insect pollinators. Currently, pollination of *E. angustifolia* inflorescences in agricultural settings is left to chance in the hopes that native pollinators are sufficiently numerous to effectively pollinate the crop. It is known that native pollinators are often not capable of effecting sufficient pollination on large stands of flowering self-infertile crops such as alfalfa (*Medicago sativum*) and sunflower (*Helianthus annuus*). In sunflower (*H. annuus*: Asteraceae), a small seed crop is produced if there is a shortage of honey bees during flowering (McGregor, 1976). In these instances, growers import supplementary, managed pollinators to their fields such as alfalfa leafcutter bees (*Megachile rotundata*) or honey bees (*Apis mellifera*) to ensure that their crop is fully pollinated. Typically, a crop that is pollinated quickly and effectively matures earlier than a poorly pollinated crop, which decreases the time from flowering to harvest so that damage from an early frost can be minimized or avoided. If the entire burden of pollination of *E. angustifolia* is to be left to native pollinators, then the identity of these native pollinators must be known. Identifying the most efficient native pollinators of *E. angustifolia* is also important so that the numbers of these insects can be monitored during flowering to gauge the amount of pollination occurring in the field. With the identity of the most efficient pollinators known, then wild areas around an *E. angustifolia* crop may be preserved or enhanced to provide suitable nesting habitats for native insects in order to maintain and increase pollinator populations and ensure a consistent supply of insects. Seed production in *E. angustifolia* fields is quite likely completely dependent on insect pollination with self-incompatibility as the usual condition in flowers of asteracean inflorescences. A well pollinated *E. angustifolia* field should provide a high number of seeds to be sold for profit, used to reseed harvested

acreages or potentially as a source of "medicinal" compounds in the same way that sunflower seeds are harvested and pressed for their oil content.

Prior information regarding the pollination requirements of *E. angustifolia* is limited. McGregor (1968) reported that *E. angustifolia* was self-infertile but did not provide any details of his experimental methods. Leuszler *et al.* (1996) found that *E. angustifolia* in North Dakota was partially self-fertile; 9% of achenes developed by geitonogamy (pollen from another flower of the same plant) and 7% from autogamy (by pollen transferred from anther to stigma of the same flower). Xenogamy resulted in 66% of achenes being viable, with increased fitness occurring due to increased achene mass, presence of an endosperm and germination capability. They also reported that *E. angustifolia* was not pollinated by wind as evidenced by the lack of its pollen captured in wind currents. Insect visitors to *E. angustifolia* in North Dakota were 12 wild bee species in four families, plus four families of Lepidoptera (butterflies and moths). The hymenopteran (bees) visitors of *E. angustifolia* were identified to the species level, and the lepidopterans were identified to family.

Clearly, insect vectors are required for proper cross-pollination of *E. angustifolia* inflorescences. *E. angustifolia* may possess the capacity for partial self-fertilization (Leuszler *et al*., 1996) but seed set percentages of 9% (geitonogamy) and 7% (autogamy) are unacceptable when the maximum number of viable seeds is required, such as in a commercial application of *E. angustifolia* seed production. This current research sought to answer one central question about the pollination of *E. angustifolia* using several methods while at the same time answering several other pollination questions. The central goal was to identify the insect pollinators of *E. angustifolia* in Saskatchewan and determine how efficiently they pollinate inflorescences. This measure of pollination efficiency (Davis, 1997) was assessed using single insect visits to virgin inflorescences. This experimental method allowed for the contribution to pollination of individual insect species to be measured at the inflorescence level. Excluder cages of different mesh sizes placed over inflorescences allowed for experiments at the field level using all available pollinators in the vicinity as study subjects. The relative contributions to the pollination of an *E. angustifolia* field by insects of different body sizes were tested using these excluder cages. Using openly-

pollinated inflorescences as controls to these excluder cages allowed for observations of the effect of visitation by all possible insects during the flowering period of an inflorescence. The control inflorescences were also a subsample of the total population of inflorescences at a particular field site and can answer the question of whether native insect pollinators are capable of pollinating an entire field of *E. angustifolia*. The effect of the absence of insects on seed set in *E. angustifolia* was also tested by excluding insects from visiting inflorescences using mesh cages. Another comparison is possible using weights and germination percentages of achenes from inflorescences excluded from insects and those from openly pollinated inflorescences. There are no consistent external cues available to identify the germination potential of an *E. angustifolia* achene, but a clue to an achene's germination potential may exist in its weight. Some *Echinacea* growers selling seed have identified that heavier achenes are of better quality than lower weight achenes and will select heavier achenes for sale (Dr. B. Barl, personal communication). With the achenes from inflorescences within excluder cages and achenes from openly pollinated inflorescences already weighed and germinated for pollination comparisons, there was an opportunity to test for relationships between achene weight and germination.

In summary, insect pollination of *Echinacea angustifolia* at three field sites in Saskatchewan were studied to discover (i) Which insects were visiting *E. angustifolia* (ii) the identity and efficiency of insect pollinators on *E. angustifolia* (iii) if a pollinator deficit was occurring at these sites. Transect observations were used to identify insect visitors to inflorescences of *E. angustifolia*, and excluder cage trials followed by achene weight and germination measurements were used to compare the contribution to pollination of large vs. small bodied insects and to identify any relationships between achene weight and germination potential. Quantification of pollen tubes at the style base and style shrivelling were used to determine the pollination efficiency of individual insects on inflorescences.

3.1.1 Excluder cage experiments

The yield and germination rate of achenes from pollination treatments were compared as an indirect determination of pollination efficiency. This method is termed

indirect as it must take into account post-pollination processes of fertilization, seed development and any abortions; pollination is only registered indirectly, through these other events (Davis, 1997). Seed yield and germination percentages are common variables to study when assessing pollination efficiency. Leuszler *et al.* (1996) used the mass and percent germination to assess the fitness (viability) of filled achenes of *E. angustifolia*. The techniques often used to properly germinate achenes of *E. angustifolia* include cold treatment and stratification (Bratcher *et al*. 1993; Feghahati and Reese, 1994). Use of these techniques should result in proper germination of viable achenes while unviable achenes that occurred as a result of inefficient pollination will also be evident.

The question to be answered is "Is there a difference in achene weights and germination among three excluder treatments?" The three treatments were as follows; (i) Openly pollinated plants representing a control where all insects are allowed to visit inflorescences; (ii) Selectively available plants, where steel mesh cages excluded large bodied insects from contacting inflorescences, but visitation by small bodied insects was allowed; and (iii) Unavailable plants, where tent mesh cages prevented all insects from contacting inflorescences. All possible insects at the field sites had full access to the inflorescences in the control treatment, so this treatment represents an openly pollinated control. It is hypothesized that achenes from inflorescences in this treatment should have the highest weight and percent germination because they will have unrestricted access to cross-pollination opportunities. With insect pollinators having full access to control inflorescences, achenes from these inflorescences should represent the full pollination potential of insects at each site. These control achenes will also indicate whether a pollination deficit exists if the average weight or germination of achenes in this treatment is low.

Achenes from inflorescences in the partial excluder treatments will not have the benefit of cross-pollination by large bodied insects but will test the pollination abilities of small bodied insects at each site where small insects should have exclusive access to these inflorescences. Weights and percent germinations of achenes from partial excluder treatments may be intermediate between the control treatment and the total excluder treatment. Inflorescences in the total excluder treatment should have no insect

visitors because the fine tent mesh screening of the total excluder cage does not allow the passage of insects. Weights and percent germination of achenes in total excluder treatments should be near zero if *E. angustifolia* florets truly need to be cross-pollinated to set seed (McGregor, 1968).

It is hypothesized that achene weights should differ significantly between the three treatments with the total excluder treatment having the lowest achene weight and the openly pollinated controls will have the highest achene weight. This judgement is based on observations made during counting, that achenes from the total excluder treatment seem not to have a viable embryo while the openly pollinated and the partial excluder treatments had achenes with viable seeds inside. Without insects to cross-pollinate florets in the total excluder cages, there should not be any viable seeds produced within the inflorescence and so the achene's weight should be lower than the weight of a fertilized achene containing a viable seed, unless wind is able to cross-pollinate over short distances. No air-borne pollen was found in a natural stand of *E. angustifolia* in South Dakota (Leuszler *et al*., 1996) but the possibility of anemophilous cross-pollination cannot be ruled out.

Florets that have been cross-pollinated should produce viable achenes that are able to germinate provided their dormancy can be broken. The estimate of pollination should be reflected by the percentage of germinated and un-germinated achenes. The hypothesis is that achenes from a well-pollinated inflorescence will have a higher germination percentage than achenes from an inflorescence that had poor cross-pollination. It is hypothesized that openly pollinated inflorescences fully accessible to insects will have a higher germination percentage than those excluded from large insects, which in turn will have a higher germination percentage as those completely excluded from insects (Open to all pollinators > partial exclusion of pollinators > total exclusion of pollinators).

3.1.2 Pollen tube counts

In cross-pollination, compatible pollen grains are transferred from the anther of one flower to the stigma of another flower. If a compatible pollen grain contacts the receptive surface of the stigma it germinates a pollen tube that penetrates the stigma and

grows down the style to deliver the microgametes (sperm cells) to the female megagamete (egg cell). As the pollen tube grows within the style, it lays down callose (β-1,3-glucan) plugs at irregular intervals as well as lining the tube walls with callose. Using aniline blue, a fluorescent dye that stains for the presence of callose, pollen tubes can be visualized within the stylar tissue (Martin, 1959; Davis, 1992 *b*). Callose plugs should fluoresce bright yellow to yellow/green against a blue or grey background of stylar tissues. Compatible pollen grains should produce a pollen tube that reaches the style base while self-pollen that germinates should be either prevented from penetrating the stigmatic surface or abort within the style and fail to reach the style base (Hiscock *et al*., 2002). If self-pollen grains germinate and penetrate the stigma surface their pollen tubes should be aborted within the transmitting tissue of the style where glycoproteins involved in self-incompatibility are known to occur (Anderson *et al*., 1986). The presence of at least one pollen tube at the base of the style should indicate that successful pollination and presumably fertilization of the ovule has occurred. Using pollen tube counts in conjunction with single insect visits to virgin inflorescences, an estimate of the efficiency of that particular insect at pollinating *E. angustifolia* inflorescences can be made. In this way, counting pollen tubes within styles can be used to directly assess the pollination efficiency of insects. The ovary of *E. angustifolia* is uniovulate, so for optimal reproductive success, technically only one pollen tube at the style base is necessary to fertilize each disc floret.

3.1.3 Shrivelled style estimate of pollination

During field observations, it was noted that stigmas and styles of disc florets of *E. angustifolia* and *E. purpurea* persisted when insects were excluded from inflorescences by the total excluder cages and mesh bags. Conversely, observations from the field season in 2003 and from experimental test crosses made in the greenhouse suggested that after cross-pollination, stigma and styles of disc florets would senesce by shrivelling and shrinking into the corolla tube. Style shriveling was also observed in *Chrysanthemum morifolium* where shriveling of pistils occurred one day after pollination and was correlated positively with seed set (De Jong and Kho, 1982). It became apparent that this visual cue could be adapted to estimate pollination of *Echinacea* inflorescences. A scientific article in press at that time, was also found that

described this phenomenon and utilized it as to measure pollen limitation in fragmented *E. angustifolia* habitats. Wagenius (2004) introduced the technique of style persistence (SP) by determining the timing of SP and shrivelling to quantify pollination in natural stands of *E. angustifolia*. During the 2004 field season, this SP technique was used to estimate pollination after a single insect visit (SIV) to a bagged, virgin inflorescence. Shrivelling of styles following pollination of receptive stigmas indicates successful cross-pollination and fertilization of the ovule. Wagenius (2004) used the term "persistent" to describe un-pollinated styles that had not shrivelled and used these persistent styles as a measure of pollen limitation in natural populations of *E. angustifolia*. Within 24-hour after cross-pollination, styles and stigmas shrivel and pull down into the corolla tube but if cross pollination does not occur, a style's turgidity can persist for up to 10 days (Wagenius, 2004; personal observation). Seed set was strongly negatively correlated with style persistence, where seed set decreases as the number of days that styles remain persistent, increases (Wagenius, 2004). This SP technique can be used in conjunction with single insect visits to virgin flowers to estimate the pollination efficiency of individual insects. A note of botanical clarification is required, however. The style shrivelling that Wagenius (2004) discusses also refers to the shrivelling of the stigma so that when speaking of shrivelled styles it is implied that the stigma has also shrivelled.

This SP technique is a valuable addition to the pollen tube counts and seed germinations to quantify pollination. Twenty-four hours after a single insect visit the numbers of shrivelled and un-shrivelled styles are counted and then the inflorescences are fixed for pollen-tube counts. This technique also had the potential to reduce or replace pollen-tube counts as the method used to determine pollination efficiency by visually identifying cross-pollinated styles that should have pollen tubes without needing to observe pollen tubes within the style. To compare these techniques (pollen-tube counts and shrivelled styles), un-shrivelled styles from the visited area and a sub sample of shrivelled styles were analyzed for pollen tubes.

3.2. Materials and Methods

3.2.1 Field sites

3.2.1.1 Prairie Plant Systems

This is the smallest field site and is located behind the greenhouses of Prairie Plant Systems Inc. (PPS), southeast of Saskatoon on highway 16. Prairie Plant Systems staff planted these *E. angustifolia* plants as an afterthought, when they had seedlings left over from a contract-growing job. Transect data, single insect visits, and excluder cage treatments were performed at this site in 2003, but in 2004 only excluder cage experiments were possible.

3.2.1.2 Meewasin Valley Authority

See section 2.2.1.1 for a description of Meewasin Valley Authority site (MVA). Transect observations, excluder cage treatments, single insect visits and alfalfa leafcutter bee (*Megachile rotundata*) trials were carried out at this site in 2003 and 2004.

3.2.1.3 Valley Road

Valley Road (VR) (see section 2.2.1.2 for site description) was employed in 2004 as a third field site where excluder cage trials, single insect visits and *M. rotundata* trials were conducted.

3.2.2. Insect diversity

3.2.2.1 Transect observations

Using a transect system allowed the diversity of insect visitors to *E. angustifolia* inflorescences to be recorded with non-invasive observation. The goal of transect monitoring was to assess the diversity and abundance of visitors during the field season at each site. At MVA and VR, transect lines with 30 mature inflorescences were set out, and insect visitors to each of these 30 inflorescences were recorded at several intervals. Insects were counted as visitors if they contacted an inflorescence of *E. angustifolia* (capitulum) and were counted as pollinators if they are known floral pollinators or possess features common to pollinating insects.

Throughout the season the inflorescences on each transect changed as inflorescences senesced and new ones were chosen to replace them. Transect locations changed as well, depending on the number of fresh or senescent inflorescences in the

area. Transect observations were made at each site at least every second day during the flowering period. There were often multiple transects per day at each site where observations were recorded in the morning, mid-afternoon, late afternoon and evening to gain an understanding of diurnal changes in insect diversity.

At PPS in 2003, 20 plants were selected for transect observation and in 2004, the small number of plants allowed for observation of all inflorescences. Pollinator diversity at each field site was assessed and frequencies of visitation determined for each insect species observed.

3.2.3 Insect pollination efficiency

3.2.3.1 Excluder cages

3.2.3.1.1 Construction

Insect-excluding cages were made from wood and mesh (120 cm x 120 cm x 120 cm) to assess the contributions to pollination made by insects of different body sizes. The control inflorescences were openly pollinated so were not caged to exclude any insects.

At each field site, two "partial excluder cages", were used to assess the pollination ability of small-bodied insects. The sides of these cages were covered with metal mesh, with 64 holes per square inch, with each square hole measuring approximately 3.1 mm (1/8 inch) x 3.1 mm (1/8 inch) or 9.6 mm^2. This treatment allowed smaller insects into the cage and the proportion of viable achenes produced in these boxes should estimate the contribution of small insects to pollination in the field. "Total excluder cages" were covered with a fine, tent screen mesh (Fig. 3.24G) that insects were unable to penetrate. Two replicates of this treatment were conducted at each field site for a total of four in 2003 (PPS, MVA) and six in 2004 (PPS, MVA, VR).

As many insects as possible were cleaned out of the excluder cages when initially placed over the plants at each site. The foliage within the total excluder cages was also sprayed once with a contact and residual insecticide (Green Cross: Creepy Crawly: D-trans allethrin 0.050%, N-Octyl bicycloheptene Dicarboximide 0.25%, Permethrin 0.200%) to ensure that it was free of insects. An exception was that at Valley Road, where the interior of the total excluder cage was not treated with

insecticide so as not to contravene organic crop regulations. The cages were staked to the ground using cord and tent pegs and dirt was heaped up around their bases to ensure that insects could not crawl underneath.

3.2.3.1.2 Placements

In summer 2003, partial and total excluder cage trials were used to help assess the pollination requirements of *E. angustifolia* at two field sites (Meewasin Valley Authority; Prairie Plant Systems Inc.) south of Saskatoon. These treatments were repeated in 2004 with the inclusion of a third field site at Valley Road, southwest of Saskatoon. Two replicates of each excluder cage treatment were conducted at each field site for a total of four each in 2003 (PPS, MVA) and six each in 2004 (PPS, MVA, VR).

Control inflorescences were chosen at random from plants near the excluder cages at each site. Ten (2003) control inflorescences were harvested near the excluder cages at MVA and PPS in 2003. In 2003, all of the inflorescences in each cage were used in weight and germination analysis (PPS partial excluder sum, n = 16; inflorescences, MVA partial excluder sum n = 25; PPS total excluder sum n = 21; MVA total excluder sum n = 22). In 2004, there were many more inflorescences per cage at MVA and VR, so a sub-sample of fifteen inflorescences was chosen at random for each cage and fifteen control inflorescences were chosen for each site. Due to the low number of plants at PPS, all of the inflorescences in each treatment were used (12 controls, 18 inflorescences in partial excluder cages, 10 inflorescences in total excluder cages).

3.2.3.1.3 Achene weights

Inflorescences from each treatment were harvested in mid-August (2003) and September 29th 2004 (VR) and 30th (MVA, PPS) when the peduncle had turned brown and its trichomes had turned white, but before achenes had begun to fall from the heads. The inflorescences were then dried for 24 hours at 30˚C in an incubator to ensure that all moisture had been removed to prevent fungal growth during storage. Achenes were separated from the inflorescences using a coffee grinder modified by duct-taping the blades. Achenes for each treatment were then counted and weighed (including the sterile achenes produced by the ray florets). To standardize each sample, the weight of the achenes from each inflorescence were extrapolated to 1000 and then compared. All

achenes per flower head were included because there are no reliable, external cues to differentiate fertilized from unfertilized achenes. This 1000 achene weight measure was then used to determine which treatments produced heavier achenes. Statistical analysis was performed to compare the mean achene weights between cages and treatments using one-way ANOVA ($\alpha = 0.05$) (SPSS) with Tukey Post Hoc tests (SPSS) to identify significant differences between samples.

3.2.3.1.4 Achene germination

Before the test achenes were germinated, a reliable and efficient pre-germination treatment needed to be found to ensure that any differences in achene germination were the result of a difference in cross-pollination and not the result of the germination methods. The literature suggests a cold stratification period of anywhere from two (Feghahati and Reese, 1994) to five (Younus *et al*., 1997) to twelve weeks (Bratcher *et al.*, 1993), where achenes are mixed with wet sand and kept in the cold under constant light. This method was tested extensively with the only consistent result being that the test achenes were overcome by fungus and would not germinate when placed in a growth chamber. Other cold stratifications on filter paper for three different time periods (2, 3½ or 5 weeks) were attempted. The stratifications met with little success with the exception of one treatment with an unexpectedly high level of germination at 4 ˚C. Most stratification experiments were overcome with fungal infections including ones sterilized with a 10% bleach solution or hydrogen peroxide solution. Stratification was deemed to be an inefficient germination method for this project based on the long period of time required for stratification, the limited success of the test stratifications and the inefficiency of this method for germinating numerous small samples of achenes. In the end a combination of techniques was used based on suggestions by Dr. B. Barl (personal communication) and the literature. All of the pre-treatment steps were performed in a laminar flow hood to minimize contamination. Subsamples of 100 achenes from each inflorescence were germinated. The achenes were first sterilized for ten minutes in a 10% bleach solution (sodium hypochlorite) (Javex™) to prevent or retard fungal growth during germination and to enhance germination (Younus *et al*., 1997). Then the achenes were rinsed and soaked in distilled water for 48 hours in the dark at 4 ˚C, before placement into an incubator in the dark at

30 ˚C for 24 hours to speed germination (Barl, 1997). Both of these steps were utilized to break dormancy and to promote synchronous germination. Achenes were then plated into a sterile polystyrene petri dish (60x15mm; Fisher®) lined with VWR filter paper, sealed with Parafilm® (Wartidiningsih and Geneve, 1994) and placed into a germination chamber at a constant temperature of 22 ˚C and a 16:8 day light: dark cycle (Schulthess *et al.*, 1991) with daytime illumination of approximately 400 ft. candles. Germination was recorded daily for 7 days and once at 14 days, because preliminary trials showed very little additional germination after the first week but enough to warrant an additional week of observation.

To compare the mean percentage germination between cages and treatments, statistical analysis was performed using one-way ANOVAs ($\alpha = 0.05$) with Tukey Post Hoc tests (SPSS) to identify significant differences between samples.

3.2.3.1.5 Comparison of achene weight and germination

The relationship between achene weight and germination was investigated by comparing 1000 achene weights with their germination percentages from sampled inflorescences of pollinator exclusion trials in 2003 and 2004. Linear regression graphs were constructed using the weights of 1000 achenes (x-axis, independent variable) and their germination percentages (y-axis, dependent variable) from field sites in 2003 (PPS and MVA) and 2004 (PPS, MVA and VR).

3.2.3.2 Determination of pollination efficiency using single insect visits

3.2.3.2.1 Controlled hand pollinations

Pollen tube growth rates were determined using hand pollinations. Hand pollination was performed by brushing receptive stigmas with the pollen mass of dehisced anthers from another plant. Selfing treatments were left unmanipulated. Sample florets were harvested at intervals of 6, 12, 24, and 48 h, and prepared for observation as described in section 3.2.3.2.3.

A shrivelled-style technique was first field-tested using controlled hand pollinations on inflorescences of *E. angustifolia* at MVA, before putting it to use in studies of single insect visits (SIVs) (section 3.3.2.2). A style was defined as shrivelled if its stigmatic lobes had retracted into the corollas tube and a persistent style was defined as being fully turgid and exserted (see Fig. 3.24E). Several inflorescences were

bagged to prevent cross-pollination by insects. Bag dimensions were 10 cm x 10 cm with a open diameter of 7.5 cm (Fig.1.1A; for mesh size, see Fig. 11 in Davis, 1992*b*). After two days under bagging, two whorls of disc florets per inflorescence were in their receptive pistillate stage and 12 florets on one and 15 florets on the other inflorescence were cross-pollinated by hand. Hand pollinations were performed using outcross pollen from dehisced anthers of another bagged plant by brushing anthers across the receptive stigmas of the receiving florets. Florets on the opposite side of the inflorescence were not cross-pollinated to serve as controls. Inflorescences were re-bagged and checked 24h and 48h after post pollination, when necessary.

To determine if pollen grains from the same inflorescences or pollen grains from inflorescences of the same plant would also produce a shrivelling reaction in the stigma and style, indicating that successful pollination had occurred, a style shrivelling trial was conducted on *E. angustifolia*. Five bagged inflorescences (replicates) of *E. angustifolia* were divided into four zones with twenty florets per zone in PP1 to PP3 phases. Each zone was subjected to one of four different pollination treatments. Florets of the first zone were pollinated by pollen of another plant (xenogamy), the next zone with pollen from another inflorescences of the same plant (geitonogamy), one zone pollinated with pollen from a staminate phase floret of the same inflorescence (within head selfing) and the final zone was unmanipulated. Inflorescences were checked at 24h and 48h post pollination and the number of shrivelled and persistent stigmas and styles were recorded *in situ*.

To determine if style persistence and shrivelling also occurred in *E. purpurea*, five plants were transplanted from the Biology garden plots into a growth chamber in the Biology greenhouse. In the growth chamber, temperature and light levels remained constant (22°C, ~400 ft candle illumination, 16:8 light: dark cycle). In two plants (replicates), an entire whorl of pistillate florets in their first day of receptivity was pollinated using four separate treatments. The whorl was divided into four zones (as described above for *E .angustifolia*), each with approximately fifteen florets. The experimental whorls were examined *in situ*, 24 and 48h post-pollination. On a third plant, half of the experimental whorl of disc florets was cross-pollinated, the other half remaining untouched (control).

3.2.3.2.2 Stigmatic loads of pollen

Germinated and un-germinated pollen grains were recorded on the stigmas of disc florets from single insect visits concurrently with counts of pollen tubes at the style base thus the same protocol (see section 3.2.3.2.3) was used to prepare stigmas. Pollen grains were identified by auto fluorescence on the stigmatic surface using the yellow filter of a Zeiss Universal epi-fluorescence microscope when the usual method of aniline blue fluorescence was insufficient to view grains. Pollen tubes of germinated grains on the stigma surface were identified by fluorescence of callose in tube walls using aniline blue staining in the same manner as pollen tubes were visualized within the style. One-way ANOVAs (SPSS, $\alpha = 0.05$) were employed to find significant differences between single insect visits and control styles with Tukey Post Hoc tests used to identify significant differences between samples.

3.2.3.2.3 Analysis of styles for pollen tubes

Single insect visits (SIV) to previously bagged virgin inflorescences were employed in 2003 to determine insect pollination ability. Inflorescences were bagged before they were mature to ensure that no insects had visited the florets. Other bagged inflorescences were used as controls to determine the effect of experimental bagging and re-bagging of heads. Some inflorescences were kept bagged for the duration of flowering while other inflorescences had their bags opened and then re-bagged after several minutes without an insect visitor. The bag was opened and watched for a potential single insect visit when at least two consecutive whorls of disc florets had become receptive. After opening the bag, a master sheet with concentric circles representing whorls of disc florets was used to record the developmental stages of each mature whorl and the path taken by visiting insects. On this sheet was recorded all of the other data collected from the solitary visit; the time interval between opening the bag and the first insect visitor was recorded; the identity of the visitor; the reward they were seeking (nectar, pollen or both); and how long they spent foraging on the inflorescence. The area on the capitulum where the insect landed was marked on a palea and ray floret with a drop of blue nail polish so that only styles from the visited area needed to be examined. After the SIV, the inflorescence was re-bagged and harvested approximately

24 hours later, to ensure that pollen tubes from any deposited and germinated pollen had ample time to reach the style base.

After harvest, stigmas and styles were fixed in 1:3 glacial acetic acid: 70% ethanol, softened with 2.5 M NaOH overnight at room temperature, stained with decolourized aniline blue (0.1%, Fisher Scientific Company) for 24 h and observed at 160x and 400x magnification under a Zeiss epifluorescence microscope with UV excitation viewed through a blue filter. Before placing styles in aniline blue, a fluorochrome that binds to callose plugs in pollen tubes, the additional step of incubation in 2.5 M sodium hydroxide (NaOH) was necessary to soften the stylar tissue so that it could be easily squashed under a coverslip and spread for easier viewing of pollen tubes (Martin, 1959). Caution is advised not to exceed a 2.5 M NaOH solution or squash the styles too vigorously or samples may be damaged.

Styles that were prepared and observed microscopically came from the area of the inflorescence that was visited by the insect. These pollen tube counts are a conservative estimate of insect efficiency because not all of the styles from each inflorescence are counted. Where possible, control styles were taken from unvisited areas on the opposite side of the inflorescence to control for the effect of bagging on pollen tube initiation and growth. The number of pollen grains and the number of germinated pollen grains per stigma were recorded to provide an estimate of the amount of pollen transferred by the single insect visitor as well as the number of pollen tubes at the base of each style.

3.2.3.2.4 Comparison of pollen tube counting and shrivelled style analysis for determining pollination efficiency

In 2004, the aniline blue fluorescence technique was again employed to compare the pollen tube counting technique with shrivelled style estimates of pollination after single insect visits. Out of the SIV samples of 2004, 17 were selected for analysis of pollen tubes based on the identity of the insect visitors and the proportion of shrivelled to persistent styles (see section 3.2.3.2.5). Where possible, an equal number of shrivelled and persistent styles were compared. Shrivelled and persistent styles were prepared separately for pollen tube analysis (see 3.2.3.2.3) and observed with UV excitation viewed through a blue filter on the Zeiss epifluorescence microscope.

Stigmatic loads of germinated and un-germinated pollen grains and the number of pollen tubes at the style base were recorded to compare differences between shrivelled and persistent styles.

To enable a comparison between SIVs in 2003 where pollen tube counts were employed, with SIVs from 2004 where shrivelled style analysis was employed, a graph illustrating the percentage of styles with at least one pollen tube at their base was constructed. Disc florets of *E. angustifolia* have one ovule so only one pollen tube is needed to reach the style base to fertilize the floret. In this way, one pollen tube at the base of the style corresponds to a style that has shrivelled due to pollination. It follows then that comparing the percentage of styles with pollen tubes to the percentage of shrivelled styles provides the same results.

3.2.3.2.5 Shrivelled style analysis

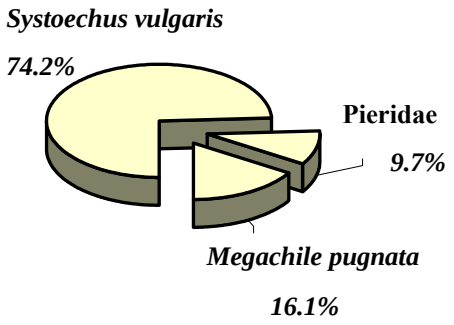
At the MVA and VR field sites in 2004, virgin inflorescences were bagged prior to anthesis to exclude insects, and then opened 2-7 days later when 2-4 whorls of florets were receptive to allow visitation by one insect (SIV). The insect's path on the inflorescence was recorded as well as the number of whorls of receptive disc florets available to receive pollen at the time of the visit. Twenty-four h after the SIV (48h during cold, overcast days), the inflorescence was harvested and taken to the lab so that the number of shrivelled and persistent styles could be counted. Stigmas and styles were then fixed and stored for later pollen tube analysis (see section 3.2.3.2.3). A percentage was calculated based on the number of styles shrivelled, divided by the total number of receptive florets at the time of the single visit. Florets with styles that persisted after an SIV were assumed not to have been cross-pollinated, whereas florets with styles that shrivelled were assumed to have been cross-pollinated. Thus, this shrivelled style technique provided a visual assessment of the pollination status of a floret. The sum of shrivelled styles divided by the sum of the total styles available during the insect visit provided a relatively rapid estimate of the pollination efficiency of that insect. Insects from SIVs were separated by species where possible; bumble bees other than *Bombus ternarius* were grouped as other *Bombus* spp. Of the 46 total SIVs, 41 and 5 were obtained at MVA and VR, respectively.

3.3 Results

3.3.1 Pollinator diversity: transect observations

Transect observations provided a non-lethal method of observing and quantifying the insect visitors to *E. angustifolia*. Potential pollinators recorded during transect observation in 2003 are summarized in Fig. 3.1 (PPS) and Fig. 3.2 (MVA). Not all floral visitors are pollinators and several species of insects were excluded from pollinator abundance graphs because they did not have the typical pollinator attributes of nectar or pollen foraging, multiple floral visits during a foraging trip, or possessing a body suited for pollen transfer. For example, omitted visitors at MVA in 2003 included 27 grasshoppers (Orthoptera: Acrididae), 201 ants (Hymenoptera: Formicidae), 68 *Lygus* bugs (Hemiptera: Miridae), 98 jagged ambush bugs, *Phymata pennsylvanica* (Hemiptera: Phymatidae), 6 yellow crab spiders (Araneae: Thomisidae) and 2 assassin bugs (Hemiptera: Reduviidae). The last three visitors are predators of pollinating insects and were observed eating bee flies, blister beetles, and butterflies. The numbers of jagged ambush bugs skewed upwards because these insects often did not move from their inflorescence and were recorded repeatedly across numerous transects. Ants were always present on the same inflorescences and were thus also recorded repeatedly across multiple transects.

FIG. 3.1: Potential pollinators at Prairie Plant Systems Inc., 2003 (n = 31)



In 2003, 31 potential pollinators were recorded on five transects at PPS representing three families of insects: *Systoechus vulgaris* Loew (Diptera: Bombyliidae), *Megachile pugnata* (Hymenoptera: Megachilidae), and sulphur butterflies (Lepidoptera: Pieridae) (Fig. 3.1). In 2004, three counts along transects were performed at PPS but

with the limited number of inflorescences (12) available, the sightings were limited. On one transect, two bee flies (*Systoechus vulgaris*) and one hover fly (Diptera: Syrphidae) were observed. On the next occasion, no visitors were recorded on the transect and with only 12 inflorescences present, the field senesced and lost its usefulness quickly. One final transect observation was possible with 8 inflorescences and the only recorded visitor was a yellow crab spider. Orange asteracean inflorescences in the area had numerous visitors and these were recorded to get an idea of the pollinator diversity at this site. There were several species of bumble bees (Hymenoptera: Apidae) (*Bombus ternarius* Say, *B. fervidus* Fabr.), some bee flies, hover flies (all of the same species), honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae), sunflower leafcutter bees (*Megachile pugnata* Say) (Hymenoptera: Megachilidae) and several golden blister beetles (*Epicauta ferruginea* Say) (Coleoptera: Meloidae). The discovery of *E. ferruginea* was interesting because this species was not observed at PPS in 2003.

In Fig. 3.3, the total number of potential pollinators at MVA is recorded based on 55 transects performed at various intervals during the day from July 17th to August 28th, 2004. The overwhelming majority (70.1%) of visitors to *E. angustifolia* were golden blister beetles (*Epicauta ferruginea*). This percentage (70.1%) representing 793 blister beetles across 55 transects (an average of 14 beetles per transect) was twice as great as in 2003, when 226 (23.4%) blister beetles were recorded across 33 transects (an average of 7 beetles per transect) from July 8th to August 5th (Fig. 3.2).

Of note in 2004 is that 110 grasshopper bee flies (*Systoechus vulgaris*) made up only 9.7% of total visitors at MVA (Fig. 3.3). This species was the dominant visitor in 2003, comprising 67.9% of the total insect visitors (Fig. 3.2).

FIG. 3.2: Potential pollinators of *Echinacea angustifolia* at Meewasin Valley Authority, 2003 (n = 966)

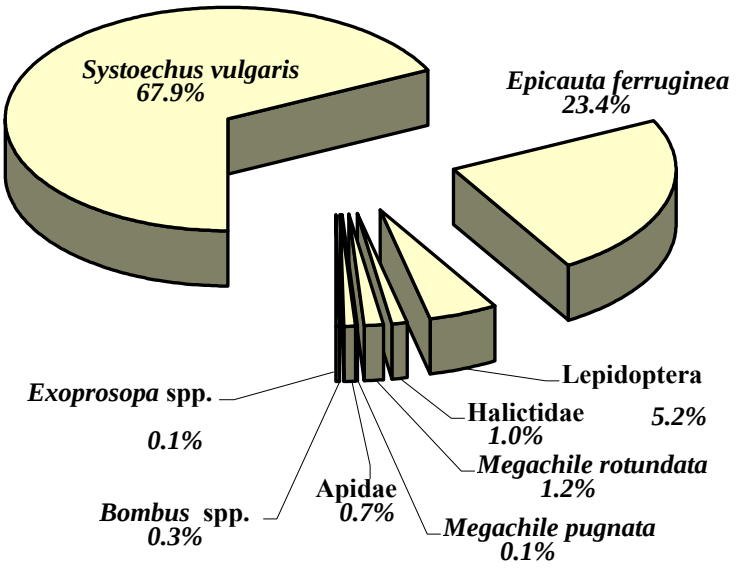
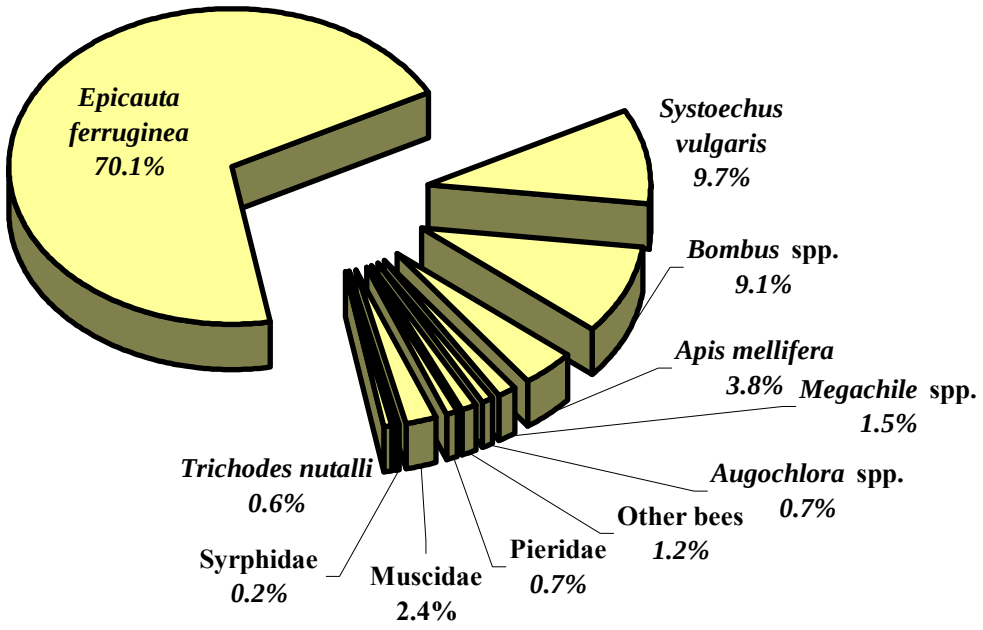


FIG. 3.3: Potential pollinators on *Echinacea angustifolia* at Meewasin Valley Authority, 2004 (n =1132)



Several species of bumble bees at MVA collectively represented 9.1% of total visitors, the third most frequent visitors in 2004 (Fig. 3.3). Bumble bees present at this site were 47 red-tailed bumble bees (*Bombus ternarius*), and a mixture of 56 other bumble bees such as *B. fervidus*, *B. vagans* and *B. nevadensis*. Most bumble bees foraged rapidly and were difficult to distinguish during field observations, thus determination of individual species was difficult on transects and single insect visits. An exception was *B. ternarius*, whose workers are distinctive, with bright red hairs on their second and third abdominal segments and a black thoracic band between their wings. *B. ternarius* were all identifiable in the field but other species were difficult to identify without catching and killing them. Killing potential pollinators may have had deleterious consequences for future single insect visits and transect observations so only small numbers of these insects were collected. Bumble bees were far more numerous in 2004 than in 2003. Three bumble bees were observed visiting *E. angustifolia* at MVA in 2003 (0.3%) (Fig. 3.2), compared to 103 (9.1%) in 2004 (Fig. 3.3). Bumble bees (similar species complement as MVA in 2004) were frequent visitors to *E. purpurea* and *E. angustifolia* in the biology garden plot in summer 2005. Honey bees (*Apis mellifera*) were present at MVA (n = 43: 3.8%) in 2004, which is significantly different from 2003 when they were not observed as visitors. Megachilid bees were grouped in 2004 (1.5%) and are comprised of the sunflower leafcutter bee (*Megachile pugnata*) (8), introduced alfalfa leafcutter bee (*M. rotundata*) (6) and *M. latimanus* (3). *Augochlora* spp. (Hymenoptera: Halictidae) and other unidentified solitary bees comprised 0.7% and 2.1% of inflorescence visitors at MVA in 2004, respectively. Halictids at MVA in 2003 comprised 1% of visitors (Fig. 3.2). The butterfly visitors were all sulphurs (Lepidoptera: Pieridae) in 2004. In 2005, both *E. angustifolia* and *E. purpurea* inflorescences in the biology garden plots were visited by many painted lady butterflies, *Vanessa cardui* (Lepidoptera: Nymphalidae).

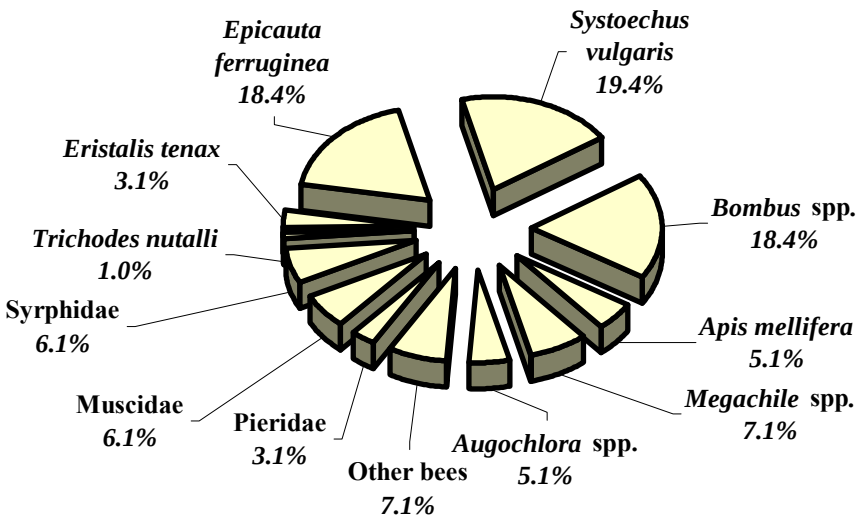
House flies (Diptera: Muscidae) and hover flies (Diptera: Syrphidae) were not recorded as visitors to *E. angustifolia* in 2003 (Fig. 3.2) but were present in low numbers (2.4% and 0.2%) in 2004 (Fig. 3.3). In 2005, 3 species of syrphid flies including drone flies, *Eristalis tenax,* and *Toxomeris* spp., were observed at the Biology garden plot visiting *E. angustifolia* and *E. purpurea* during July and August in much higher numbers

than in previous years. *Trichodes nutalli*, the red-blue checkered beetle (Coleoptera: Cleridae), was found several times (0.6%) eating pollen from staminate florets at MVA (3.3).

Visitors to inflorescences of *E. angustifolia* omitted at MVA in 2004 included 63 *Lygus* bugs, five assassin bugs, 10 jagged ambush bugs, one stink bug (Hemiptera: Pentatomidae), 43 grasshoppers, four ants, and two yellow crab spiders. At VR, a similar complement of visitors were omitted: 58 *Lygus* bugs, one assassin bug, one stink bug, 46 grasshoppers, seven ants, two yellow crab spiders and one harvestman (Opilionae: Phalangodidae).

Figure 3.4 illustrates the potential pollinating insects recorded on capitula of *E. angustifolia* at VR in 2004 across 22 transects from July 23rd to August 25th. *Epicauta ferruginea* (18.4%), *Systoechus vulgaris* (19.4%) and bumble bees (*Bombus* spp.) (18.4%) were found in nearly equal proportions. Honey bees (5.1%) were also observed visiting inflorescences, as were megachilid bees (7.1%). The megachilids were grouped together but they appeared in these proportions: *Megachile pugnata* (4), introduced *M. rotundata* (2), and *M. latimanus* (1). *Augochlora* spp. comprised 5.1% of inflorescence visitors at VR, whereas other unidentified bees were observed as 7.1% of visitors. Hover flies (Syrphidae) and house flies (Muscidae) each comprised 6.1% of all visitors to inflorescences of *E. angustifolia* at VR, whereas white and sulphur butterflies (Pieridae) made up 3.1%. Just a single red-blue checkered beetle, *Trichodes nutalli* (1.0%), was recorded at VR.

FIG 3.4: Potential pollinators on *Echinacea angustifolia* at Valley Road, 2004 (n=90)



3.3.2 Insect pollination efficiency

3.3.2.1 Excluder cages

3.3.2.1.1 Achene weights

Achene weights were used as an indicator of the quality of seed produced in each excluder-cage treatment. In 2003, the mean (± S.E.) number and range of achenes per inflorescence collected at PPS was 197 ± 9 (47 to 405; n = 47 inflorescences) and from MVA, mean number and range of achenes per inflorescence was 184 ± 8 (39 to 324; n = 57 inflorescences). In 2004, mean (± S.E.) number of achenes per inflorescence was 177 ± 11 at PPS (56-327; n = 40), 234 ± 7 at MVA (116-423; n = 75), and 204 ± 5 at VR (123-309; n = 75).

Plants were divided into three treatments at each site: openly pollinated controls; partial excluder cages in which large-bodied insects were excluded from inflorescences; and total excluder cages wherein all insects were excluded from inflorescences. Achene weights at PPS in 2003 are illustrated by Fig. 3.5. Owing to the similar achene weights per cage type, it was possible to pool the data from PPS's two partial excluder cages (P = 0.792) and also to combine data from the two total excluder cages (P = 0.281) to increase sample sizes. This situation was repeated at MVA (Fig. 3.6), where achene weights were pooled in the same manner for partial (P = 0.612), and total (P = 0.247)

excluder cages. Letters on graphs indicate which treatment means were statistically different from each other ($\alpha = 0.05$). The number of inflorescences per treatment is represented by "n". 1000 achenes at PPS from openly pollinated inflorescences were over twice as heavy (2.5 g) as achenes in both partial excluder cages (0.94 g) and total excluder cages (1.02 g).

A slightly different situation was found at MVA, where achene weights in each treatment were all significantly different (Fig. 3.6). Achenes weights at PPS and MVA are compared in Fig. 3.7.

FIG. 3.5: Pooled mean (± S.E.) 1000 achene weights of inflorescences (n) of *Echinacea angustifolia* from Prairie Plant Systems Inc, 2003

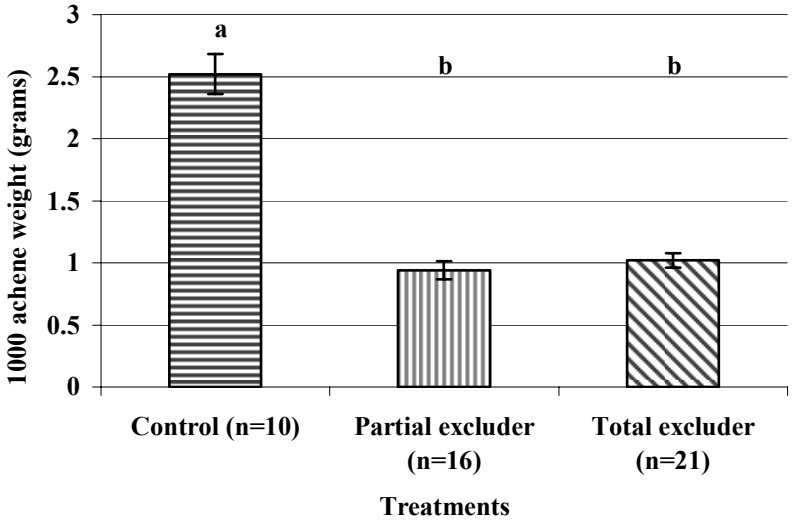


FIG: 3.6: Pooled mean (± S.E.) 1000 achene weights of inflorescences (n) of *Echinacea angustifolia* at Meewasin Valley Authority, 2003

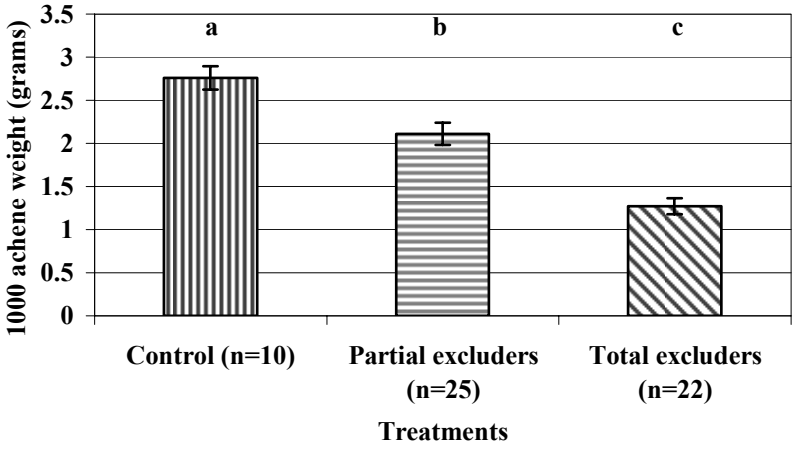
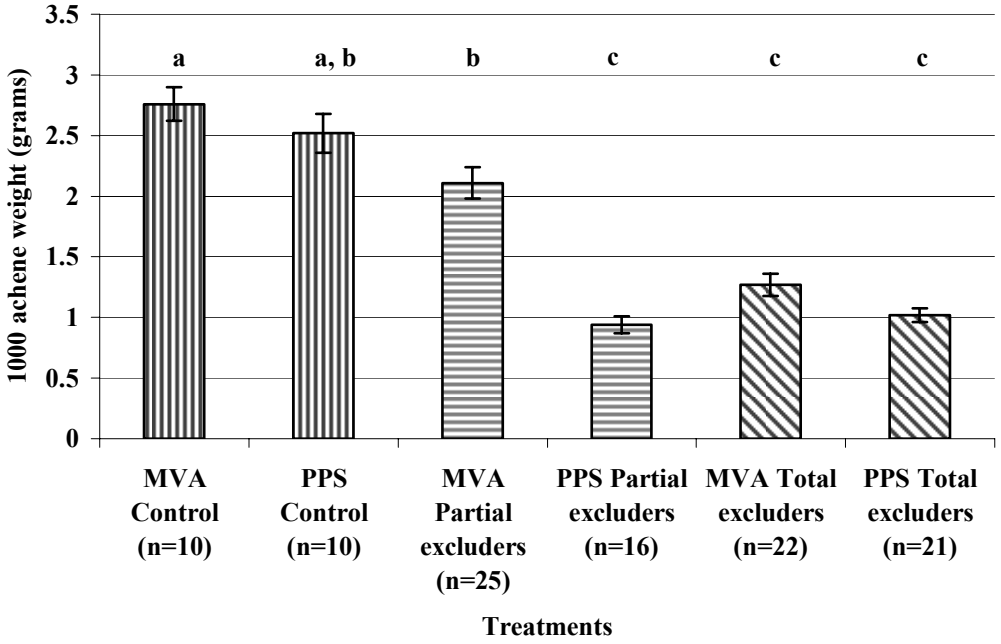


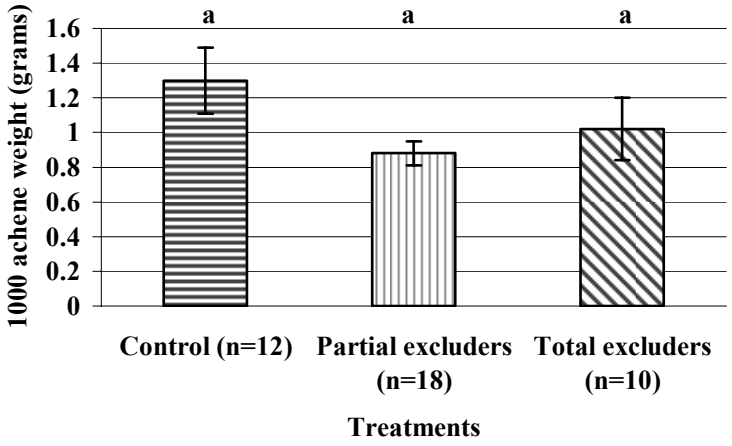
FIG. 3.7: Comparison of mean (±S.E.) 1000 achene weights from Meewasin Valley Authority with those of Prairie Plant Systems Inc., 2003



Control inflorescences from MVA and PPS produced achenes with average weights that were not significantly different (P = 0.698) (Fig. 3.7). Interestingly, partial excluder treatments at MVA produced achenes with mean weights that were not significantly different from control achenes at PPS (P = 0.164) and achene weights from partial excluder treatments at PPS were significantly lower than controls and partial excluders at MVA. Partial excluders from PPS had low achene weights that were not significantly different from the uniformly low achene weights in total excluder cages (P = 0.349).

In 2004, achene weights at PPS (Fig. 3.8) were uniformly low with openly pollinated control inflorescences having a slightly higher mean 1000 achene weight (1.3 g) than pooled partial excluder treatments (0.88 g) and total excluder treatments (1.02 g). Mean 1000 weights of treatments are not significantly different from each other ("a" P = 0.089). Achene weights were not significantly different in partial (P = 0.703) and total (P = 0.969) excluder cages, which allowed data to be pooled.

FIG. 3.8: Mean (± S.E.) 1000 weight comparison between cage treatments at Prairie Plant Systems Inc., 2004



Sample sizes across treatments were equal for excluder trials at MVA and VR in 2004, so partial excluder treatments and total excluder treatments were not pooled, to maintain equal sample sizes for statistical comparisons. Control inflorescences at MVA had mean 1000 achene weights that were twice as heavy as those in the total excluder cages and those in the partial excluder cages (Fig. 3.9). The control mean was significantly different from all other means ($P = 0.000$). There was no significant difference between the two partial excluder cages or between the two total excluder cages ($P = 0.59$).

At VR, achenes from control inflorescences openly pollinated by insects were over twice as heavy than the mean weight of achenes from inflorescences protected completely from insects (Fig. 3.10). Mean weight of achenes from partial excluder cage #5 was not significantly different from the mean weight of achenes in the total excluder treatments ($P = 0.994$), whereas achene weights from partial excluder cage #6 were significantly different from other treatments.

FIG. 3.9: Mean (± S.E.) 1000 achene weight of inflorescences (n) of *Echinacea angustifolia* between cage treatments at Meewasin Valley Authority, 2004

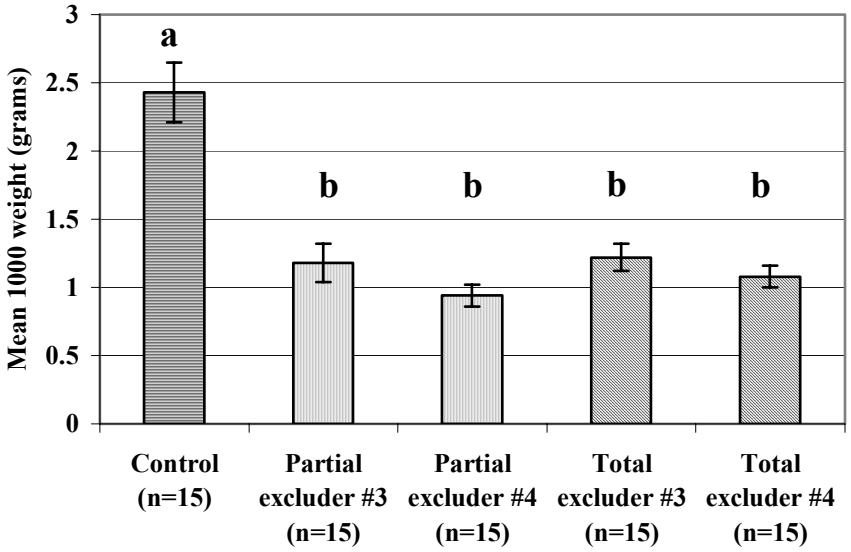


FIG. 3.10: Mean (± S.E.) 1000 achene weights of inflorescences (n) of *Echinacea angustifolia* between cage treatments at Valley Road, 2004

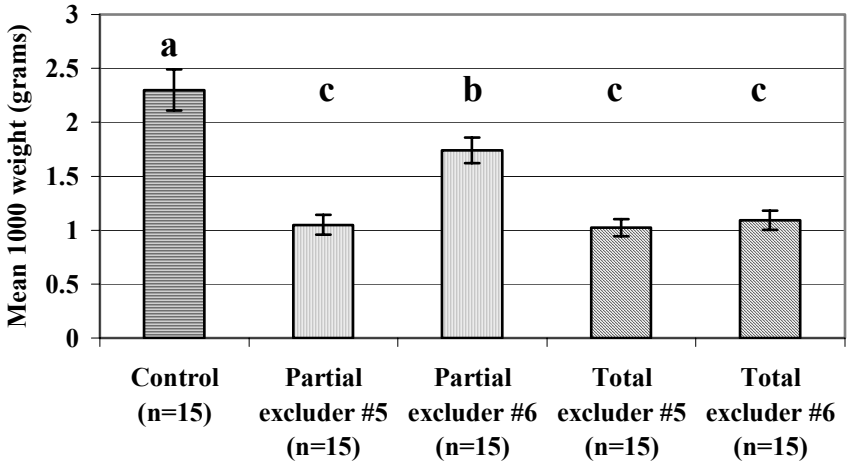


FIG. 3.11: Combined mean (± S.E.) 1000 achene weights of inflorescences (n) of *Echinacea angustifolia* from all three field sites, 2004

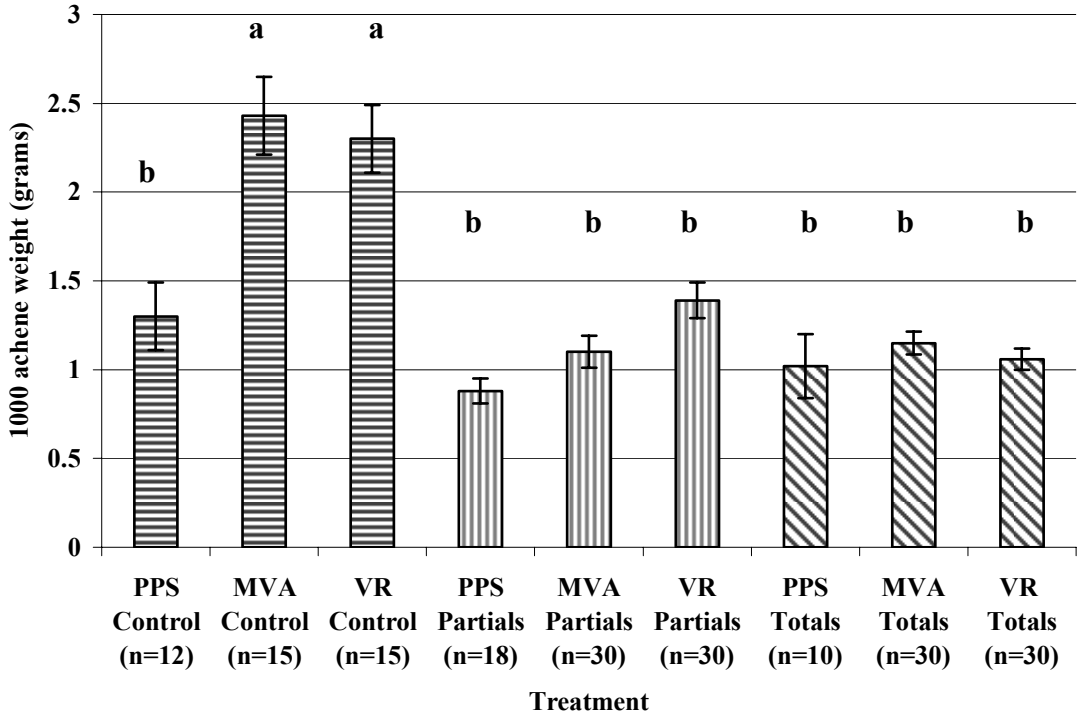


Fig. 3.11 illustrates site differences between treatments in 2004. Data from partial excluder treatments at each site were grouped together even though there were significant differences between cages at VR. Data from total excluder cages were pooled in the same manner without difficulty because there were no significant differences among cages at any of the sites. All excluder treatments were compared and grouped into two significantly different homogenous subsets indicated by "a" (P = 0.998) including the heavy achene samples of MVA and VR controls and "b" (P = 0.077) including PPS control inflorescences and all partial and total excluders (Fig. 3.11). MVA and VR control inflorescences had mean achene weights that were not significantly different (P = 0.999), but were significantly different from the controls at PPS (P = 0.000). Partial excluder means from all sites were grouped into the same subset but were not all significantly lower than each other. Achene weights from partial excluders at PPS were significantly lower than partial excluders at VR (P = 0.026).

Achene weights from MVA partial excluders were not significantly different from PPS (P = 0.959) or VR (P = 0.236) partial excluders. MVA partial excluders had mean 1000 achene weights that were not significantly different from 1000 achene weights in any of the total excluder treatments whereas PPS partial excluder treatments produced 1000 achene weights with means lower than total excluder treatments at MVA and VR. Inflorescences within total excluder cages produced mean 1000 achene weights that were not significantly different from each other (P = 0.578).

3.3.2.1.2 Achene germinations

Fig. 3.12 displays the percent germination of achenes from PPS in 2003. The percent germination of control achenes was significantly greater than the partial excluder treatment means and the total excluder treatment means (P = 0.000). The differences in mean achene germination are not significant between the partial excluder treatments and total excluder treatments (P = 0.751).

FIG. 3.12: Mean (± S.E.) achene germination of inflorescences (n) of *Echinacea angustifolia* from Prairie Plant Systems Inc., 2003

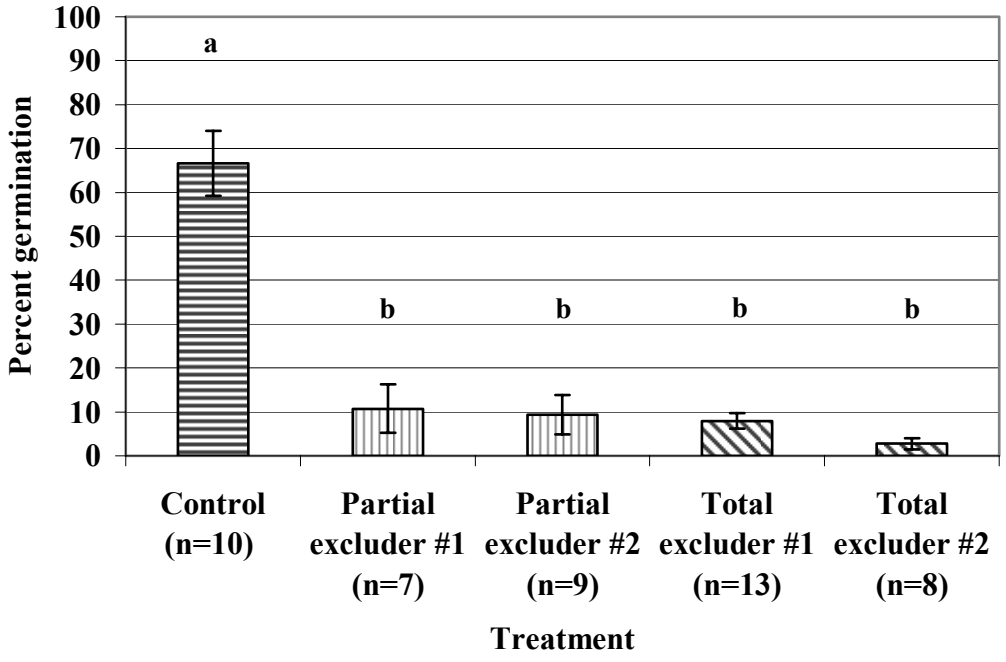
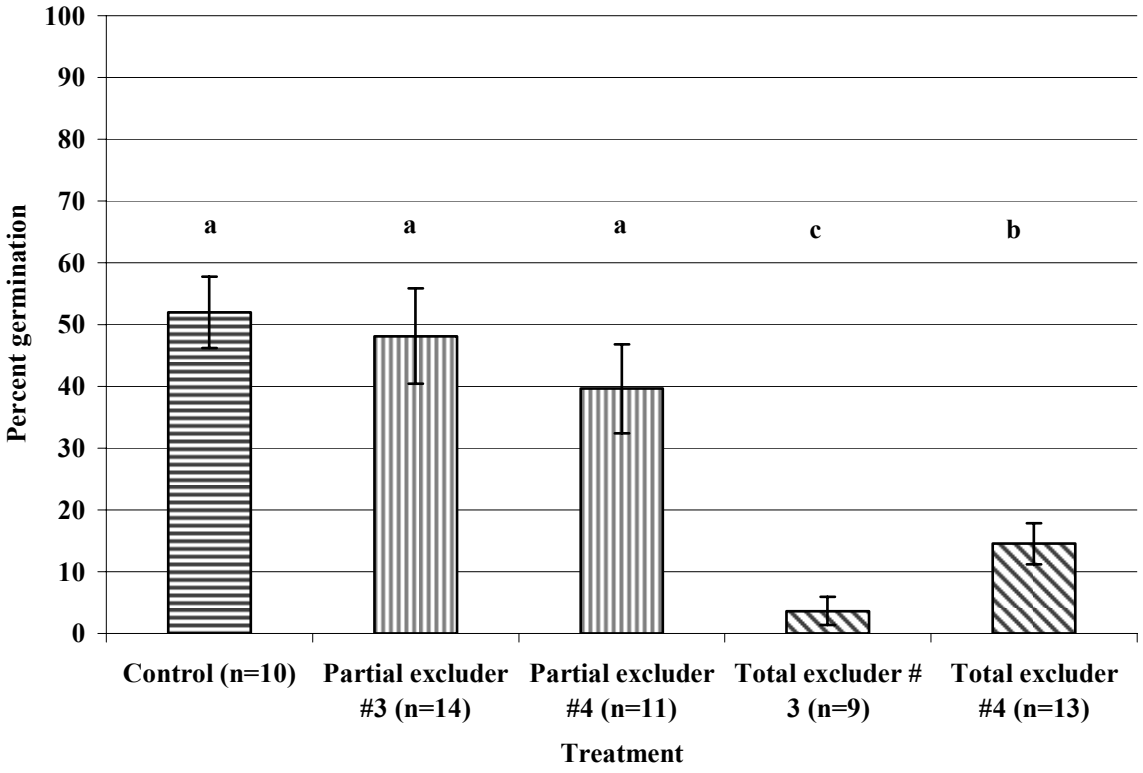


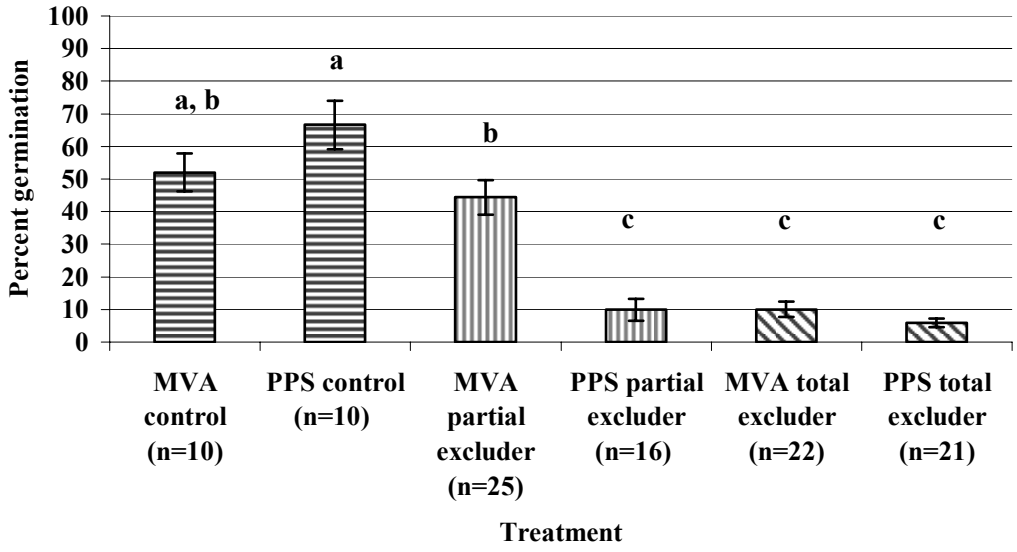
Fig. 3.13 represents the percent germination of achenes from MVA over a 14-day period. On average, 52.0% of achenes from control inflorescences germinated while achenes from partial excluder treatments germinated at 48.1% (#3) and 39.6% (#4). These mean germinations were not significantly different (Fig. 3.13 "a", P = 0.613). The total excluder treatments not only had lower germination than the control and partial excluder treatments, but also had percent germinations that were significantly different (P = 0.023).

FIG. 3.13: Mean (± S.E.) achene germination of inflorescences (n) of *Echinacea angustifolia* from Meewasin Valley Authority, 2003



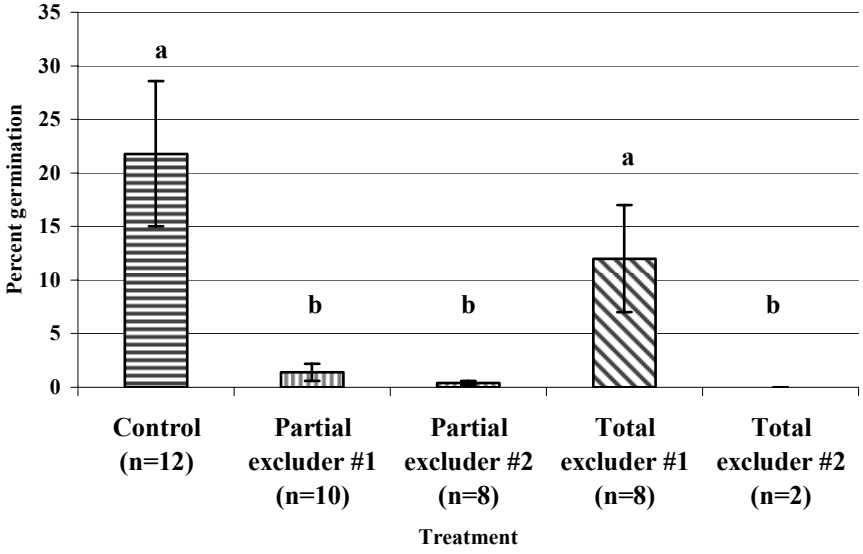
Control inflorescences from PPS and MVA produced achenes with germinations that were not significantly different (Fig. 3.14 "a", P = 0.451). Germination of achenes in partial excluders was significantly different (P = 0.000) between sites (Fig. 3.14). Total excluders from each site produced achenes with low germination that were not significantly different (P = 0.973) from each other or from PPS partial excluders (Fig. 3.14 "c", P = 0.988).

FIG. 3.14: Mean (± S.E.) germination comparison of achenes from inflorescences (n) of *Echinacea angustifolia* from MVA and PPS, 2003



The percent germination of achenes from inflorescences of *E. angustifolia* at PPS in 2004 is illustrated in Fig. 3.15. The control bar represents all of the inflorescences exposed to insect pollination at this site.

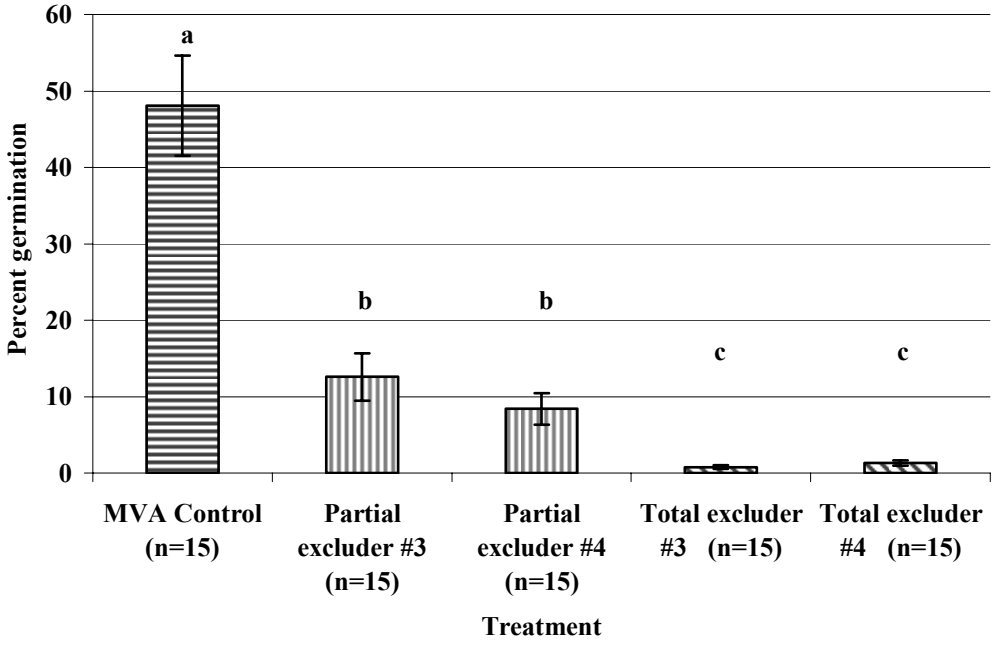
FIG. 3.15: Mean (± S.E.) achene germination of inflorescences (n) of *Echinacea angustifolia* from Prairie Plant Systems Inc., 2004



Germination in partial excluder cages #1 and #2 was extremely low at PPS, with averages of 1.4% and 0.4%, respectively (Fig. 3.15). No germination was recorded for

the two surviving inflorescences of total excluder cage #2 and an average of 12% germination occurred in inflorescences from total excluder #1. The differences between partial excluder #1 and #2 and total excluder #2 were not significantly different (Fig. 3.15 "b" P = 1). The control mean was significantly higher than mean germination in these three cages (P = 0.000). Mean germination of controls and total excluder #1 were not significantly different (P = 0.589).

FIG. 3.16: Mean (± S.E.) achene germination of inflorescences (n) of *Echinacea angustifolia* at Meewasin Valley Authority, 2004

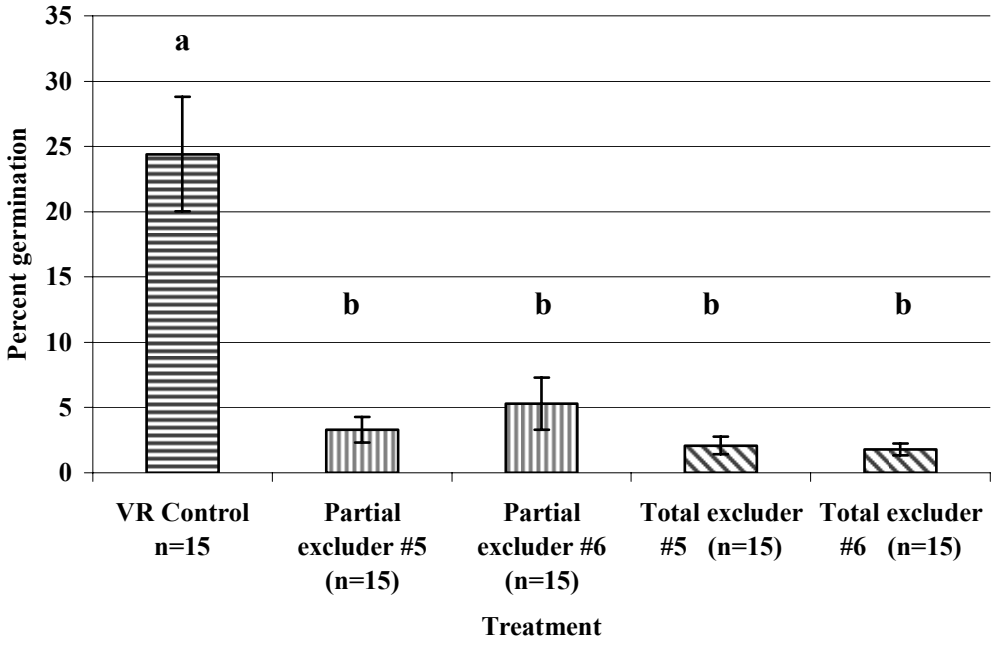


Mean germination of achenes from the three cage treatments at MVA in 2004 was significantly different (P = 0.000) (Fig. 3.16). Partial excluders were significantly different from controls (P = 0.000) and from total excluders (P = 0.017) but were not significantly different from each other (P = 0.903). Total excluder treatments in 2004 had very low percent germinations of 1.3% and 0.8%, respectively, and were not significantly different (P = 1).

Control inflorescences at VR in 2004 had a mean percent germination of 24.4%, which was significantly greater than the partial excluder treatments and the total excluder treatments (P = 0.000, Fig. 3.17). Mean percent germinations of achenes in the

partial excluder treatments were not significantly different from each other (P = 0.969) or from mean percent germination of achenes in the total excluder treatments ("b", P = 0.805, Fig 3.17).

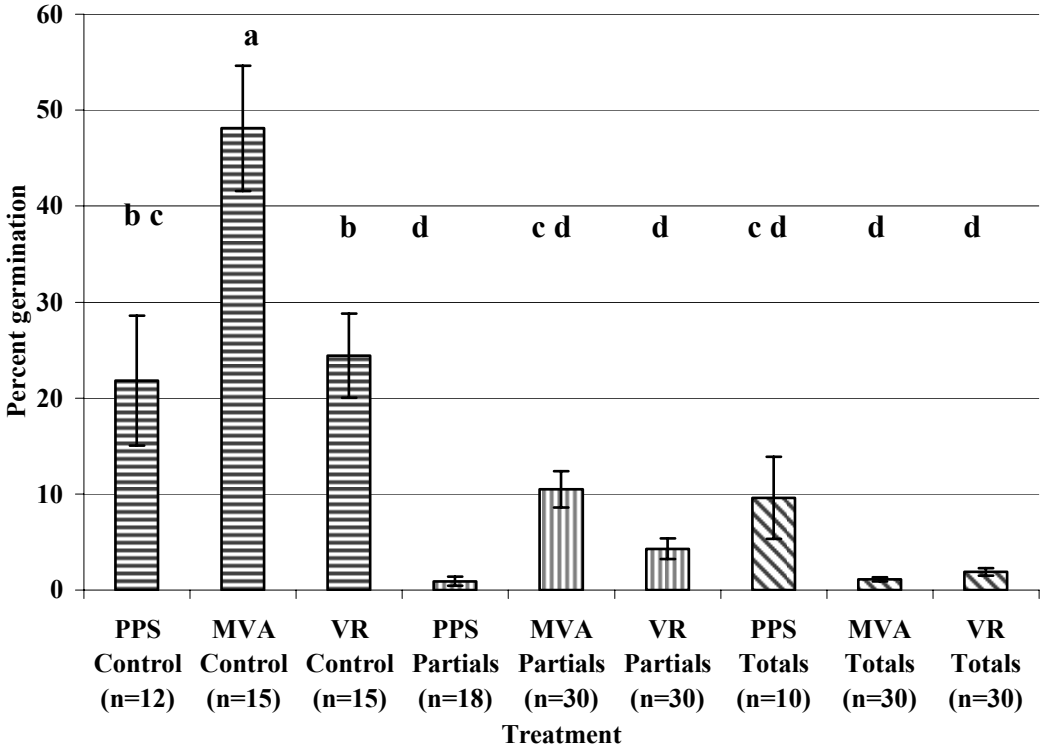
FIG. 3.17: Mean (± S.E.) achene germination of inflorescences (n) of *Echinacea angustifolia* at Valley Road, 2004



All of the partial excluder treatments from each site have been pooled so that trends between sites can be visualized (Fig. 3.18). Mean percent germinations were not significantly different, so this was possible. Total excluder treatments have also been pooled per site even though the differences between total excluder cages at PPS were significantly different. PPS total excluder cage #2 had a sample size of two inflorescences and their percent germination (0%) was identical to the mode of percent germinations (0%) in PPS total excluder cage #1 so pooling in this circumstance was appropriate. Mean germinations of achenes from control inflorescences at MVA were significantly higher than those at PPS and VR (P = 0.000, Fig. 3.18), but controls at PPS and VR were not significantly different from each other (P = 0.999, Fig 3.18). Mean germinations of achenes from partial and total excluders was low and are grouped into a homogenous subset (P = 0.278, Fig. 3.18) with two exceptions of MVA partials and PPS

totals having achene germinations that were not significantly different from PPS control germinations (P = 0.55, Fig. 3.18).

FIG. 3.18: Combined mean (± S.E.) percent germination of achenes from *Echinacea angustifolia* inflorescences (n), 2004



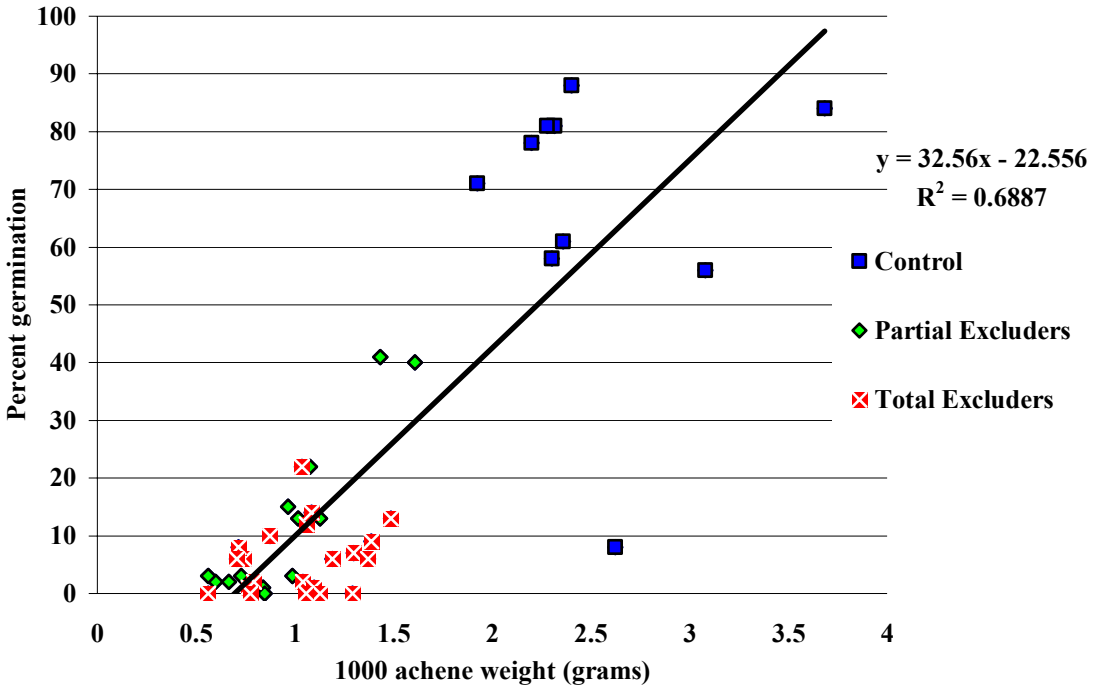
3.3.2.1.3 Comparison of achene weight and germination

1000 achene weights and their corresponding germinations at each field site in each year were compared using linear regressions. An R^2 value was calculated to determine how closely the data points reside to the best-fit line. Each data point represents the achenes from one inflorescence. Data points marked as blue squares represent achenes from control treatments, green diamonds are achenes from partial excluder treatments and red X squares represent achenes in total excluder treatments.

At PPS in 2003 (Fig. 3.19), the regression coefficient (R^2 value) of achene weight vs. germination is $R^2 = 0.6887$, indicating a positive relationship between achene weight and seed germination. Samples from inflorescences in total excluder cages are

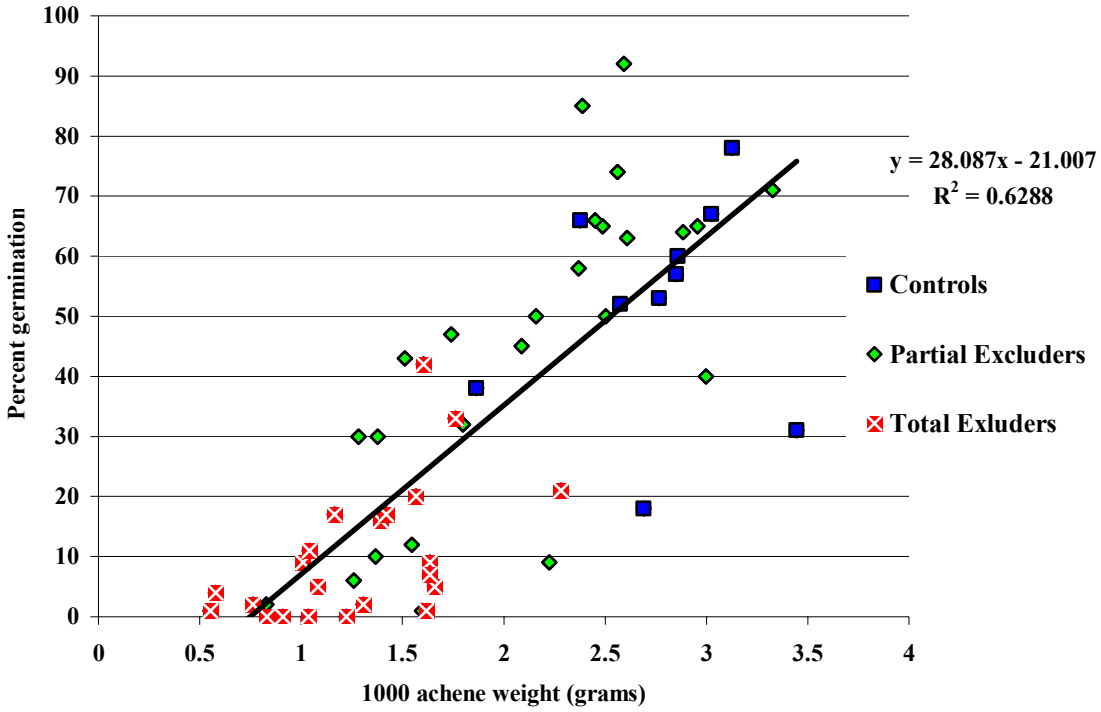
clustered at the bottom of the graph with low weights and low germinations, whereas control inflorescences that were allowed insect visitation aggregated mostly at the top end of the graph with achene weights over 2.0 g and germinations greater than 50% (Fig. 3.19).

FIG. 3.19: Percent germination vs. 1000 achene weight of *Echinacea angustifolia* inflorescences from Prairie Plant Systems Inc., 2003 (n = 47)



At MVA in 2003 (Fig. 3.20), the R^2 value (0.6288) also indicated a positive relationship between achene weights and germination. Of particular note is that the weights and germinations of individual inflorescence samples from the partial excluder treatment (Fig. 3.20), compared to the open-pollinated controls, were intermediate in 1000 achene weight (Fig. 3.6) and just as high in achene germination (Fig. 3.13).

FIG. 3.20: Percent germination vs. 1000 achene weight of *Echinacea angustifolia* inflorescences from Meewasin Valley Authority, 2003 (n = 57)



The weights and germination percentages from achenes in 2004 also showed a positive correlation between 1000 achene weights and percent germination. Most achenes at PPS (2004) had low weights and low germination percentages. The linear regression is positive with an R^2 value of 0.5258 (Fig. 3.21). Fig. 3.21 also demonstrates the low germination percentages and low weights of achenes at PPS, where the majority of data points showed 0% germination and fell along the x-axis.

Weights and germination percentages of achenes at MVA in 2004 had an R^2 value of 0.4647, again indicating a positive relationship between achene weight and germination (Fig. 3.22). Of note was that control achenes at MVA (2004, Fig. 3.22) had excellent germination compared to both PPS (Fig. 3.21) and VR (Fig. 3.23) control achenes.

FIG. 3.21: Percent germination vs. 1000 achene weight of *Echinacea angustifolia* inflorescences from Prairie Plant Systems, 2004 (n = 40)

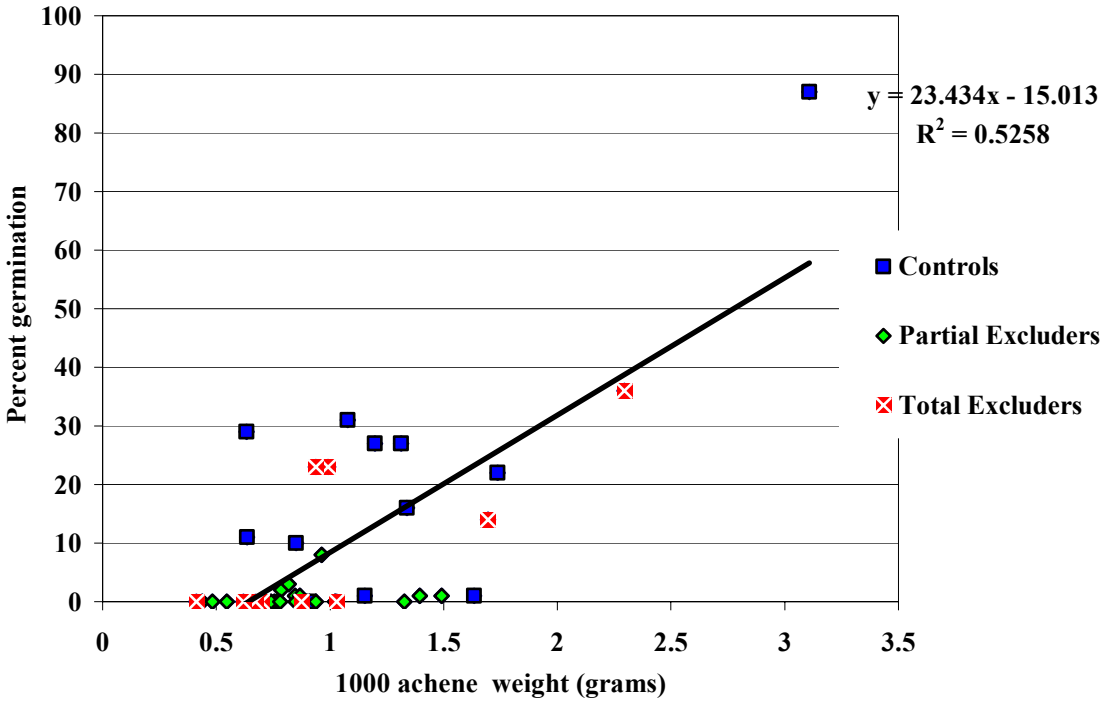
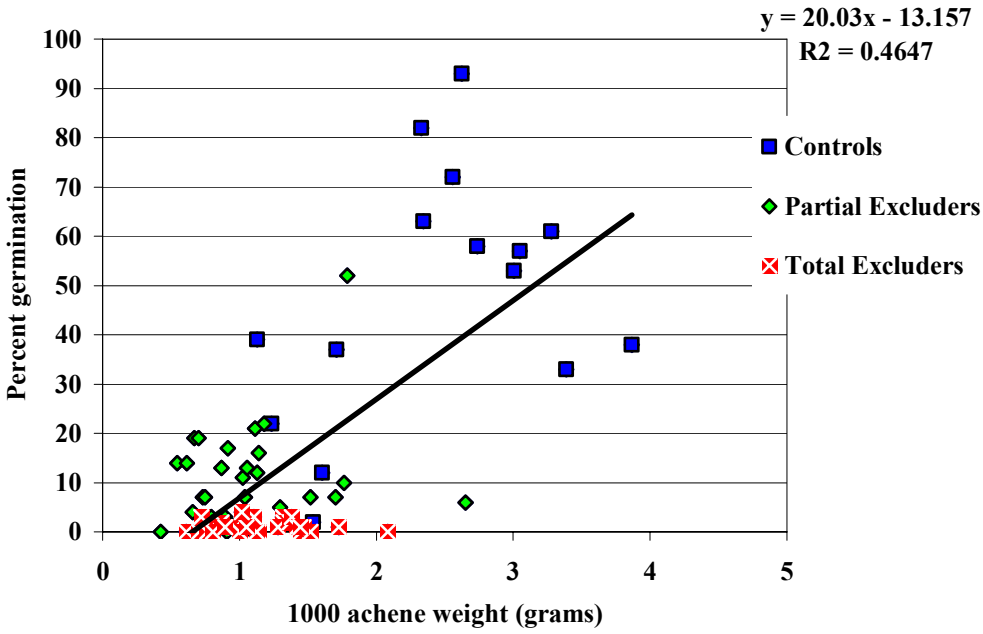
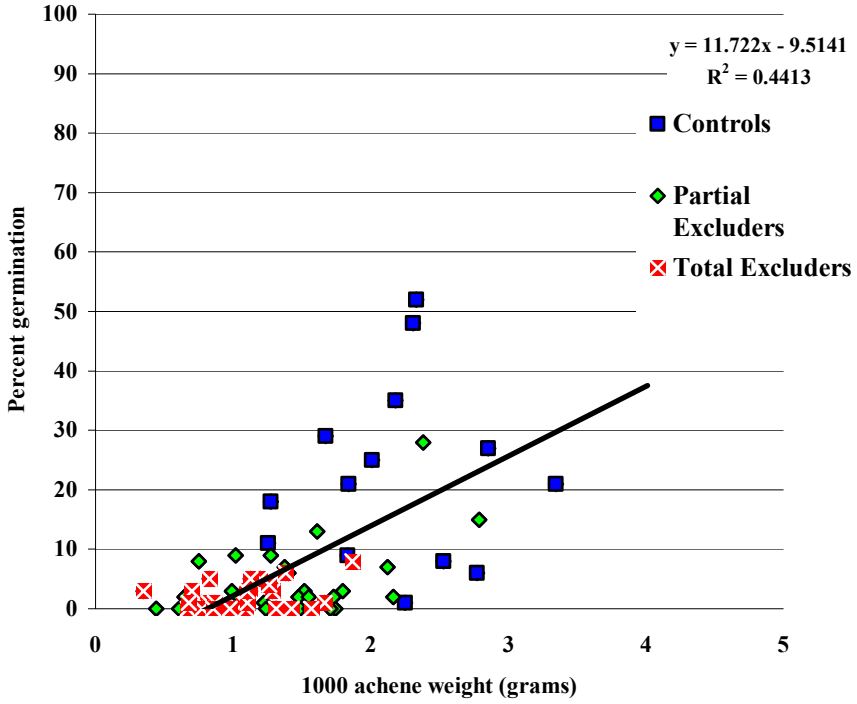


FIG. 3.22: Percent germination vs. 1000 achene weight of *Echinacea angustifolia* inflorescences from Meewasin Valley Authority, 2004 (n = 75)



A positive relationship existed between percent germination and 1000 achene weight of achenes at Valley Road (Fig. 3.23), with an R^2 value of 0.4413.

FIG. 3.23: Percent germination vs. 1000 achene weight of *Echinacea angustifolia* inflorescences from Valley Road, 2004 (n = 75)



3.3.2.2 Single insect visits

3.3.2.2.1 Controlled hand pollinations

After microscopic examination of styles harvested from controlled hand pollinations, it was determined that pollen tubes were present at the style base between 12 h and 24 h of the event of cross-pollination. A 24 h growth window was thus established for harvesting inflorescences for pollen tube counts after single insect visits. It was also observed however, that contrary to what was expected, self pollen grains that were transferred from anthers to stigmas of the same floret would germinate, although the actual proportion of germinated self grains was not noted. Germination of self pollen grains occurred in florets of *E. angustifolia* and *E. purpurea*.

Cross pollination experiments with shrivelled styles were performed on *E. angustifolia* before this technique was employed to estimate insect pollination efficiency

in the field (see 3.2.3.2.1). In the first two trials initiated on July 20, 2004, there was no shrivelling of styles below cross-pollinated stigmas after 24 h (Table 3.1) and they appeared as turgid and un-shrivelled as the unmanipulated control stigmas. After 48 h another check of these two inflorescences revealed that all styles of the cross-pollinated florets had shrivelled (100%), while none of the styles of the control florets of the same whorl were shrivelled. Two more inflorescences were tested in the same manner on July 24, 2004, and after 24 h all styles of the cross-pollinated florets had shrivelled while the styles of control florets remained turgid and exsert (persistent). Differences in the weather occurred where trials 1 and 2 were conducted under cloudy, cool and wet conditions, whereas trials 3 and 4 were conducted under sunny and hot conditions.

Table 3.1: Percent styles shrivelled following controlled cross-pollination by hand or without interference (control) in bagged inflorescences of *Echinacea angustifolia*

Plant	Post-pollination interval	% styles shrivelled		Weather conditions
		Xenogamy	Control	
1	24 h	0	0	cloudy/cool/rainy
	48 h	100	0	cloudy/cool/rainy
2	24 h	0	0	cloudy/cool
	48 h	100	0	cloudy/cool
3	24 h	100	0	sunny/hot
	48 h	100	0	sunny/hot
4	24 h	100	0	sunny/hot
	48 h	100	0	sunny/hot

A pollination trial investigating style responses to pollen from four donor treatments was then performed (see 3.2.3.2.1). Within 48 h, most cross-pollinated florets (xenogamy) had shrivelled styles (mean = 99%) (Table 3.2) and unmanipulated controls had 0% shrivelled styles. The effect of weather was seen again with a 24 h period insufficient to promote full shrivelling during periods of cool, cloudy weather. It was not possible initially to experiment with a geitonogamous treatment where donor pollen came from anthers of another inflorescence of the same plant until *E. angustifolia* plants produced multiple inflorescences, which they tend to do in abundance in later years of growth. Surprisingly, geitonogamous pollination (mean = 27%) produced

shrivelling of styles in percentages greater than selfing within the inflorescence (mean = 5.2%) and unmanipulated controls (mean 0%) (Table 3.2). Selfed stigmas that shrivelled tended to be from the most advanced florets (i.e., PP3 stage) when pollen applied, rather than younger stages (i.e., PP1, PP2).

Table 3.2: Percent styles shrivelled following four controlled pollination treatments by hand or without interference (control) on bagged inflorescences of *Echinacea angustifolia*

Plant	Time	% styles shrivelled				Weather conditions
		Xenogamy	Geitonogamy	Selfing within head	Control	
1	24 h	85	0	0	0	Cloudy/warm
	48 h	100	5	0	0	Sunny/hot
2	24 h	35	0	0	0	Part.Cloudy/warm
	48 h	100	25	0	0	Cloudy/cool
3	24 h	75	0	0	0	Sunny/warm
	48 h	100	0	0	0	Cloudy/warm
4	24 h	65	65	0	0	Cloudy/warm
	48 h	95	90	20	0	Cloudy/warm
5	24 h	82	0	0	0	Sunny/hot
	48 h	100	15	6	0	Cloudy/warm

Upon cross-pollination of *E. purpurea* florets on plants within a growth chamber, all styles shrivelled within 24 h whereas styles of control florets not cross-pollinated by hand remained turgid for approximately eight days (Table 3.3).

Table 3.3: Percent styles shrivelled following four controlled pollination treatments by hand or without interference (control) on inflorescences of *E. purpurea* in a growth chamber

Plant	Time	% styles shrivelled			
		Xenogamy	Geitonogamy	Selfing within head	Control
1	24 h	100	30	27	0
	48 h	100	30	45	0
2	24 h	100	0	0	0
	48 h	100	13	6	12
3	12 h	100	-	-	0

Of note is that geitonogamous pollination (pollen from the same plant, but a different head) and self-pollination (pollen from within the same head), resulted in 13-30% and 6-45% of styles shrivelling, respectively, within 48 h (Table 3.3). Also, 12% of non-manipulated florets (controls) had shrivelled styles after 48 h (Table 3.3).

3.3.2.2.2 Stigmatic loads of pollen

Analysis of the number of pollen grains (un-germinated and germinated) was conducted on stigmas of *E. angustifolia* concurrently with quantification of pollen tubes at the style base from samples involving single insect visits. The number of germinated grains can then be compared to the number of pollen tubes at the style base. A summary of data collected by fluorescence microscopy following single insect visits to inflorescences of *E. angustifolia* is presented in Table 3.4. The number of styles examined after apparent contact by the visitor was not consistent across visits, therefore percentages were used to best illustrate differences between visits. The designation (% germ. grains) represents the percentage of grains per sample that have germinated compared to the total number of grains per sample. The number of germinated grains is a conservative estimate of the number of viable grains transferred during the single insect visit. The literature suggests that in the Asteraceae, the sporophytic self-incompatibility system operates to prevent the germination of self pollen grains (Brewbaker, 1957) but that this system often fails and self pollen grains can germinate on stigmas (Hiscock, 2000; Hiscock *et al*., 2002; Hiscock *et al.,* 2003). Often it was observed that on unmanipulated or self-pollinated stigmas of *E. angustifolia*, where self pollen should be the only grains present, there were sites of localization of callose within stigmatic papillae at sites where pollen tubes from germinated grains contacted receptive papillae (Fig. 3.24B, C). Originally it was thought that cross pollination could be estimated by comparing the percentage of germinated grains between species making single insect visits. However, germination of self-pollen grains confounds any patterns that may be present and there was no significant difference between the percentage of germinated pollen grains per insect species ($P = 0.948$) or between the number of germinated grains per style ($P = 0.272$).

From Table 3.4, it is evident that not every germinated pollen grain successfully produced a pollen tube that reached the style base. On most stigmas, there were more germinated grains than there were pollen tubes at the style base. Most SIVs in 2003 involved *Systoechus vulgaris* and *Epicauta ferruginea*, which were the two most abundant species visiting *E. angustifolia* at MVA based on transect observations (Fig. 3.2).

Table 3.4: List of single insect visit data from 2003
Abbreviations: SIV = Single Insect Visit, *S.v.* = *Systoechus vulgaris*, *Ex.* sp. = *Exoprosopa* sp., *E.f.* = *Epicauta ferruginea*, *P.s.* = *Phoebis sennae*, *M.p.* = *Megachile pugnata, M.r.* = *M. rotundata*, H. spp. = Halictidae sp., C = control

		Pollen grains				**Pollen tubes at style base**		
Visitor and SIV no.	**No. styles**	**Total no.**	**No. per style**	**Total no. (%) germ**	**No. germ per style**	**Total no.**	**No. per style**	**No. (%) styles with tubes**
S.v. 1	56	376	6.7	70 (18.6)	1.3	158	2.8	48 (85.7)
S.v. 2	47	205	4.4	26 (12.6)	0.6	8	0.2	5 (10.6)
S.v. 3	61	780	12.8	120 (15.4)	2.0	53	0.9	36 (59.0)
S.v. 4	39	226	5.8	31 (13.7)	0.8	11	0.3	10 (25.6)
S.v. 6	29	784	27.0	143 (18.2)	4.9	37	1.3	14 (48.3)
S.v. 8	34	384	11.3	7 (1.8)	0.2	1	0.0	1 (2.9)
S.v. 25	59	458	7.8	12 (2.6)	0.2	17	0.3	12 (20.3)
S.v. 29	90	1835	20.4	69 (3.8)	0.8	15	0.2	11 (12.2)
S.v. 31	46	349	7.6	23 (6.6)	0.5	7	0.2	6 (13.0)
S.v. 47	84	201	2.4	41 (20.4)	0.5	12	0.1	8 (9.5)
S.v. 49	59	162	2.7	31 (19.1)	0.5	6	0.1	4 (6.8)
S.v. 52	39	41	1.1	36 (87.8)	0.9	9	0.2	8 (20.5)
S.v. 54	81	287	3.5	94 (32.8)	1.2	1	0.0	1 (1.2)
S.v. 55	40	57	1.4	18 (31.6)	0.5	7	0.2	6 (15.0)
S.v. 56	43	31	0.7	10 (32.3)	0.2	0	0.0	0 (0.0)
S.v. 57	32	26	0.8	12 (46.2)	0.4	5	0.2	4 (12.5)
S.v. 59	54	54	1.0	20 (37.0)	0.4	7	0.1	6 (11.1)
S.v. 61	36	108	3.0	58 (53.7)	1.6	16	0.4	10 (27.8)
S.v. 62	47	115	2.4	63 (54.8)	1.3	20	0.4	18 (38.3)
S.v. 63	39	40	1.0	16 (40.0)	0.4	5	0.1	4 (10.3)
Ex. sp. 48	63	219	3.5	25 (11.4)	0.4	9	0.1	6 (9.5)
E.f. 5	100	1382	13.8	808 (58.5)	8.1	267	2.7	96 (96.0)
E.f. 10	124	2478	20.0	155 (6.3)	1.3	156	1.3	71 (57.3)
E.f. 11	145	1502	10.4	118 (7.9)	0.8	64	0.4	84 (57.9)
E.f. 13	44	582	13.2	55 (9.5)	1.3	6	0.1	5 (11.4)
E.f. 36	53	2024	38.2	160 (7.9)	3.0	45	0.8	14 (26.4)
E.f. 38	39	411	10.5	29 (7.1)	0.7	0	0.0	0 (0.0)
E.f. 43	47	287	6.1	139 (48.4)	3.0	68	1.4	29 (61.7)

P.s. 7	55	217	3.9	47 (21.7)	0.9	69	1.3	42 (76.4)
P.s. 20	22	536	24.4	23 (4.3)	1.0	8	0.4	4 (18.2)
M.p. 30	72	834	11.6	9 (1.1)	0.1	7	0.1	7 (9.7)
M.p. 32	41	192	4.7	12 (6.3)	0.3	5	0.1	4 (9.8)
M.p. 40	28	300	10.7	72 (24.0)	2.6	46	1.6	15 (53.6)
M.p. 41	14	13	0.9	9 (69.2)	0.6	10	0.7	3 (21.4)
M.p. 50	38	145	3.8	57 (39.3)	1.5	4	0.1	2 (5.3)
M.r. 12	48	429	8.9	78 (18.2)	1.6	79	1.6	4 (85.4)
H. sp. 60	36	6	0.2	3 (50.0)	0.1	5	0.1	5 (13.9)
H. sp. 64	57	478	8.4	29 (6.1)	0.5	23	0.4	16 (28.1)
C 9	81	176	2.2	3 (1.7)	0.0	3	0.0	3 (3.7)
C 23	57	1769	31.0	29 (1.6)	0.5	28	0.5	17 (29.8)
C 33	38	185	4.9	9 (4.9)	0.2	0	0.0	0 (0.0)
C 35	11	81	7.4	19 (23.5)	1.7	0	0.0	0 (0.0)
C 42	33	21	0.6	13 (61.9)	0.4	3	0.1	2 (6.1)
C 44	46	155	3.4	30 (19.4)	0.7	6	0.1	6 (13.0)
C 53	42	382	9.1	274 (71.7)	6.5	1	0.0	1 (2.4)
C 61	36	108	3.0	58 (53.7)	1.6	16	0.4	10 (27.8)

Epicauta ferruginea was the most thorough visitor to inflorescences of *E. angustifolia* with an average of nearly 80 florets visited per capitulum and deposited the greatest number of pollen grains per stigma (16.0) (Table 3.8). *Phoebis sennae* had the second highest number of pollen grains per stigma (14.2) after an SIV followed by control inflorescences where there were nearly 8 grains per stigma (Table 3.8). *E. ferruginea* had an average of 2.6 germinated grains per stigma while *P. sennae*, *Megachile pugnata* and *Systoechus vulgaris* each had approximately one germinated pollen grain per stigma following their SIV. Control inflorescences had comparatively high numbers of pollen grains (7.7) and germinated pollen grains (1.5) per stigma, compared to *M. pugnata* (6.3, 1.0), the two halictid bees (4.3, 0.3) and the two species of bee flies, *S. vulgaris* (6.2, 1.0) and *Exoprosopa* sp. (3.5, 0.4). There were no significant differences between these means, however (bottom row, Table 3.5).

Table 3.5: Mean ± S.E. (range) of pollen grains on stigmas following single insect visits to inflorescences of *E. angustifolia* separated by insect species, 2003

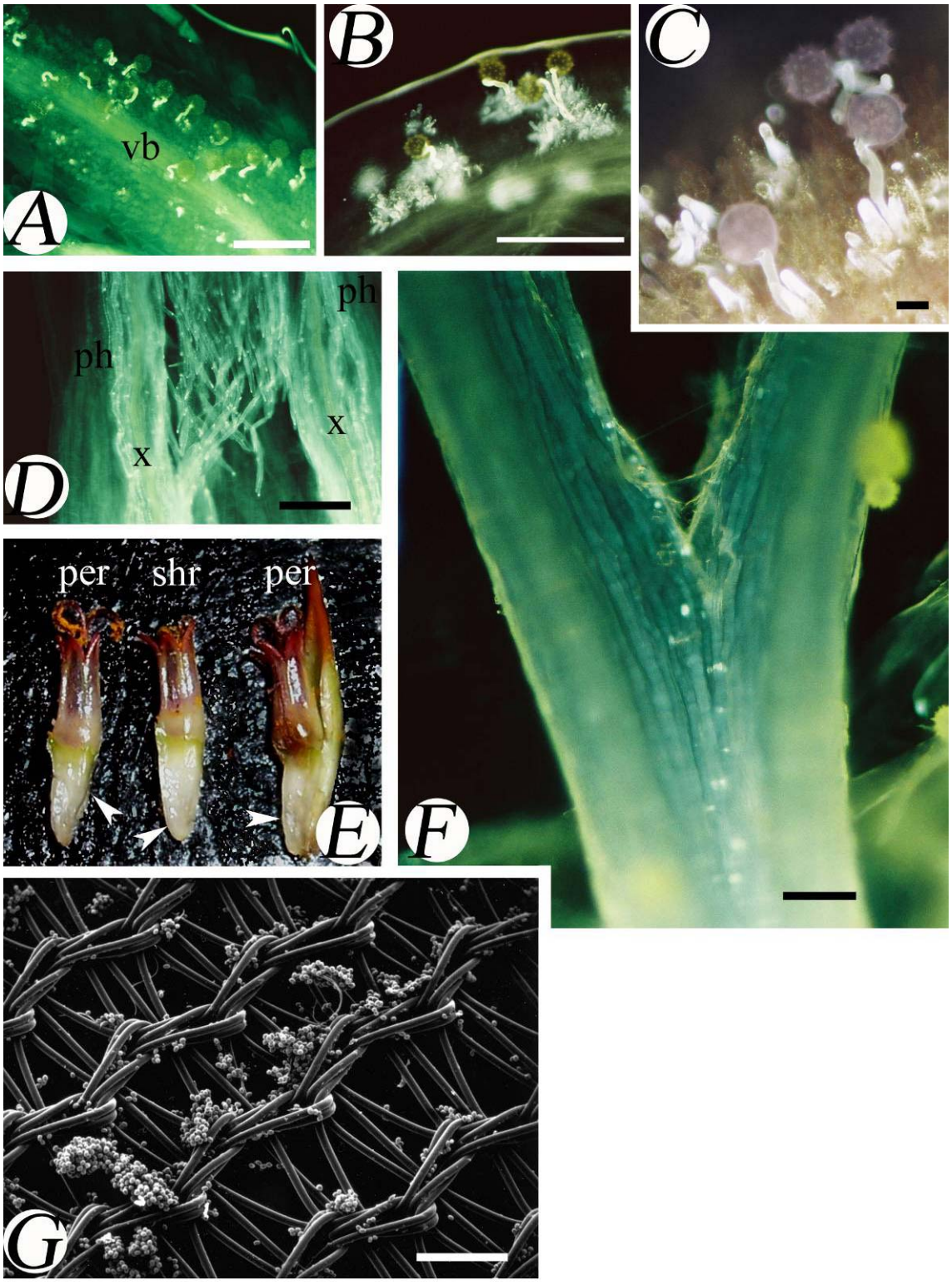
Order (Family) *Species*	**No. single insect visits**	**Estimated no. virgin florets visited per SIV**	**No. pollen grains per stigma per SIV**	**No. pollen grains germinated per stigma per SIV**	**Percent of total pollen grains per stigma that germinated per SIV**
Coleoptera (Meloidae) *Epicauta ferruginea*	7	78.9 ± 16.4 (39 – 145)	16.0 ± 4.0 (6.1 – 38.2)	2.6 ± 1.0 (0.7 – 8.1)	20.8 ± 8.5 (6.3 – 58.5
Diptera (Bombyliidae) *Exoprosopa* sp.	1	63.0	3.5	0.4	11.4
Systoechus vulgaris	20	50.8 ± 3.9 (29 - 90)	6.2 ± 1.6 (0.7 – 27.0)	1.0 ± 0.2 (0.4 – 4.9)	27.5 ± 4.8 (1.8 – 87.8)
Hymenoptera (Halictidae) *Augochlora* sp.	2	46.5 ± 10.5 (36 – 57)	4.3 ± 4.1 (0.2 – 8.4)	0.3 ± 0.2 (0.1 – 2.6)	28.1 ± 22.0 (6.1 – 50.0)
(Megachilidae) *Megachile pugnata*	5	38.6 ± 9.6 (14 – 72)	6.3 ± 2.1 (0.9 – 11.6)	1.0 ± 0.5 (0.1 – 2.6)	28.0 ± 12.3 (1.1 – 69.2)
Megachile rotundata	1	48	8.9	1.6	18.2
Lepidoptera (Pieridae) *Phoebis sennae*	2	38.5 ± 16.5 (22 – 55)	14.2 ± 10.3 (3.9 – 24.4)	1.0 ± 0.1 (0.9 – 1.0)	13.0 ± 8.7 (4.3 – 21.7)
	No. inflorescences	**No. florets examined per capitulum**	**No. pollen grains per stigma**	**No. pollen grains germinated per stigma**	**Percent of total pollen grains per stigma that germinated**
CONTROLS	8	43.0 ± 7.1 (11 – 81)	7.7 ± 3.5 (0.6 – 31.0)	1.5 ± 0.8 (0.0 – 6.5)	29.8 ±10.1 (1.6 – 71.7)
Between group significance ($\alpha = 0.05$)			P = 0.172	P = 0.347	P = 0.948

3.3.2.2.3 Analysis of styles for pollen tubes

Counting the number of pollen tubes that reached the style base indicated the amount of cross-pollination that occurred during an SIV. A compatible pollen grain germinates on the stigma by producing one pollen tube from one of three pores. The tube tip then grows between the papillae (Fig. 2.2F) on the receptive surface of the stigma (Fig. 3.24A) and travels beneath the receptive layer until it reaches the transmitting tract of the style (Fig. 3.24F). Callose plugs form at intermittent intervals within the pollen tube (Figs 3.24D, F). Aniline blue binds primarily to callose plugs and

secondarily to the wall of the pollen tube. Fluorescence amongst samples was often inconsistent with callose plugs and wall material of pollen tubes sometimes appearing bright yellow or green against the dark background of the transmitting tract and at other times fluorescing only weakly from callose plugs. Pollen tubes were counted when they appeared at the style base, indicating that the pollen tube had successfully reached the ovule for fertilization (Table 3.4). Xylem within the vascular bundles was usually observed fluorescing weakly while phloem was very evident by the fluorescence of callose in the sieve plates of the end walls between sieve elements and callose lining pores of sieve areas in the walls of sieve elements (Figs 2.3B, 3.24D). Isolated phloem that occurred outside of the vascular bundles was often found obscuring the transmitting tract (Figs 2.3B, C) and would closely mimic the appearance of pollen tubes in samples that were not well cleared. Care had to be taken to distinguish sieve tubes from pollen tubes.

FIG. 3.24: A-F Light micrographs. A-D, F Aniline blue fluorescence of stigmas and styles of *Echinacea angustifolia*. (A) Pollen grains germinating on the surface of a compatible stigma. Vascular bundle (vb). Scale bar = 50 µm. B-C Callose deposition in stigmatic papillae (bright areas) to prevent penetration of an incompatible pollen tube. (B) Wide callose deposition beneath incompatible pollen tubes. Scale bar = 50 µm. (C) Callose deposition localized to papillae adjacent to pollen tubes. Scale bar = 10 µm. (D) Numerous pollen tubes halted at the top of a cross-pollinated stigma by early fixation. Note the equally bright fluorescence of phloem (ph) sieve tubes exterior to weakly fluorescing xylem (x) vessels. Scale bar = 50 µm. (E) Pistillate phase disc florets of *E. angustifolia* illustrating style shrivelling (shr) and style persistence (per). Note the unfilled nature of persistent and shrivelled ovaries (arrows). (F) Pollen tube represented by callose plugs (bright dots) entering style from left stigma lobe. Scale bar = 50 µm. (G) SEM of *E. angustifolia* pollen grains on tent mesh of a total excluder cage. Scale bar = 0.5 mm.

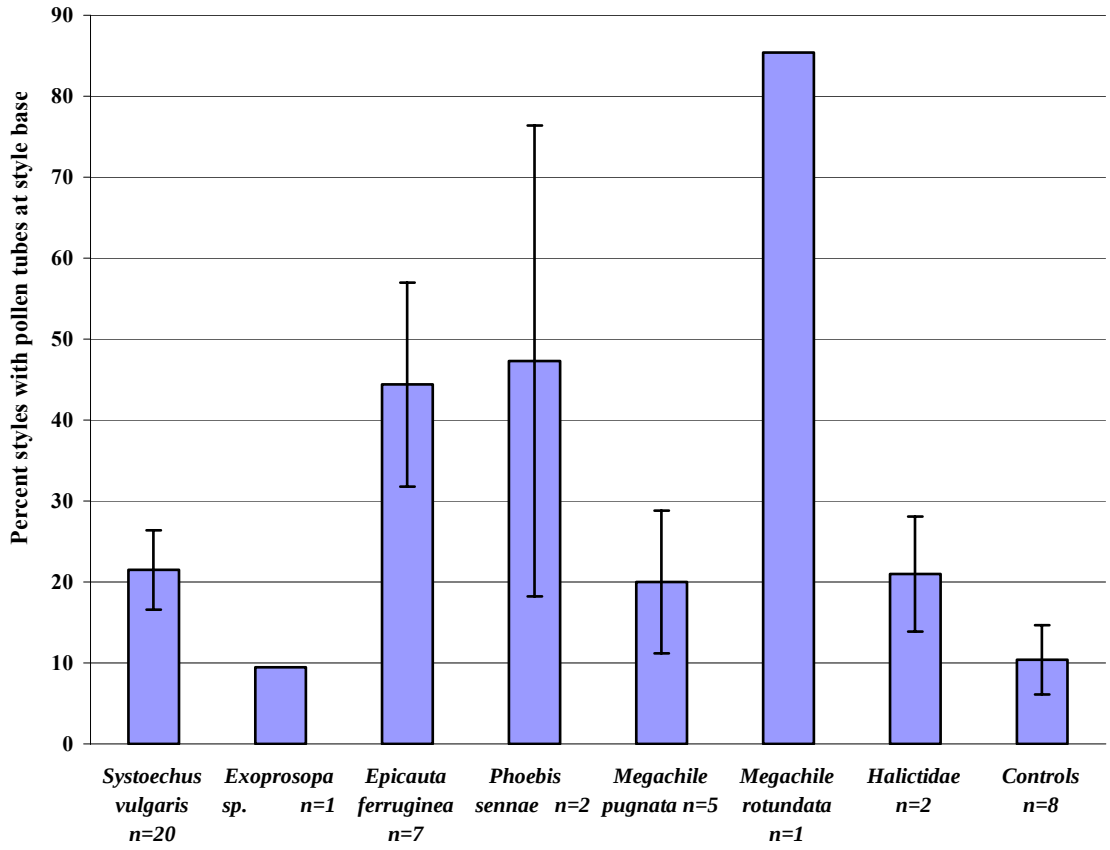

A
vb
B
C
D
ph
ph
x
x
E
per
shr
per
F
G

Pollen tube counts were employed in 2003 at PPS and MVA to determine the pollination efficiency of individual insects on *E. angustifolia* following SIVs. There were 36 documented SIVs at MVA and 10 at PPS including control inflorescences. A single pollen tube at the style base indicates successful pollination and likely fertilization of a disc floret. Several pollen tubes at the style base are redundant in terms of fertilization because there is only one ovule per floret but an increased number of pollen tubes indicated that the visiting insect transferred many compatible grains.

In order that pollen tube counts were presented in a comparable way to shrivelled style data from SIVs in 2004, the number of styles per inflorescence with pollen tubes was employed (Fig. 3.25). Percentages were calculated by taking the number of styles with pollen tubes and dividing by the number of styles in the visited area that were analysed for pollen tubes (right most column of Table 3.4). This method gave a conservative estimate of pollination efficiency per insect on the visited area of the inflorescence. In some instances, insects traversed the entirety of an inflorescence and had the opportunity to contact and cross-pollinate every receptive floret, but for most SIVs, only florets from the visited area had the potential to be pollinated, so styles from only these florets were analysed.

After 20 grasshopper bee fly (*Systoechus vulgaris*) visits, a mean of 21.5% of styles from the visited area showed at least one pollen tube (Figure 3.25). The duration of bee fly visits ranged from 10 s to 24 m 31 s. The only single insect visit by a progressive bee fly (*Exoprosopa* sp.) resulted in 9.5% of styles with at least one pollen tube in its short visit (10 s). Seven golden blister beetles (*Epicauta ferruginea*) had visit lengths that ranged from 10 s to 1 h and produced a mean of 44.4% of style bases possessing at least one pollen tube. Two cloudless sulphur butterflies (*Phoebis sennae*) visited for 13 s and 2 m 35 s with a mean percentage of style bases with pollen tubes of 44.4%. Five sunflower leafcutter bees visited from 5 s to 2m 15 s and introduced compatible pollen grains to 20% of visited disc florets. Only one alfalfa leafcutter bee (ALB) (*Megachile rotundata*) visited an experimental inflorescence of *E. angustifolia* in 2003.

FIG. 3.25: Percentage (mean ± S.E.) of styles with at least one pollen tube at the style base following single insect visits (n) to *Echinacea angustifolia*, 2003.



The one ALB transferred enough pollen for 85.4% of visited florets to have pollen tubes at their style bases. Two small halictid bees visited inflorescences of *E. angustifolia* and 21% of visited styles showed pollen tubes after their visits of 30 s and 2 m and 35 s. Control styles were taken from inflorescences after failure of an SIV to occur, wherein the capitula were rebagged and later harvested (n=6); or from inflorescences that matured in the bag, never having been unbagged (n=2). Table 3.6 gives the range of data for each insect to illustrate the within species difference in pollination efficiency.

Table 3.6: Range of percentages of total styles analysed from the visited region of an experimental capitulum, that possessed at least one pollen tube at the style base after a single insect visit, 2003.

Species	Range
Grasshopper bee fly: *Systoechus vulgaris*	0 - 85.7%
Progressive bee fly: *Exoprosopa* spp.	9.50%
Golden blister beetle: *Epicauta ferruginea*	0 – 96.0%
Cloudless sulphur butterfly: *Phoebis sennae*	18.2 - 76.4%
Sunflower leafcutter bee: *Megachile pugnata*	5.3 -53.6%
Alfalfa leafcutter bee: *Megachile rotundata*	85.4%
Sweat bee: Halictidae	13.9 - 28.1%
Controls: bagged/rebagged and never unbagged	0 - 29.8%

3.3.2.3 Comparison of pollen tube counting and shrivelled style analysis for determining pollination efficiency

It was noted that after cross-pollination of disc florets of *E. angustifolia*, the stigma and style would shrivel and retract into the floral tube (Fig. 3.24E "shr"). This shrivelling of stigma and style was a clear, visual cue that cross-pollination had occurred in the floret, whereas the persistent turgidity of a stigma and style indicated that the floret was not cross-pollinated (Fig. 3.24E "per"). By assessing the number of stigmas and styles that shrivelled after an SIV and comparing those to the total number of florets available, the pollination efficiency of visiting insects was estimated. This shrivelled/persistent style technique provided pollination efficiency estimates in a much shorter period of time than quantifying pollen tubes after SIVs.

Style shrivelling indicates that at least one pollen tube reached the style base in order for shrivelling to occur. In this way, insect pollination efficiency can be determined by counting the percentage of styles with pollen tubes or by counting the number of styles shrivelled after an SIV. Shrivelled styles should have more pollen tubes at their style bases than persistent styles. These two methods were compared by counting the number of pollen tubes at the style base of shrivelled vs. persistent styles to identify if there was a significant difference between them (Fig. 3.26).

The number and percentage of germinated grains was also utilized as an indicator of cross-pollination for shrivelled and persistent styles and used in conjunction with pollen tube counts to indicate differences in the pollination status of stigmas and styles (Tables 3.7, 3.8). The number of germinated grains was standardized for different numbers of styles by expression as a percentage. No significant difference was found

between percentages of germinated pollen grains of shrivelled vs. persistent styles (P = 0.109) or the number of germinated grains per style (P = 0.089) but as established in section 3.3.2.2.2, this was a problematic test due to potential germination of self-pollen grains.

Table 3.7: Comparison of stigmatic pollen loads and pollen tubes at the style base between shrivelled and persistent styles from single insect visits in 2004. Abbreviations: SIV = Single insect visit, S = shrivelled styles, P = persistent styles, *E.f. = Epicauta ferruginea*, *S.v. = Systoechus vulgaris*, *B.t. = Bombus ternarius*, *B.* spp. = *Bombus* species, *A.m. = Apis mellifera*

			Pollen grains				**Pollen tubes at style base**		
Visitor and SIV no.	**Style state**	**No. styles**	**Total no.**	**No. per style**	**Total no. (%) germ**	**No. germ per style**	**Total no.**	**No. per style**	**No. (%) styles with tubes**
E.f. 2	S	29	422	14.6	77 (18.2)	2.7	40	1.4	21 (72.4)
	P	30	208	6.9	34 (16.0)	1.1	5	0.2	4 (13.3)
E.f. 9	S	21	1015	48.3	637 (62.8)	30.3	123	5.9	20 (95.2)
	P	23	405	17.6	242 (59.8)	10.5	70	3.0	20 (87.0)
E.f. 22	S	12	807	67.3	328 (40.6)	27.3	70	5.8	12 (100)
	P	12	172	14.3	29 (16.9)	2.4	7	0.6	4 (33.3)
E.f. 25	S	33	138	4.2	58 (42.0)	1.8	21	0.6	13 (39.4)
	P	25	131	5.2	12 (9.2)	0.5	8	0.3	5 (20.0)
E.f. 26	S	18	72	4.0	29 (40.3)	1.6	19	1.1	10 (55.6)
	P	21	111	5.3	5 (4.5)	0.2	4	0.2	4 (19.0)
S.v. 8	S	31	124	4.0	9 (7.3)	0.3	7	0.2	5 (16.1)
	P	18	42	2.3	8 (19.0)	0.4	1	0.1	1 (5.6)
S.v. 11	S	38	284	7.5	5 (1.8)	0.1	3	0.1	3 (7.9)
	P	20	70	3.5	3 (4.3)	0.2	0	0.0	0 (0.0)
B.t. 34	S	24	411	17.1	142 (34.5)	5.9	59	2.5	20 (83.3)
	P	34	370	10.9	72 (19.5)	2.1	19	0.6	11 (32.4)
B.t. 40	S	36	123	3.4	11 (8.9)	0.3	15	0.4	7 (19.4)
	P	31	958	30.9	43 (4.5)	1.4	3	0.1	3 (9.7)
B.t. 44	S	24	295	12.3	120 (40.7)	5.0	42	1.8	18 (75.0)
	P	24	220	9.2	41 (18.6)	1.7	5	0.2	2 (8.3)
B.t. 50	S	18	401	22.3	277 (69.1)	15.4	62	3.4	17 (94.0)
	P	18	71	3.9	30 (4.2)	1.7	11	0.6	4 (22.0)

B. spp. 47	S	25	172	6.9	15 (8.7)	0.6	6	0.2	6 (24.0)
	P	25	105	4.2	23 (21.9)	0.9	5	0.2	2 (8.0)
B. spp. 48	S	10	320	32.0	249 (77.8)	24.9	37	3.7	10 (100)
	P	10	246	24.6	157 (63.8)	15.7	7	0.7	3 (30.0)
B .spp. 55	S	15	543	36.2	369 (68.0)	24.6	48	3.2	14 (93.0)
	P	15	408	27.2	228 (55.9)	15.2	1	0.1	1 (7.0)
B. spp. 64	S	25	290	11.6	27 (9.3)	1.1	10	0.4	6 (24.0)
	P	25	222	8.9	18 (8.1)	0.7	4	0.2	2 (8.0)
B. spp. 66	S	20	203	10.2	7 (3.4)	0.4	3	0.2	3 (15.0)
	P	20	168	8.4	13 (7.7)	0.7	0	0.0	0 (0.0)
A.m. 57	S	15	68	4.5	44 (64.7)	2.9	18	1.2	11 (73.0)
	P	15	54	3.6	21 (38.9)	1.4	1	0.1	1 (7.0)

There were no significant differences between means of pollen grains per stigma on shrivelled styles between insect visitors or persistent styles (Table 3.8). Persistent styles were harvested from the same whorls of florets as shrivelled styles and thus had equal opportunity to receive pollen grains during the visit.

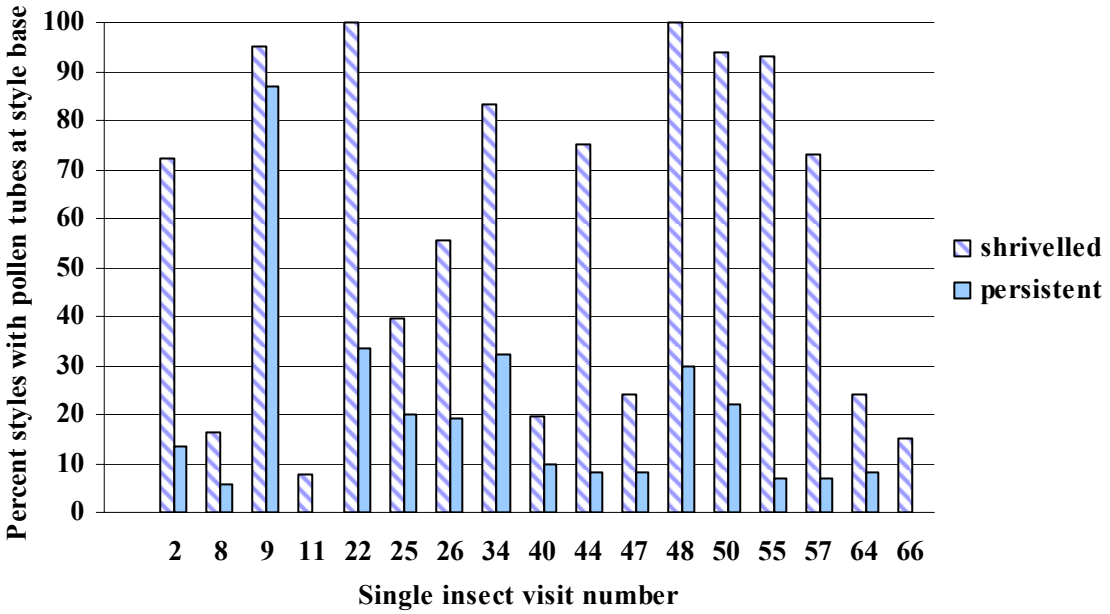
Table 3.8: Mean ± S.E. (range) of pollen grains per stigma of shrivelled and persistent styles from 17 randomly selected SIVs, 2004

No. single insect visits (n) **Order** (Family) *Species*	**No. florets examined per SIV**	**No. pollen grains per stigma on shrivelled styles per SIV**	**No. pollen grains germinated per shrivelled style per SIV**	**Percent of total pollen grains per stigma of shrivelled style that germinated per SIV**
5 **Coleoptera** (Meloidae) *Epicauta ferruginea*	22.6 ± 3.8 (12.0 - 33.0)	27.7 ± 12.8 (4.0 - 67.3)	12.7 ± 6.6 (1.6 - 30.3)	40.8 ± 7.1 (18.2 – 62.8)
2 **Diptera** (Bombyliidae) *Systoechus vulgaris*	34.5 ± 3.5 (31 – 38)	5.8 ± 1.8 (4.0 – 7.5)	0.2 ± 0.1 (0.1 - 0.3)	4.6 ± 2.8 (1.8 – 7.3)
4 **Hymenoptera** (Apidae) *Bombus ternarius*	25.5 ± 3.8 (18 – 36)	13.8 ± 4.0 (3.4 – 22.3)	6.7 ± 3.2 (0.3 – 15.4)	38.3 ± 12.4 (8.9 – 69.1)
5 **Hymenoptera** (Apidae) *Bombus* spp.	19.0 ± 2.9 (10 – 25)	19.4 ± 6.1 (6.9 – 36.2)	10.3 ± 5.9 (0.4 – 24.9)	33.4 ± 16.2 (3.4 – 77.8)
1 **Hymenoptera** (Apidae)	15	4.5	2.9	64.7

Apis mellifera				
Persistent styles from each SIV				
17	21.5 ± 1.6 (10 – 34)	11.0 ± 2.2 (2.3 – 30.9)	3.3 ± 1.2 (0.2 – 15.7)	21.9 ± 4.9 (4.2 – 63.8)
Between group significance (α=0.05)		P = 0.228	P = 0.260	P = 0.166

The percentages of pollen tubes in shrivelled vs. persistent styles were compared for 17 SIVs from 2004. In florets that had shrivelled styles, a mean of 60% of the styles had pollen tubes at their base and persistent styles had a mean of 17% of styles with pollen tubes at their base. These means are significantly different (P = 0.000), indicating that there are more pollen tubes at the base of shrivelled styles than there are in persistent styles. A significant difference (P = 0.006) existed as well between the number of pollen tubes at the style base (right most column of Table 3.7) of shrivelled vs. persistent styles. Fig. 3.26 illustrates the individual differences in percentages of styles with pollen tubes between shrivelled and persistent styles per SIV. The counting of pollen tubes at style bases was consistent with shrivelled style analysis.

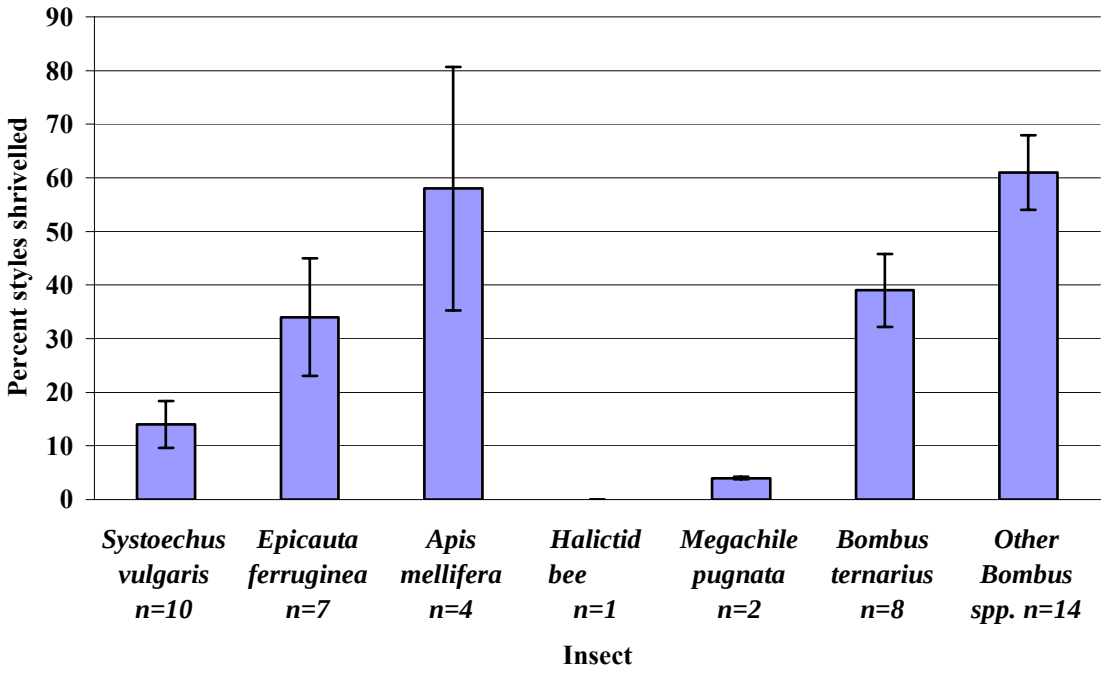
FIG. 3.26: Percentage of styles with pollen tubes at their bases in shrivelled vs. persistent styles located within capitula following 17 randomly-selected single insect visits, 2004



3.3.2.4 Shrivelled style analysis

Shrivelled vs. persistent styles were used to assess pollination efficiency of insects on *E. angustifolia* in 2004 in conjunction with SIVs. Percentages were calculated based on the number of florets with shrivelled styles compared to the total number of florets receptive during each single visit and illustrated in Fig. 3.27. Insects from SIVs were separated by species where possible; bumble bees other than *Bombus ternarius* were grouped as other *Bombus* spp. (Fig. 3.27). Of the 46 total SIVs, 41 and 5 were obtained at MVA and VR, respectively. After single insect visits by several species of bumble bees, 61% of available styles shrivelled while after honey bee visits, 58% of styles shrivelled (Fig. 3.27). Single visits by red-tailed bumble bees and golden blister beetles caused shrivelled styles with percentages of 39 and 34, whereas grasshopper bee flies and sunflower leafcutter bees had low percentages of 14 and 4, respectively (Fig. 3.27), and the single sweat bee visit did not lead to any shrivelled styles.

FIG. 3.27: Percentage (mean ± S.E.) of styles shrivelled per inflorescence of *Echinacea angustifolia* after single insect visits at Meewasin Valley Authority and Valley Road, 2004



Common names of the insects and the range of shrivelled style percentages obtained are shown in Table 3.9. The range of percent shrivelling varied widely within each insect species (Table 3.9).

Table 3.9: Range of percentages of shrivelled styles after single insect visits, 2004

Insect	Range of shrivelled styles
Golden blister beetle: *Epicauta ferruginea*	4-88%
Grasshopper bee fly: *Systoechus vulgaris*	2-41%
European honey bee: *Apis mellifera*	6-100%
Sunflower leafcutter bee: *Megachile pugnata*	4-4.5%
Red-tailed bumble bee: *Bombus ternarius*	22-74%
Other bumble bees: *Bombus fervidus*, *B. nevadensis, B. vagans*	21-94%
Sweat bee: Halictidae	0%

3.3.2.5 Duration of visits by insects to a capitulum

Mean visit duration and rewards sought were also recorded for SIVs in 2003 and 2004 to determine which floral rewards were exploited by insects and if there was a difference in time per visit for each reward sought (Table 3.10). Bee flies foraged either for nectar alone or nectar and pollen and their mean visit times were different between seasons (285.7 s in 2003, 39.8 s in 2004) but not quite significantly different ($P = 0.06$, $\alpha = 0.05$). Blister beetles foraged solely for pollen and spent the longest time on inflorescences as any insect (Table 3.10). For insects that foraged for more than one reward type, there was no significant difference between the times spent per inflorescence for each reward (*S. vulgaris*, $P = 0.100$, *B. ternarius* $P = 0.242$, Other *Bombus* spp. $P = 0.655$), except for *A. mellifera* ($P = 0.007$). However, sample sizes for these comparisons are low and may not accurately reflect trends.

Table 3.10: Mean times (± S.E.) of insect visits to inflorescences of *Echinacea angustifolia* and the floral rewards sought, 2003 and 2004

Insect visitor	**Reward sought**						
2003	**Nectar alone**		**Nectar + Pollen**		**Pollen alone**		
	No. visits	**Time (s)**	**No. visits**	**Time (s)**	**No. visits**	**Time (s)**	**Mean visit time (s)**
Systoechus vulgaris	18	212.9 ± 91.6	6	502.8 ± 93.5			285.7 ± 76.3
Exoprosopa sp.	1	10					
Epicauta ferruginea					8	1859.3 ± 573.4	
Phoebis sennae	2	84 ± 71					
Megachile pugnata					5	34.6 ± 15.8	
Megachile rotundata			1	179			
Halictidae					2	87.5 ± 57.5	
2004	**Nectar alone**		**Nectar + Pollen**		**Pollen alone**		
	No. visits	**Time (s)**	**No. visits**	**Time (s)**	**No. visits**	**Time (s)**	**Mean visit time (s)**
Systoechus vulgaris	7	37.6 ± 16.7	2	47.5 ± 27.5			39.8 ± 13.6
Epicauta ferruginea					7	1027.0 ± 23.0	
Megachile pugnata					2	21.5 ± 0.5	
Halictidae					2	56.0 ± 24.0	
Apis mellifera	1	12	3	61 ± 2.1			48.8 ± 12.3
Bombus ternarius	2	44.0 ± 19	4	86.0 ± 41.6	2	384.5 ± 294.5	150.1 ± 78.3
Other *Bombus* spp.	7	205.0 ± 55.7	7	245.6 ± 68.9			225.3 ± 42.9

3.4 Discussion

3.4.1 Transect observations

The first transect performed on July 8, 2003, recorded 208 grasshopper bee flies (*Systoechus vulgaris*) foraging on 30 inflorescences, with densities of up to 15 bee flies per inflorescence. There are no previous references for this particular bee fly on any species of Asteraceae but other bee fly species have been observed on numerous asteracean inflorescences (Johnson and Midgley, 1997; Souza-Silva *et al*., 2001). Typically bee flies are skittish feeders and will fly away if disturbed while foraging, but at these high densities, bee flies were so intent on feeding that it was possible to touch

them repeatedly without having them abscond from the inflorescence. It seemed like the competition for resources induced a "feeding frenzy". Later on in the season and in 2004, their skittish behaviour surfaced and the approach of another bee fly would often disturb them sufficiently to cause them to cease feeding and abscond. Bee flies foraged for nectar for the most part, but on many occasions during single insect visits, bee flies were observed feeding on pollen from whorls of florets in the dehiscent staminate phase. This feeding behaviour is surprising because the bee fly proboscis is long, thin and "straw-like", but has been described previously for female bee flies, of *Peocilognathus punctipennis*, feeding on pollen of *Commelina erecta* and *Tradescantia roseolens* (Deyrup, 1988).

The golden blister beetle, *Epicauta ferruginea,* is not often described as a great pollinator (Mani and Saravanan, 1999), but it was found in large numbers foraging on *E. angustifolia* at MVA. Golden blister beetles were present in small numbers on inflorescences of *E. angustifolia* at VR but were not seen as inflorescence visitors at PPS, suggesting that they would not be consistent pollinators of *E. angustifolia* across Saskatchewan or even across growing seasons.

The abundance of bee flies and blister beetles at MVA (2003, 2004) was likely a product of the large grasshopper population whose cycle peaked in 2003. Grasshopper bee flies (*S. vulgaris*) and golden blister beetles (*E. ferruginea*) are both larval predators of grasshopper eggs as well as adult pollinators, and are thus doubly beneficial in the prairie ecosystem and in an agricultural planting of *E. angustifolia* (Gillott *et al.,* 2003). *E. angustifolia* inflorescences remain in flower for an extended length of time with fields having mature inflorescences for six weeks or more. These long-lived inflorescences (10-12 days) provide a continual food source of both nectar and pollen for adults of *S. vulgaris* and *E. ferruginea*, which keeps these insects in the vicinity of the *E. angustifolia* crop and nutritionally maintains these beneficial adults through till oviposition upon grasshopper eggs. It is necessary for oviposition by *S. vulgaris* and *E. ferruginea* to overlap with grasshopper oviposition because female *S. vulgaris* and *E. ferruginea* adults lay their eggs in grasshopper egg beds where eggs develop rapidly and soon hatch. Larvae actively search for grasshopper egg pods on which to feed. A single larva of either species can destroy an entire egg pod (about 25 eggs) of smaller

grasshoppers such as the clear-winged (*Camnula pellucida*) and lesser migratory (*Melanoplus sanguinipes*), or about half the eggs in the pod of the larger, two-striped grasshopper (*M. bivittatus*) (Swan and Papp, 1972). The interaction of a constant nectar and pollen source for adult bee flies and blister beetles at MVA and an abundance of grasshopper eggs as a larval food source culminated in the large populations seen in 2003 and 2004. By 2004, the population of bee flies was already beginning to decline as was evidenced by their lower numbers on *E. angustifolia* inflorescences, while the blister beetle population increased in 2004.

Lepidopteran visitors to transect inflorescences decreased from 50 individuals (5.2%) in 2003 to 8 (0.7%) in 2004 at MVA. This change is surprising because gardening literature often recommends planting *E. purpurea* (with a very similar inflorescence) in flower gardens to attract butterflies. However, environmental conditions across Saskatchewan may not have been favourable for butterfly development because it seemed that butterfly populations were low compared to the previous year. The appearance of painted lady butterflies on both *E. purpurea* and *E. angustifolia* at the biology garden plots in 2005 reinforces the attractiveness of *Echinacea* inflorescences to butterflies. Painted ladies usually perched on paleae at the top of the disc florets to imbibe nectar and only their proboscis came into contact with mature disc florets. Painted lady populations were extremely high across the prairie region of Saskatchewan in 2005 and their larvae were observed defoliating nearly every Canada thistle plant that was seen in July.

At first glance at the insect numbers at VR (2004), it might seem that only a small number of observations along transects were performed, but this was not the case. In spite of fewer transect observations at VR (22 compared to 55 at MVA), there indeed were significantly fewer insects visiting *E. angustifolia* inflorescences at this site compared to MVA. There are several explanations for the low number of visitors. There may have been more inflorescences at this site than the local pollinators could saturate, which would indicate a native pollinator deficit. Often only one insect was observed working hundreds of inflorescences in the *Echinacea* rows. Low numbers of inflorescence visitors were also evident by the long waiting periods during exposure of previously-bagged inflorescences to a single insect visit (mode of 30 m, 5 times).

Waiting for a visitor to a newly-exposed capitulum was usually aborted after 0.5 h; unfortunately, this scenario occurred frequently. Another explanation for the low number of potential pollinators is that the large number of weedy flowers at VR competed for visitor attention. There were many competing flowers apparently more attractive to insects than *E. angustifolia*, such as yellow sweet clover (*Melilotus officinalis*) (~75% of competing bloom in field), perennial sow thistle (*Sonchus arvensis*) (~15%), Canada thistle (*Cirsium arvense*) (~5%), common blue lettuce (*Lactuca pulchella*) (~1%), prickly lettuce (*L. serriola*) (~2%), goat's beard (*Tragopogon dubius*) (~1%) and dandelion (*Taraxacum officinale*) (~1%). The field plant composition was about half *E. angustifolia* and half yellow sweet clover and perennial sow thistle, with an understory vegetation of blue burr (*Lappula squarrosa*). The north end of the field near the alfalfa leafcutter bee hut (see section 4.2) was composed of 75% perennial sow thistle. The field itself then appeared as a sea of yellow and purple from the weeds and *E. angustifolia*. It often occurred that many insects were observed in the field but were visiting only the yellow flowers of weeds and all but ignored *E. angustifolia* inflorescences. There were many golden blister beetles at VR but they were visiting perennial sow thistle (yellow asteracean flowers) almost exclusively, except for the few beetles found on transects. Honey bees and alfalfa leafcutter bees were similarly more attracted to the yellow flowers of perennial sow thistle than they were to *E. angustifolia*. Yellow sweet clover (*Melilotus officinalis*), as a legume and a close relative of alfalfa (*Medicago sativa*), was far more attractive than *E. angustifolia* to the introduced alfalfa leafcutter bees. It seemed that flowers of the weedy species out competed *E. angustifolia* for the attraction of potential pollinators.

A much greater abundance of bumble bees was observed visiting *E. angustifolia* in 2004 than in 2003. The reasons for this change in population are unknown but bumble bees were frequent visitors to inflorescences of *E. angustifolia* at MVA and VR in 2004, were present at PPS, and also visited *E. angustifolia* and *E. purpurea* in the biology garden plot in July and August of 2005. Bumble bee behaviour was indicative of that of a good pollinator on *E. angustifolia* inflorescences, in that they foraged for nectar and pollen, flew swiftly between inflorescences and showed a high fidelity to *E. angustifolia*. Based on their abundance and cosmopolitan range at all field sites and

across the Saskatchewan prairie (Curry, 1984), it is likely that bumble bees will contribute a great deal to *E. angustifolia* pollination in natural and agricultural settings. Bumble bees (*Bombus* spp.) and honey bees (*Apis mellifera*) were frequent visitors to *E. tennesseensis* (Hemmerly, 1976; Drew, 1991).

3.4.2 Excluder cages

At achene maturity, the ovary wall hardens into the achene fruit wall regardless of whether cross-pollination has occurred (Wagenius, 2004; personal observation). It is therefore difficult to use visual cues to determine the fertilization status of an achene. Weights of achenes, however, should indicate the presence of an embryo with heavier achenes having a fully formed embryo and lighter achenes being unfilled. A viable achene is an indirect method of determining if a floret was cross-pollinated. Achene weights have previously been used to determine cross-pollination in *Echinacea angustifolia* (Leuszler *et al.,* 1996) and sunflower, *Helianthus annuus* (Langridge and Goodman, 1981). Germination of achenes is an additional technique used to indirectly assess pollination in *E. angustifolia* (Leuszler *et al*., 1996; Wagenius, 2004), and sunflower, *Helianthus annuus* (Langridge and Goodman, 1981). Poor germination of achenes indicates poor pollination of disc florets.

3.4.2.1 Control inflorescences

Thousand achene weights were calculated for achenes to compare pollination between inflorescences in insect excluder trials in 2003 and 2004, as an initial estimate of pollination. Achenes were then germinated as an additional test to compare pollination between treatments. In 2003, 1000 achenes from openly pollinated control inflorescences at two field sites (PPS and MVA) were twice as heavy as 1000 achenes from inflorescences excluded from insects by total excluder cages. Germination of these achenes revealed that heavier achenes (approximately 2.5 g per 1000 achenes) from control inflorescences had much higher germination rates than lighter achenes (approximately 1 g per 1000 achenes) from total excluder treatments. The mean germination percentages in the controls would have been higher if the one or two inflorescences, whose achenes exhibited low germination, were ignored. Most of the control inflorescences in 2003 had achene germination percentages between 55-89% at

PPS, whereas one inflorescence had only 8% germination of its achenes. Of the MVA control inflorescences, seven had achenes germinating between 53 and 79%, with the other three replicates between 18 and 38%. Lighter achenes of *E. angustifolia* were deemed as "unfilled" and had significantly less mass and lower germination (8% vs. 92%) than "filled" achenes (with an embryo) (Leuszler *et al*., 1996). Wagenius (2004) reports that seed set of individual unmanipulated inflorescences ranged from 0 to 83%, with population means of 0 to 54% and 0 to 63% across two seasons, in wild stands of *E. angustifolia*. The designation "seed set" is identical to the designation "percent germination" employed by this study and the values of percent germination were remarkably similar to those of seed set with germination of achenes from control inflorescences ranging from 0 to 92% and population means of 22 to 66% across both seasons.

The pattern of heavier achenes having good germination occurred again in 2004, where heavier achenes from control inflorescences had greater percent germination than achenes from partial and total excluder treatments. However, the effect was not as pronounced in 2004 as it was in 2003, with heavy (2.3 g per 1000 achenes) germination of control achenes at VR having relatively poor achene germination of 24%. Such a discrepancy may illustrate a shortcoming of using germination as an indicator of pollination. There is also a possibility that the late senescence of inflorescences in the field due to a late start in July affected the proper maturation of seeds. Environmental factors such as the early frost that struck the *E. angustifolia* crop before the inflorescences had fully matured may have affected the germination potential of achenes which then gives a false impression that pollination of control inflorescences was low. It is possible that the germination pre-treatment protocol was inadequate for breaking dormancy of achenes from control inflorescences at VR. However, the same protocol was used with success in 2003 and on other achenes in 2004, so unless achenes from VR were more genetically prone to dormancy or climatic conditions played a role in initiating dormancy, then the treatment protocol should have been sufficient for seed germination.

In 2004, a pronounced site and year effect was evident from inflorescences at PPS. Achene weights were uniformly low at PPS in 2004, with control inflorescences

producing achenes having a mass that was not significantly different from partial and total excluder treatments. Control inflorescences at MVA and VR in 2004 produced achenes that were 1 g heavier than controls at PPS (2.4 g MVA, 2.3 g VR and 1.3 g PPS). A year effect was evident with control inflorescences in 2003 having mean achene weights of 2.5 g compared to 1.3 g in 2004. The low number of plants at PPS may be responsible for the lower weights and germinations of control achenes due to pollen limitation in this population. The 12 *E. angustifolia* plants were spread at large distances from each other amongst many pockets of weeds. Insects were rarely observed visiting inflorescences and were not observed travelling between inflorescences. Self-incompatible plants that rely on a generalist pollination system, like *E. angustifolia,* growing at low densities, such as occurred at PPS in 2004, are likely to suffer low pollination due to limited pollen flow from a reduction in the quantity and quality of pollinator visits (Wagenius, 2004; Kunin, 1993).

Small populations of flowers are less attractive to pollinators so if an inflorescence is visited it is less likely that a pollinator will be carrying pollen from another *E. angustifolia* inflorescence and will be less likely to visit another *E. angustifolia* head afterwards thus limiting pollen flow between inflorescences. 2004 control weights and germinations were higher at PPS than achene weights and germinations from partial and total excluder treatments but still low compared to 2003 germinations. This slight difference in weight and higher germination suggested that some cross-pollination of disc florets occurred on PPS control inflorescences. Low germination from openly pollinated inflorescences at VR, compared to MVA, may indicate a pollination deficit at this site even though 1000 achene weights indicated otherwise. Transect observations at VR indicated that potential pollinator visits to *E. angustifolia* were low and that a pollination deficit was likely. The lack of pollinators may be reflected by low germination of control achenes.

3.4.2.2 Inflorescences from partial excluder cages

Inflorescences in partial excluder treatments were used to indicate the contribution of small bodied insects to pollination at each field site. In the field, it was observed which small bodied insects could pass through the coarse wire mesh (3.1 x 3.1 mm squares; see section 3.2.3.1.1) of the partial excluder cage. Golden blister beetles,

small solitary bees such as halictids and small flies were capable of passing through the mesh whereas insects such as bee flies, butterflies and larger-bodied bees ranging in size from alfalfa leafcutter bees to bumble bees, were incapable of traversing the mesh.

During the field season these cages were observed to see which insects had gained access to the covered inflorescences, but across all field sites there were not many insects observed. Several small flies, a few blister beetles, several grasshoppers, a moth, and a few lygus bugs were observed within cages but did not seem to be foraging on *E. angustifolia* inflorescences. Pollen masses presented on anthers were always still present by the end of the day in comparison to inflorescences open to insects that typically had their pollen masses removed before the end of the afternoon. Uncollected pollen masses indicated that few pollen-collecting insects were foraging within partial excluder cages.

A site effect was evident in 2003 between weights and germination percentages of achenes from partial excluder treatments at MVA and PPS. Inflorescences in partial excluder cages at MVA had achene weights that were as heavy as achenes from control inflorescences at MVA and were significantly heavier than achenes from inflorescences in PPS partial excluder treatments. Germination percentages of these heavy achenes from MVA partial excluder cages were more similar to those of control inflorescences than they were to those from partial excluder cages at PPS. At MVA, a far greater number of small-bodied insects, including golden blister beetles and small bees, were observed visiting inflorescences during transect observations, while at PPS these insects were not evident. These small-bodied insects were providing as much pollination service at MVA as larger-bodied insects like bee flies (*Systoechus vulgaris*), butterflies (Lepidoptera: Satyridae and Pieridae) and large-bodied bees. In 2003 at PPS, only insects of large body sizes were recorded on transects and single insect visits. Small bees, and especially golden blister beetles, were absent. In both 2003 and 2004, inflorescences in partial excluder treatments at PPS produced consistently low achene weights and low germination percentages. The contribution to *E. angustifolia* pollination by small-bodied insects at PPS in both 2003 and 2004 was quite small and thus the low achene weights in the partial excluder treatments resulted from a lack of insect pollination.

A pronounced difference in achene weights and germinations between partial excluder treatments at MVA from 2003 and 2004 was evident, with significantly heavier achenes produced by inflorescences in 2003. The low weight of achenes from partial excluder cages at MVA in 2004 is surprising because of the high number of golden blister beetles at this site. It was thought that golden blister beetles were responsible for much of the pollination that occurred in partial excluder cages at Meewasin in 2003. Blister beetles can easily pass through the steel mesh of the partial excluder cages, but for some reason they did not seem to be doing so in 2004. There may have been sufficient open inflorescences to support foraging beetles that caged inflorescences were not overly attractive, even though they produced pollen that was inaccessible to large pollen foragers like bumble bees. There was a great increase in the number of inflorescences in 2004 with plants often producing multiple inflorescences. Perhaps that even though beetles and other small insects such as halictid bees could physically pass through the mesh, it posed a visual or tactile barrier that prevented their entry. Achenes from inflorescences within partial excluder cages at VR had the greatest mean weight, exceeding the mean of MVA by 0.29 g, but were intermediate in germination between PPS and MVA but still rather low (4.3%). It seemed that very little if any cross-pollination occurred in partial excluder cages at VR. With a visible shortage of potential pollinators on uncaged inflorescences at VR, it is likely that there was little pressure for insects to enter partial excluder cages in search of nectar or pollen.

Achene 1000 weights and germinations from achenes in the majority of partial excluder treatments at PPS, MVA and VR were similar to those of the total excluder treatments, suggesting that the partial excluder cages were acting as total excluder cages, and excluding most to all insects even though mesh sizes were quite different.

3.4.2.3 Inflorescences from total excluder cages

As predicted, the total excluder treatment produced achenes with the lowest weights and percent germination, which suggests that these fruits were formed without cross-pollination. Achenes from inflorescences from total excluder treatments showed nearly uniformly low weights and germinations in 2003 and 2004, with the exceptions of total excluder cage # 1 at PPS (2004) and total excluder cage #4 at MVA (2003). The mean germination of 12% in total excluder cage #1 at PPS was unusual but not

extraordinary because a similar situation occurred in 2003 where achenes from one inflorescence in total excluder cage #4 at MVA germinated at 44% and skewed the mean upwards. Percent germinations from total excluder cage #1 at PPS in 2004 were 36%, 23%, 23% and 14%, with achenes from the remaining 4 inflorescences failing to germinate. The two inflorescences that germinated at 23% were from the same plant and the inflorescences with 36% and 14% were also both from the same plant. If multiple inflorescences occur per plant, they typically do not mature at the same time so it is less likely that an insect breached the total excluder cage and cross-pollinated both inflorescences. That the inflorescences producing achenes that germinated are genetically identical suggests that in the case of these two inflorescences, the sporophytic self incompatibility system is not absolute and may have broken down and allowed for self pollination and fertilization of some florets. Sporophytic self-incompatibility is "flexible" in *Senecio squalidus* (Asteraceae) and self-pollination can occur (Hiscock, 2000). In sunflower (*Helianthus annuus*), when florets remain unpollinated their stigmatic lobes curve downward to touch their receptive inner surface onto the style to collect any remaining self pollen (Free, 1993). However, there is no evidence that this method of self–pollination is effective. With the typically short period of viability of trinucleate pollen grains (Brewbaker, 1967), well exhibited in *E. purpurea* with initial viability of grains at 32% one hour after presentation which then decreased to 2% in pollen grains remaining on PP3 florets, any self grains adhering to the receptive surface of the stigma may have lost their viability days earlier. McKeown (1999) stated that the sporophytic self-incompatibility system in *Echinacea* is not perfect and cites McGregor (1997, as cited in McKeown, 1999) that all *Echinacea* can self-pollinate to a certain degree. Low levels of germination without cross-pollination were also reported for *E. angustifolia* in South Dakota (Leuszler *et al*. 1996) at 9% (geitonogamous pollination) and 7% (autogamous pollination). No achenes were produced by *E. tennesseensis* when insects were excluded from inflorescences (Hemmerly, 1976), which supports the notion that insects are essential for cross-pollination in the genus *Echinacea*.

There are other possibilities that could explain germination of achenes in total excluder treatments. It is possible that insects were capable of infiltrating total excluder

cages. A tunnelling insect may have been able to dig its way under the cage and pollinate at a low level. Ants would be capable of this and they were recorded visiting un-caged inflorescences at MVA during transect observations but antibiotics present on the ant exoskeleton drastically reduces the viability of pollen grains even after a brief period of contact (Beattie *et al*., 1984). However, insects were rarely observed within total excluder cages. Thrips (Thysanoptera) were cosmopolitan on every inflorescence in the fields and may have been contributing to cross-pollination or self-pollination within the inflorescence. Inflorescences bagged to exclude insects for single insect visits still had an active complement of thrips and so it is conceivable that germination in the total excluder cages was a result of thrips cross-pollination. Thrips may have gained entry to the mesh bags through small openings where the mouth of the bag did not fully meet the peduncle of the inflorescence or thrips may have present on the inflorescences prior to bagging. A thrips contribution to pollination is discussed for several other asteracean species where female thrips move up and down the corolla tube to feed on pooled nectar and to oviposit at the stylar base (Gopinathan and Varatharajan, 1983). Mani and Saravanan (1999) refute the claims of earlier authors that thrips are true pollinators of other asteracean flowers due to their poor ability to fly between inflorescences and those pollen grains transferred to stigmas of other florets within the inflorescences should be incompatible and not germinate. However, germination of self-pollen grains in *E. angustifolia* and *E. purpurea* occurred and style shrivelling indicating fertilization occurred at 0 – 45% (Tables 3.2, 3.3) in controlled crosses of florets within the inflorescence suggesting that thrips may be contributing to self-pollination of florets within an inflorescence. Leuszler *et al*., (1996) observed the production of viable achenes at 9% in florets of *E. angustifolia* that were pollinated by another floret on the same inflorescence. Low achene weights and percent germinations from MVA and VR total excluder treatments (n = 120 inflorescences) confirm that insect cross-pollination is required for *E. angustifolia* florets to set a reasonable amount of seed.

Anemophilous (wind) pollination could account for some of the increased germination and achene weights of inflorescences under total excluder conditions. *E. angustifolia* has the potential for limited pollen dispersal through abiotic factors like wind. Pollen grains of *E. angustifolia* are on average 25 μm in diameter so *E.*

angustifolia grains may be small enough to travel a short distance on wind currents. *E. angustifolia* pollen grains can be blown at least one metre by a strong gust of air (personal observation) and *E. angustifolia* pollen was found on trap slides covered with petroleum jelly and placed at MVA and VR sites in 2004. Limited wind pollination may have been occurring within partial and total excluder cages with pollen donors either from within the cage or from outside the cage. Scanning electron micrographs show that *E. angustifolia* pollen grains were small enough to pass through the tent mesh material of the total excluder cages (Fig. 3.24G). Wind pollination may be a confounding factor in instances where achene weights and germinations in partial excluders were slightly greater than in total excluder cages. The tent mesh of total excluder cages blocked much of the wind and may prevent pollen from transferring between inflorescences within cages. The steel mesh of the partial excluder cages allows more air passage so there is greater potential for wind gusts to transfer pollen grains between inflorescences from the interior and exterior of the cages. Wind pollination within mesh cages was implicated in pollination of self-incompatible and primarily insect pollinated plants of *Linanthus parviflorus* (Polemoniaceae) (Goodwillie, 1999).

The controls from each field site had better germination than did all of the total excluder treatments and most of the partial excluder treatments. Achene weights and percent germinations seemed to agree in most instances, except with VR controls. Based on achene weights, it seems that VR controls were well pollinated by all insects, partially-excluded inflorescences were slightly pollinated by small insects and totally-excluded inflorescences were not pollinated. When germination is considered, however, the differences between treatments were not as pronounced with fairly low germination for control inflorescences especially considering their achene weights. Germination of achenes from partial and total excluder treatments at VR were quite low which was predicted by low achene weights of 1.4 and 1.1 g, respectively. Most evidence indicated that a pollination deficit at VR was most likely responsible for low germination of control achenes. Pollinators were infrequent on inflorescences which is most likely due to floral competition from weedy flowers. Native pollinators were unable to effectively pollinate this large plot of *E. angustifolia*. Insect pollinators were not limited at MVA in either year and small bodied-insects were able to effectively pollinate florets of *E.*

angustifolia when they chose to enter partial excluder cages in 2003. Pollinator limitation was also responsible for low achene weights and germination of controls at PPS in 2004, with pollination limitation arising from a small number of plants and possibly from floral competition from other asteracean flowers nearby.

A higher weight and percent germination of control inflorescences overall indicates that more cross pollination and presumably more insect visits were occurring on control inflorescences than on inflorescences in partial and total excluder cages. Insect pollination is thus essential for consistent production of quality achenes with a good ability to germinate.

3.4.2.4 Comparison of achene weight and germination

A link between achene weight and achene viability exists. Low achene weight for a sample indicated that the viability of achenes was also low. This trend was observed at PPS in 2004 where achenes had a low mean weight of 1.3 g and low germination of 21.8%, whereas in 2003, high weights of control achenes of 2.5 g were followed by a high mean germination of 66.6%. Control inflorescences at VR produced achenes that were twice as heavy as those in total excluder cages, indicating that cross pollination occurred in the field. Germination of achenes from control treatments at VR should have been on par with germination of achenes from controls at MVA, based on their similar 1000 achene weights. However, germination was comparatively low (24.4%) suggesting that the trend of higher weights indicating better germination ability may not hold true in all instances.

The linear regression graph allowed for a direct comparison between achene weight and germination of achenes from a single inflorescence. Positive R^2 values indicated that as the weight of achenes increased, so did their tendency to germinate. Usually inflorescences with heavier achenes had a higher germination percentage than those with achenes of lower weight. It can be concluded that achene weight is a fairly accurate predictor of achene germination. Achene weights may be able to predict germination percentages before achenes are even planted. It seems that on average, a minimum 1000 achene weight of 2.0 g is necessary before 50% germination is achieved. Growers could use this information to estimate the number of achenes that must be planted in order to produce a full crop. However, although there are no incidences of

achenes with 1000 achene weights of less than 2.0 g having greater than 50% germination, there were numerous samples where 1000 achene weight exceeded 2.0 g but achenes failed to achieve 50% germination. Therefore, any determinations based on weight would be conservative estimates only. It is possible that samples with 1000 achene weights greater than 2.0 g had confounding factors that contributed to low germination. It may simply be possible that larger inflorescences produced proportionally large achenes whose mean 1000 achene weights reached 2.0 g due to the weight of the achene walls alone.

When insects were excluded completely from inflorescences in the total excluder trials, achenes had the lowest weights and germinations and were found to cluster around the base of the graphs. Achenes in the total excluder treatment had both low 1000 achene weights and very low germination, showing that without insect cross pollination achenes remained lighter compared to the openly pollinated achenes of the control treatments. These light achenes also have poor germination compared to the heavier achenes of the openly pollinated treatments. In all cases, the control inflorescences (those allowed full insect visitation) had greater average weight and germination than those in either excluder-cage trial. Even in the total and partial excluder treatments, those inflorescences with achenes of greater weights had higher germination. There are a finite number of achenes that can be produced in a field based on the number of disc florets present and achenes are sold based on weight. In order for a grower of *E. angustifolia* to increase the value of his seed crop, it is necessary to increase the weight of achenes by ensuring an adequate supply of insects. Achene weight and percent germination more than doubled with insect cross pollination, so the need for insect pollination in seed production is evident.

3.4.3. Single insect visits

3.4.3.1 Controlled hand pollinations

Pollen tubes from xenogamous pollen reached the style base within 24 h in controlled hand pollinations of *E. angustifolia* florets, which established the time length from visit to inflorescence harvest for SIVs in 2003.

Pollination trials were performed on *E. angustifolia* inflorescences to determine if cross-pollination resulted in stigma and style shrivelling within a 24 h period. A weather dependent difference in the time to shrivelling following cross pollination of 24 h was observed. Under cool, cloudy and sometimes rainy conditions, cross-pollinated styles took closer to 48 h to shrivel while sunny, hot weather resulted in shrivelling of cross-pollinated styles within 24 h or hand pollinations. The difference in time to shrivelling between the two trials is probably a result of environmental conditions like temperature, sunlight and humidity at the time of the trials. Pollen germination and pollen growth within the style are both dependent on temperature, where increased humidity inhibits germination of pollen grains while pollen tube growth is slowed during periods of low temperature (Corbet, 1990). Low temperature and humidity either affected germination of pollen, growth rate of pollen tubes or the subsequent physiological changes that initiate stigma/style shrivelling. Any further pollination studies using the style's persistent turgidity and shrivelling to determine pollination should therefore take into account the climatic conditions and adjust the duration from cross-pollination to floret harvest and pollen counts, accordingly. Wagenius (2004) did not record any weather effects on style shrivelling in initial trials, but did observed that style shrivelling was delayed on cool day (Dr. S. Wagenius, pers. comm.). However, for the purposes of his pollen limitation study, 72 h elapsed between each inflorescence observation, thus climactic effects on style shrivelling would not have been evident or of consequence, as styles shrivel within 48 h of cross-pollination even under cool, cloudy conditions. Further controlled hand pollinations confirmed that shrivelling of the stigma and style in *E. angustifolia* occurred between 24 h and 48 h after cross pollination and the stigma and style from unmanipulated florets did not shrivel within this time period. A high proportion of stigmas and styles had shrivelled after 24 h in these trials, where conditions were often warm but overcast and so based on this finding and the 12 – 24 h growth rate of pollen tubes within the style, inflorescences from SIVs in the field (2004) were also harvested 24 h after visitation, taking into account climatic conditions, where the length of time between insect visitation and inflorescence harvest was extended when inclement weather occurred after SIVs.

Pollen donation from closely related florets was also investigated using controlled pollinations by hand. Geitonogamous treatments, where donor pollen came from a different inflorescence of the same plant, produced shrivelling of 27% styles, which indicated either that these pollen grains were sufficiently genetically dissimilar to fertilize ovules in a limited capacity or that the incompatibility of the stigma was altered to finally allow self-pollen. The latter hypothesis is supported by observations that older florets (PP3) were more apt to shrivel following self pollinations within the inflorescence than were younger florets (PP1). Shrivelling of a small number of styles (5.2%) following selfing within the inflorescence indicated that limited pollination from closely related florets of the same head can occur. Viable achenes (9% of total) have been produced from controlled geitonogamous (selfing within the inflorescence) hand pollinations of *E. angustifolia* (Leuszler *et al.,* 1996).

Inflorescences that remain bagged for the entirety of their flowering period will eventually possess shrivelled styles in the majority of their disc florets if the particular florets are older than approximately eight to ten days (Leuszler *et al*., 1996; Wagenius 2004). Leuszler *et al*. (1996) observed that most stigmas in geitonogamous hand pollinations were persistent ("remained fleshy and turgid") (39 of 46 florets) 24 h after pollination, while stigmas following xenogamous hand pollinations shrivelled within 24 h of cross-pollination. Wagenius (2004) also tracked unmanipulated florets excluded from insects to determine that style persistence is time sensitive. To properly utilize shrivelled style analysis with SIVs to determine pollination efficiency, requires that florets must not be receptive for longer than 6 days before the SIV is allowed or the event of shrivelling of styles over time will confound results by skewing shrivelled style counts upwards.

Stigmas and styles of *E. purpurea* shrivelled in the same manner as those of *E. angustifolia*, suggesting that comparing shrivelled vs. persistent (turgid) styles after single insect visits would be an appropriate technique to study the pollination efficiency of insects on inflorescences of *E. purpurea*, as well. Shrivelling of some of the stigmas of florets of *E. purpurea* occurred during geitonogamous hand pollination treatments, although geitonogamy in the instance of *E. purpurea* was defined as pollen from another inflorescence of the same plant and not as pollen from another floret of the same

inflorescence. After 48 h, 13-30% of geitonogamously pollinated stigmas led to shrivelled styles suggesting that pollen from the same plant but another inflorescence is capable of effecting limited self pollination. Achenes were not examined for embryos and weights were not recorded so the quality of the achenes produced by these geitonogamous pollinations is unknown. Autogamy sometimes resulted in a few shrivelled styles where florets were either unmanipulated (controls) or where pollen was transferred from the anther of a disc floret in the staminate phase to a near-neighbour floret in the first day of the pistillate phase (PP1). In one of two trials, selfing within the inflorescence resulted in 6% of styles shrivelling while the unmanipulated controls shrivelled at 12%. In the other trial, selfed and unmanipulated styles did not shrivel. It would be expected that no shrivelling of stigmas or styles would occur in any of the self pollination trials, but the unexpected shrivelling of these styles suggests that in some instances self pollen may be capable of fertilization. Interestingly, it seemed that older pistillate phase florets (PP3, PP4) of both *Echinacea* species were more receptive to pollination by self pollen grains (from florets of the same head) than were younger pistillate florets (PP1, PP2), as evidenced by more shrivelling of self-pollinated styles from older whorls of florets. A mixed-pollination system may be occurring in *E. purpurea* in the same manner as was described for *E. angustifolia* (Leuszler *et al.*, 1996) based on the shrivelling of stigmas and styles in geitonogamous and selfed treatments. Both *E. angustifolia* and *E. purpurea* appear to be self compatible to a small degree.

3.4.3.2 Stigmatic loads of pollen

Pollen grains on the surface of the stigma (germinated and ungerminated) were recorded while conducting counts of pollen tubes within the styles of florets from single insect visits, to estimate the amount of pollen grains transferred by an insect. The number of pollen grains that germinated was compared to the total number of pollen grains on the stigma in an effort to differentiate between viable, xenogamous pollen and self-pollen and to provide another method of estimating pollination efficiency after an SIV. It was hoped that the sporophytic self-incompatibility systems employed by asteracean florets would prevent the germination of self-pollen on the stigma, but after several hand pollination trials it was evident that self pollen grains would often germinate. *E. angustifolia* appears to follow a pattern similar to *Senecio squalidus*,

where it was reported that self or incompatible pollen grains often did not germinate on the sporophyticly self-incompatible stigma, but that many self pollen grains did germinate and send pollen tubes towards the stigma, some of which penetrated (Hiscock *et al*., 2002). Germination of self-pollen grains followed by penetration of the stigma by self-pollen tubes occurred in *Cosmos bipinnatus* and *Helianthus annuus* (Elleman *et al*., 1992). Germination of self pollen confounded any effort to use germinated cross-pollen grains as a tool to evaluate insect pollination efficiencies using SIV samples. ANOVA results were not significantly different when the number of germinated grains per stigma was compared between SIV insect groups or between shrivelled and persistent styles from the same SIV, suggesting that using germinated grains to estimate cross - pollination is a problematic technique. The great variability in the number of pollen grains transferred and the number of pollen grains germinated within visits from the same insect species may also confound the use of germinated pollen grains as a tool for estimating pollination efficiency.

Self pollen-grains transferred by hand to the stigmas of *E. purpurea* and *E. angustifolia* were observed to germinate and pollen tubes were observed contacting the stigmatic papillae. On control stigmas (hand pollinations), persistent stigmas (2004), and stigmas from control inflorescences, callose deposition was observed within stigmatic papillae at the site of penetration of pollen tubes from presumably incompatible pollen grains. The localization of callose at sites of pollen tube contact is a mechanism to prevent penetration of self-pollen tubes into the stigma. This barrier method was employed to prevent penetration of self-pollen tubes by asteraceans such as *Helianthus annuus* (Vithanage and Knox, 1977), *S. squalidus* (Hiscock *et al.*, 2002) and species of other families such as *Ipomoea trifida* of the Convolvulaceae (Kowyama *et al.,* 2000). It may be possible to use this callose reaction of stigma papillae to differentiate between germinations of self and cross pollen grains in future pollination studies of Asteraceae and in turn use germination of cross-pollen grains as a tool to estimate pollination efficiency.

3.4.3.3 Comparison of methods for determining pollination efficiency

It was important to compare the pollen tube counting technique employed in 2003 with the shrivelled style analysis developed in 2004 to ensure that both estimates

of pollination efficiency on *E. angustifolia* were comparable. Shrivelled and persistent styles were compared for the number of germinated pollen grains per stigma, the number of pollen tubes at the style base and the percentage of styles with pollen tubes at the style base, to identify if shrivelled styles manifested more features indicative of cross-pollination than persistent styles.

It was hypothesized that shrivelled styles will have a greater proportion of germinated pollen grains on their stigmatic surface and a greater number of pollen tubes at their style bases, than will persistently turgid styles. If a style shrivelled after an SIV, then presumably it was cross-pollinated by at least one pollen grain and should have at least one pollen tube at the style base.

There were significantly more shrivelled than persistently turgid styles with pollen tubes at their bases. This significant result indicates that utilizing shrivelled styles or pollen tube counts are both acceptable methods of estimating pollination efficiency on *E. angustifolia* inflorescences. Pollen-tube counting is a well established method of determining pollination efficiency (Davis, 1992 *b*; Kearns and Inouye, 1993), but it was slow and labour-intensive for use on asteracean inflorescences. Comparing the number of shrivelled to persistently turgid styles promised to provide a relatively rapid method for estimating pollinator efficiency. An estimate of efficiency was made within 24 to 48 h of the single insect visit with a count of shrivelled styles vs. persistent styles being completed in approximately ten minutes.

In theory, persistent styles should not contain pollen tubes at their style bases if shrivelling of the stigma and style indicate successful cross-pollination and fertilization of the ovule. The presence of low numbers of pollen tubes within persistent styles was therefore unexpected but can be explained in several ways. It was demonstrated through controlled hand pollinations that self-pollen grains will germinate on the stigma surface and that self-pollen can initiate style shrivelling (section 3.3.2.2.1), so it seems likely that *E. angustifolia* is at least partially self-compatible. Self-pollination may account for some of the pollen tubes at the style base in persistent styles. It was also demonstrated that specific environmental conditions must be met in order for style shrivelling to occur within a 24 h period. It is conceivable that these styles with pollen tubes were in fact cross-pollinated but that shrivelling had not yet been initiated due to the retarding effects

of low temperature and high humidity. If these samples had been left in the field longer, for 48 h, these persistent styles with pollen tubes may have shrivelled. Shrivelling may also have been initiated but had not progressed to a point where it was possible to distinguish between these and truly persistent styles.

More difficult to explain was the absence of pollen tubes at the base of some shrivelled styles. Other factors known to induce style senescence are the passage of time, and wounding of the style by insect damage. It was noted in *Petunia hybrida* that pollination and stigma wounding both induced ethylene production, with the end result being style senescence (Woltering *et al*., 1997). However, these florets should not have reached an age where shrivelling due to time would be a factor and the excluder bags prevented all insects but thrips from contacting the stigma/style. Thrips, however, suck sap and may induce some level of style senescence through wounding. There may also have been some human error if mistakes were made when judging styles as shrivelled or persistent where a persistent style was mistaken for a shrivelled style.

3.4.3.4 Estimates of pollination efficiency

The percentage of styles with at least one pollen tube at their base and stigma loads of pollen grains were employed with SIVs in 2003, and the percentage of styles that shrivelled compared to the total number of styles available and some data on stigma loads of pollen (2004) were employed to determine pollination efficiency of individual insects. These techniques were comparable and produced similar estimates of pollination efficiency for several insect visitors common between field seasons. Disc florets of *E. angustifolia* have one ovule, so only one pollen tube was needed to reach the style base to fertilize the floret. In this way, one pollen tube at the base of the style should represent the successful cross-pollination and likely fertilization of that floret. Several pollen tubes at the style base were redundant in terms of floret reproduction because only one ovule is present for fertilization, so the main comparison was the percentage of styles with at least one pollen tube at their base so that shrivelled-style estimates were comparable to pollen-tube estimates in 2003. The percentage of pollen tubes at each style base was then consistent with shrivelled-style analysis, because if a style shrivelled after an insect visit, then there was at least one pollen tube that reached the style base to induce shrivelling. Accordingly, insect pollination efficiency was

determined by counting the total number of pollen tubes at the base of the style (2003) and by counting the number of styles shrivelled after an SIV (2004).

Even though self-pollen grains germinated on receptive stigmatic surfaces and self-pollen tubes may penetrate into the style if *E. angustifolia* is similar to *S. squalidus* (Hiscock *et al*., 2002), any self-pollen tubes should have aborted before reaching the style base. It seems that in some instances, self-pollen tubes were able to reach the style base and fertilize the ovule but this occurrence cannot be differentiated from cross-pollination using pollen tube quantifying or shrivelled style analysis. Quantification of pollen tubes at the style base still provided a conservative estimate of the pollination abilities of insect visitors to *E. angustifolia* during SIVs. Control inflorescences were used to identify the presence of pollen tubes in the absence of insect visitation, and so were treated the same way as visited SIV inflorescences with the exception that they did not have insect visitors. The presence of a mean of 10.4 % of styles with pollen tubes in control styles (Fig. 3.25) indicates that there may be pollen tubes in SIV inflorescences that were not initiated by pollen transferred from an insect visitor. There are several potential reasons for the presence of pollen tubes in control treatments. Compatible pollen grains may have been transferred to control inflorescences by an insect resting on bagged inflorescences, because the bags are impermeable to insects but not to *E. angustifolia* pollen. During a night transect, an unidentified moth was seen feeding on a bagged inflorescence with its proboscis fully inserted through the fine mesh. Pollen tubes could be initiated by self grains or grains from another floret on the same inflorescence. Human error may have occurred where errant phloem sieve tube elements may have been misidentified as pollen tubes. Phloem sieve tube elements have callose in their sieve plates that take up the aniline blue stain and fluoresce and often mimicked callose plugs of pollen tubes when overlying tissue prevented a sharp focus of the sieve tube. Much care was taken to avoid misidentification of pollen tubes but it must have occurred to some degree.

Control inflorescences had pollen tubes in 10% of their styles (Fig. 3.25). This percentage represents the effect of bagging on experimental inflorescences of *E. angustifolia*, and is similar to the 10-20% of achenes produced by sunflower (*Helianthus annuus*) inflorescences bagged to exclude insects that Free (1993), directly attributed to

the effects of bagging. When bags were kept from touching florets, the number of achenes produced by bagged inflorescences decreased to 1% (Free and Simpson, 1964). Bags can rub across anthers and stigmas and transfer pollen grains among florets of the same inflorescences, and this within-head selfing was demonstrated to produce shrivelled styles in 6 and 20% of florets in 2 of 5 pollination experiments on bagged inflorescences of *E. angustifolia* (Table 3.2). It is conceivable that pollen tubes within control styles resulted from within-head selfing of florets due to the effect of bagging inflorescences.

Systoechus vulgaris was the most abundant visitor of *E. angustifolia* in 2003 and this is reflected by their abundance at SIVs accounting for 53% of 38 SIVs in 2003. Individually, *S. vulgaris* was only 21.5% efficient at cross-pollinating florets in receptive areas of the inflorescence that were visited. This percentage is twice as high as that of control inflorescences (Fig. 3.25). This 2003 efficiency percentage was higher than the 14% styles that shrivelled after 10 bee fly visits in 2004 (Fig. 3.27), but visit lengths need to be considered for yearly comparisons. *S. vulgaris* spent more time on SIV inflorescences in 2003 than 2004 so each insect had more opportunity to pollinate florets. Individually *S. vulgaris* might not be the most efficient pollinator but, in 2003, the sheer number of bee flies would have compensated for the lack of efficiency of individual insects with each visit contributing a small amount to the pollination total. In 2004, however, bee flies did not occur in great numbers and their contribution to pollination of *E. angustifolia* was probably much less than it was in 2003. In years with lower *S. vulgaris* numbers such as 2004, however, these insects may not be effective pollinators of an *E. angustifolia* field and probably will only reach significant population levels for pollination in areas with abundant grasshopper eggs to feed bee fly larvae.

The progressive bee fly (*Exoprosopa* spp.) was not an effective pollinator and was present in such low numbers (1 in 2003, 0 in 2004) that it may only be an occasional visitor and would contribute very little to overall pollination of *E. angustifolia*. This progressive bee fly species had a pollen tube percentage lower than the mean percentages of pollen tubes at style bases in control inflorescences (Fig. 3.25).

Golden blister beetles (*Epicauta ferruginea*) produced the third highest number of styles with pollen tubes at 44.4% (Fig 3.25) and seemed to be efficient pollinators of

E. angustifolia. Golden blister beetles were fairly efficient individual pollinators in 2004 as well, with a mean of 34% of styles shrivelled after a single visit (Fig. 3.27). Blister beetles also foraged for longer periods on inflorescences than most other insects and travelled around the capitulum, which increased the number of stigmas that they encountered. The high numbers of *E. ferruginea* in 2004 combined with their pollination efficiency of 34% per insect would make this species an efficient pollinator of *E. angustifolia*. It was noted, however, that golden blister beetles preferred to visit yellow asteracean flowers such as perennial sow thistle (*Sonchus arvensis*) when they were present, rather than visit inflorescences of *E. angustifolia*. Meloidae beetles are known to feed on pollen and yellow petals of ray florets of asteracean inflorescences (Mani and Saravanan, 1999), indicating a preference for yellow over other colours. The majority of golden blister beetles observed at VR were found on sow thistle inflorescences and no blister beetle visitation was observed at PPS, or the Biology garden plots, so their contribution to *E. angustifolia* pollination across Saskatchewan may actually be minimal. Large numbers of blister beetles on *E. angustifolia* inflorescences were only observed at MVA, where there were few competing yellow flowers. Thus, this phenomenon of beetle pollination of *E. angustifolia* may be sporadic and probably only occurs near sites with grasshopper egg beds. Interestingly, blister beetles are often considered as detrimental to seed production due to their tendency to feed on flowers (Mani and Saravanan, 1999), and *Epicauta fabricii* have been recorded destroying more than 80% of flowers and young seed pods of *Baptisia australis* (Leguminaceae) (Evans *et al.,* 1989). *E. ferruginea* was observed only to feed on the yellow pollen grains of *E. angustifolia* florets and did not damage achenes or ray florets.

Insufficient pollination of *Chrysanthemum cinerariaefolium* (Asteraceae) by coleopteran and dipteran visitors resulted in unfilled achenes that were low in pyrethrin (Kroll, 1961). Safflower, *Carthamus tinctorius* (Asteraceae), is visited primarily by honey bees (*Apis mellifera*) (90%) in Canada, but is also visited at 7% by flies such as syrphids (Diptera: Syrphidae) (Boch, 1961). Not many records exist for coleopterans as pollinators of asteracean crop flowers.

Sulphur and white butterflies, especially cloudless sulphurs, *Phoebis senna* (Lepidoptera: Pieridae), were fairly abundant visitors according to transect observations

in 2003. The two that were observed visiting *E. angustifolia* during SIVs had very different pollination efficiencies (Fig. 3.25). One butterfly produced pollen tubes in 76.4% of available styles and was therefore a very effective pollinator, whereas the other produced only 18.2%. No SIV were recorded in 2004, so a shrivelled-style estimate of *P. sennae* efficiency was not possible. A larger sample size would most likely have allowed the true efficiency of these butterflies to be seen. It is unclear whether these butterflies are individually efficient on *E. angustifolia* based on these figures but it can be said that they show a variation in their pollination efficiency that may be natural for the species. Other insects also showed a wide range of pollination efficiencies based, on pollen-tube counting and shrivelled-style analysis. Nymphalids such as painted ladies (*Vanessa cardui*) were *E. angustifolia* visitors but were not recorded during SIVs. Pearl crescent butterflies (Nymphalidae) were observed visiting *E. angustifolia* in Minnesota, U.S.A. (Dr. S. Wagenius, pers. comm.). Nymphalidae and Pieridae (sulphurs and whites) butterflies were considered to be primary visitors of *E. tennesseensis* (Hemmerly, 1976; Drew, 1991). Mani and Saravanan (1989) claim that as a family, asteraceans are shifting pollination syndromes from bee dominated to butterfly pollination based on floral economics. They suggest that bee visitation can be damaging to asteracean florets and drain inflorescences of pollen and nectar, whereas butterflies do no damage to florets while feeding and consume only a small amount of nectar for sustenance, such that bees are more costly floral visitors than butterflies. However, these arguments do not address which order of insects is the most efficient at pollinating asteracean inflorescences.

Sunflower leafcutter bees, *Megachile pugnata,* are discussed in the literature as asteracean specialists and excellent pollinators of sunflower (*Helianthus annuus*) (Parker and Frohlich, 1983), but results from their SIVs indicate that they are not very efficient pollinators of *E. angustifolia*, with a mean of 20% of styles with pollen tubes after 5 visits in 2003 (Fig. 3.25) and only 4% of styles shrivelled after 2 visits in 2004 (Fig.3.27). Compared to 10% of control styles with pollen tubes, a value of 20% was not high. These leafcutter bees were fairly abundant at PPS in 2003 and seem to be a fairly regular floral visitor around Saskatoon, being found at all three field sites across both years. *M. pugnata* may be less efficient at pollinating *E. angustifolia* because on all

visits it foraged solely for pollen and its visits were typically short. The foraging strategy of *M. pugnata* was to circle the staminate whorl (SP stage) and collect pollen as quickly as possible. There was a visible reduction in the amount of pollen on the anther tubes after a visit but this foraging strategy did not bring the bee into contact with the majority of receptive florets in PP1 or PP2 whorls. It was hoped that these bees would be excellent *Echinacea* pollinators based on their ability to pollinate sunflower (*Helianthus annuus*) (Parker and Frohlich, 1983, 1985) and to lend support to testing the alfalfa leafcutter bees (*M. rotundata*) as managed pollinators of *E. angustifolia*. It seemed that *M. pugnata* contributed little to pollination of *E. angustifolia* in either year. Although not recorded on an SIV, *M. latimanus* was observed on transects in low numbers and this leafcutter bee was also recorded as a visitor to inflorescences of *E. angustifolia* as well as one other leafcutter bee, *Heriades carinata*, in Douglas County, MN in 2004 (Dr. S. Wagenius pers. comm.).

The single alfalfa leafcutter bee (*M. rotundata*) visit recorded during an SIV in 2003 (Fig. 3.25) produced a large number of styles with pollen tubes (84.5%) and would seem to be an efficient individual pollinator. However, a much larger sample size would be required to make suitable inferences of individual pollination efficiency. Unfortunately, these bees were not attracted to *E. angustifolia* inflorescences and were more loyal to legume weeds, so their potential as *E. angustifolia* pollinators is low.

Two sweat bees (Halictidae) were observed foraging for pollen on *E. angustifolia* inflorescences but they were only captured on camera, so identification to species proved difficult. Visits by these bees led to as many pollen tubes as *S. vulgaris* and twice as many styles with pollen tubes as control inflorescences (Fig. 3.25), so they were definitely contributing to pollination in 2003. In 2004, sweat bees made up a small percentage of *E. angustifolia* visitors on transects, and they were only observed once during SIVs but no shrivelled styles were evident after this solitary visit. Halictids foraged solely for pollen and the small body size of these bees may limit their usefulness to *E. angustifolia* pollination, as the bees contact only staminate whorls while foraging and are also probably too small to reach nectar from the corolla tube of pistillate florets. Sweat bees probably played only a minor role in pollination at field sites due to their low numbers and low pollination efficiency. Of note is that these bees are small enough to

pass easily through the mesh of partial excluder cages, and may be part of the small-bodied insect group responsible for production of achenes that germinated in partial excluder treatments. Five genera of Halictids (sweat bees) were recorded visiting *E. angustifolia* in Douglas County MN (U.S.A.) in 2004 (Dr. S. Wagenius, pers. comm.), however, honey bees (*Apis mellifera*) were not indicated as inflorescence visitors.

European honey bees (*Apis mellifera*) were observed on *E. angustifolia* in 2004, but not in 2003. Visits by 4 honey bees resulted in the shrivelling of styles on 54% of receptive florets (Fig. 3.27). A wide range of efficiencies (6-100%) from individual visits, resulted. Honey bees seem to be relatively effective pollinators based on the number of shrivelled styles resulting from visits by three of four honey bees. The inflorescence on which the honey bee visited that resulted in 100% style shrivelling, had only one receptive whorl and as the bee collected pollen from the staminate whorl, it contacted and pollinated all of the styles in the only receptive pistillate whorl (PP1). Honey bees are often used as managed pollinators of sunflower (*Helianthus annuus*) and were thus the most abundant visitors in Australia (99%) (Langridge and Goodman, 1974, 1981) and the U.S.A. (83%) (Freund and Furgala, 1982).

The late summer of 2004 brought an abundance of bumble bees (Hymenoptera: Apidae) as visitors to *E. angustifolia* inflorescences. Bumble bees were rarely observed in 2003 (1 specimen) so for this reason alone, 2004 was a very different season for insect visitation. It was difficult to identify bumble bees to species in the field except in the case of *Bombus ternarius*, the red-tailed bumble bee, which was easily identified by the red colouration of the pile on its 2nd and 3rd abdominal tergites (Curry, 1984). Results from *B. ternarius* were therefore kept separate from those of other bumble bees. The bumble bee group was composed primarily of *B. fervidus*, with others such as *B. vagans* and *B. nevadensis* also present. *B. ternarius* (n = 8) caused an average of 39% of receptive florets to shrivel after their SIVs (Fig. 3.27), with a wide range of 22-74%. Other species of bumble bees (n = 14) were even more efficient at pollinating florets based on their mean of 61% of styles shrivelled per inflorescence (range 21-94%). Bumble bees as a group seemed to be efficient pollinators of *E. angustifolia* based on the percent of receptive styles shrivelled after a single visit and their foraging behaviour on inflorescences. On each SIV, observed bumble bees always flew from another *E.*

angustifolia inflorescence and after leaving the sample inflorescence they always flew to another *E. angustifolia* inflorescence. This foraging behaviour was not surprising because individual bumble bees typically show a high fidelity for the flowers they visit. Based on this data set (n = 46 SIVs), bumble bees and honey bees individually were efficient pollinators of *E. angustifolia* florets in the 2004 season. Beetles of *E. ferruginea* were also relatively efficient at individually pollinating florets and their large numbers made them important pollinators at MVA, but less important at VR and unimportant at PPS (2003 and 2004).

Butterfly pollination efficiency was difficult to observe due to their skittish nature. Butterflies were reluctant to approach humans and were thus difficult to capture on an SIV even though observers kept their distance from experimental inflorescences. It is anticipated that butterfly contribution to pollination of *E. angustifolia* across Saskatchewan is greater than indicated by this study. The literature suggests that the capitulum type inflorescence of asteracean plants evolved in conjunction with bee (Hymenoptera: Apoidea) pollination (Mani and Saravanan, 1999), but that butterfly pollination of asteraceans is also quite important. Results from 2004 suggested that large-bodied bees in the family Apidae (honey bees and bumble bees), when present, are frequent visitors to *E. angustifolia* inflorescences where they collect both nectar and pollen and efficiently cross-pollinate disc florets. Individual insects varied in their pollination efficiency during SIVs to virgin flowers as evidenced by wide ranges of percentages of styles with pollen tubes and shrivelled styles per insect group, but on average, apid bees were good individual pollinators. Variation in individual pollination efficiency was caused by several factors; including the insect's fidelity to *E. angustifolia* inflorescences, the number of inflorescences previously visited before the virgin inflorescence, the number of pollen grains present on the insect's body at the time of the visit and possibly visit duration. Some insects such as honey bees and bumble bee workers exhibited a pollen cleaning behaviour where pollen grains were combed from body hairs and stored in corbiculae on their hind legs. Pollen grains stored in corbiculae were no longer available for pollination and clean bees will have reduced free pollen to transfer to another disc floret. Golden blister beetles were also observed exhibiting cleaning behaviour where the foreleg was used to comb pollen grains from their

antennae and bodies (personal observation). Grasshopper bee flies as a group will contribute significantly to *E. angustifolia* pollination in years when their population is high due to abundant grasshopper eggs and golden blister beetles will tend to follow a similar pattern. If an abundance of competing yellow asteracean flowers is not present it is likely that a large population of golden blister beetles would contribute significantly to *E. angustifolia* pollination. Small-bodied bees such as halictids visited *E. angustifolia* inflorescences but showed very little individual pollination efficiency according to the small number of SIV assessments that occurred.

Chapter 4: SUITABILITY OF *MEGACHILE ROTUNDATA* AS A MANAGED POLLINATOR OF *ECHINACEA ANGUSTIFOLIA*

4.1 Introduction

In the 1960's, the alfalfa leafcutter bee (ALB), *Megachile rotundata* (Hymenoptera: Megachilidae), was found to be a much better pollinator of alfalfa (*Medicago sativum*) than the honey bee. Alfalfa leafcutter bees have the capacity for domestication and require less effort to manage than a honey bee colony. Leafcutter bees are relatively easy to manage and require little care during their adult phase. Bohart (1972) suggested several biological features that make alfalfa leafcutter bees dependable pollinators of alfalfa, and these features make *M. rotundata* a possible candidate for the pollination of other crops. *M. rotundata* requires a cold treatment to break pupal diapause, so that adult emergence can be controlled to coincide with flowering of selected crops simply by raising the incubation temperature of pupae (Richards, 1984). Leafcutter bees are solitary but they prefer to nest closely, making the development of artificial domiciles possible. Females tend to nest in man-made environments and can be found in nail holes, copper tubing, between shingles or in any area with the proper dimensions for nest building. This tendency towards nesting in man-made enclosures coupled with their communal nesting habits allow thousands of bees to nest in boards fabricated for them. Nesting boards formed by drilling holes in wooden or polystyrene blocks are enclosed in shelters for protection from the environment. Females actively forage for both nectar and pollen to provision their nest cells, such that flowers with an abundance of nectar and pollen should be attractive to *M. rotundata* females. Female leafcutter bees cut sections of leaves and use them to line their nests. They prefer soft, supple leaves like alfalfa or buckwheat. Flower petals of some plants may be cut and utilized as well. Cut leaves or flowers of *E. angustifolia* may

not be desirable for growers however, so this behaviour would be a potential drawback if it occurred.

Research in the last forty years has focused on the use of *M. rotundata* to enhance cross-pollination of important crops other than just alfalfa. For example, Fairey and Lefkovitch (1991) found that *M. rotundata* effectively pollinates clover (*Trifolium* spp.) and *Brassica campestris*. Alfalfa leafcutter bees also provided sufficient pollination of *Brassica napus* (Soroka *et al*., 2001) and increased fruit set by 30% in lowbush blueberry (*Vaccinium angustifolium*) (MacKenzie *et al*., 1997). Recently, Goerzen (2000) found that bees of *M. rotundata* were excellent cross-pollinators of strawberries and melons in California. Discovering new crops that are pollinated well by *M. rotundata* will add demand for the leafcutter bee stocks exported by Canadian and especially Saskatchewan growers. Canadian leafcutter bees are of good quality and are exported all over the world. Canadian leafcutter bees have low larval mortality rates and low incidences of parasitism and disease and these attributes give them a valued reputation in the world market (Richards, 1984).

Megachilid bees (*Megachile latimanus* and *Ashmeadiella bucconis*) were observed visiting *E. angustifolia* in South Dakota (Leuszler *et al*., 1998), and *A. bucconis* was thought to be a specialist of asteracean flowers. The presence of megachilid bees on *E. angustifolia* may indicate that *Megachile rotundata* (used in Saskatchewan in alfalfa seed production) would also be attracted to *Echinacea* inflorescences and may function as a managed pollinator for *E. angustifolia* crops.

These solitary bees have proven to be more efficient pollinators of crops in Saskatchewan like alfalfa, than honey bees (McGregor, 1976). Also, other leafcutter bees exhibit a preference for species of the Asteraceae, the plant family to which both *E. angustifolia* and sunflower (*Helianthus annuus*) belong. The sunflower leafcutter bee, *Megachile pugnata,* was found to be a more efficient pollinator of sunflower than were honey bees (Parker and Frolich, 1983, 1985). Their findings indicate that a few hundred *E. pugnata* females per acre pollinated a sunflower field as effectively as several colonies of honey bees (Parker and Frolich, 1983). Sunflower and *Echinacea* have similar inflorescence and flower structure and maturation pattern. Furthermore, sunflower cannot set seed effectively without insect pollination (Free, 1964) and it

seems that *E. angustifolia* will set only a very low number of seeds without cross-pollination (McGregor, 1968; Leuszler *et al*., 1996). These similarities favour the hypothesis that ALBs will be excellent pollinators of *E. angustifolia*. If this hypothesis is supported, this research may open up a new market for the use of ALBs on *E. angustifolia* in Saskatchewan, thus increasing the demand for and economic value of *M. rotundata* reared in Saskatchewan.

These field tests to study pollination by *M. rotundata* will also allow comparison with the pollination efficiency of the native visitors to heads of *Echinacea angustifolia*.

4.2 Materials and Methods

On July 18, 2003, approximately 20,000 ALBs (*Megachile rotundata*) were released at MVA (Chief Whitecap Park) into a field of *E. angustifolia*. Bees were supplied by Wayne Goerzen and Gaylord Mirau (alfalfa seed producer at Dalmeny, SK.) of the Saskatchewan Alfalfa Seed Producers Association (SASPA). One plastic bee hut was employed to protect the four nesting boards. The hut was placed on the west side of the *E. angustifolia* field in close proximity to many inflorescences already in flower. *E. angustifolia* plants within 100 m (ALB foraging distance from nest) (Tasei and Delaude, 1984) of the nesting boards were the sample group. The pollination efficiency of leafcutter bees was to be tested using a single insect visit method where individual inflorescences of *E. angustifolia* were bagged to exclude insects and then opened to allow a single leafcutter bee to visit the inflorescence when it was at an appropriate stage of development (see section 3.2.3.2 for details). Pollen tubes initiated following the insect visit were used to gauge how many outcross pollen grains the ALB transferred on its visit. Inflorescences utilized for these single-visit experiments were those bagged near the ALB shelter, to increase the chances of an ALB visit.

On July 5, 2004, approximately 10,000 ALBs were released into MVA and another 10,000 ALBs were released at the agricultural planting of *E. angustifolia*, at Valley Road (VR). ALBs were again supplied by SASPA. However, inflorescences had not yet reached anthesis due to a cool wet, spring. A plastic bee hut with nesting boards was placed in the same location at MVA as it was in 2003, and an additional bee hut and nesting boards were placed in the northwest corner at VR. Single insect visits

were again used to assess the pollination efficiency of these bees on *E. angustifolia* inflorescences and transect observations (see section 3.2.2.1) were used to estimate the number of ALB visits to unbagged inflorescences.

Two excluder cages constructed with tent screen material were placed over approximately fifteen flowering plants of *E. angustifolia* at MVA from July 26 until August 6, 2004. A small trap-nesting board and two Petri dishes, each with 100 nest cells, were placed into each cage and observations conducted as soon as bee emergence was complete. Open inflorescences were identified with marking tape so that they could be harvested when the inflorescence was mature. Achenes from inflorescences that flowered during the time that ALBs were caged were scheduled to be harvested, weighed and germinated in the same way as previously used in excluder cage trials. However, test inflorescences were lost due to an unexpected harvest of achenes by Meewasin staff at the MVA field site.

4.3 Results and Conclusions

Plant material from *E. angustifolia* did not seem to be suitable as nesting material, since no leaves or flowers were cut by ALBs during either of the field seasons. This finding eliminates any question of potential damage caused by ALB females foraging for nesting material.

In summer of 2003, only one single insect visit by an ALB was recorded. This ALB visit produced 79 pollen tubes in 41 of 48 styles of receptive florets (85.4%), indicating that ALB females may be efficient at pollination of *E. angustifolia* inflorescences. ALBs were recorded visiting inflorescences along transects set out in the field, but only for a short period (1 week) during the flowering season after the bees were released. Thereafter, the ALBs were no longer found on *E. angustifolia*. Initially it was thought that this problem arose because the bees were released several weeks after *E. angustifolia* had begun to flower and perhaps the field had progressed beyond the capacity to support 20,000 bees. In this case, the inflorescences would not provide an adequate source of pollen and nectar and the female ALBs would have to forage elsewhere. It was also thought that the bees were foraging in the tree margins because there were many cut leaves found along the margins. Bees were never seen there

however, but the presence of cut leaves suggests that leafcutter bees were visiting the area. These leaves may also have been cut by a native bee species like the sunflower leafcutter bee (*Megachile pugnata*), but these insects were not recorded on any of the transects or single insect visits that season. Later it was thought that competing flowers were responsible for the low number of bees on inflorescences of *E. angustifolia*, and similar results from 2004 support this hypothesis.

Due to a cold, wet spring in 2004, the flowering period of *E. angustifolia* fields was delayed by two weeks (July 15 to September 1 compared to June 27 to August 12, 2003) and the ALBs were released before *E. angustifolia* flowered. There were enough weedy flowers in these two fields to provision the *M. rotundata* populations before *E. angustifolia* flowered. These additional flowering species may have initiated the first problem. The bees acclimatized to foraging on plants other than *E. angustifolia*. In the summer of 2004, no SIVs by ALB were recorded and leafcutters were rarely seen on *E. angustifolia* inflorescences. All recorded ALBs on *E. angustifolia* inflorescences were resting and not foraging and this behaviour was observed 12 times on 33 transects.

Caging experiments showed initial promise with male and female ALBs visiting inflorescences and collecting pollen. For the first few days of the caged bee trials, pollen from SP florets was removed by the end of the afternoon indicating that ALBs were foraging on *E. angustifolia* inflorescences. However, nest cells were not initiated within the provided blocks and the ALBs eventually escaped from the cages. The unexpected disappearance of test inflorescences did not allow for any comparisons to be made.

At MVA in 2004, ALBs were followed to their forage flower of choice, alfalfa (*Medicago sativa*), and not to competing flowers in the forest like was hypothesised in 2003. The entire area owned by the Meewasin Valley Authority along the riverside had been rented to an alfalfa grower who planted the entire area to alfalfa. The alfalfa crop was harvested before *E. angustifolia* flowered but many alfalfa plants grew back and flowered. In 2003, likely it was these plants that competed with *E. angustifolia* for the ALBs attention and eventually drew all of the bees away. At the end of 2003, most of the volunteer alfalfa crop was treated with the herbicide glyphosate, (Roundup^{TM}), except for a thin row of plants (2-3 metres deep) that surrounded the *E. angustifolia*

field. The ALBs in 2004 foraged only on these volunteer alfalfa plants and ignored *E. angustifolia* completely. At VR, there were no alfalfa plants to distract ALBs but there were hundreds of yellow sweet clover (*Melilotus officinalis*) plants that held the attention of the majority of ALBs. Perennial sow thistle (*Sonchus arvensis*) grew heavily around the ALB hut and its yellow dandelion like flowers also attracted many leafcutter bees. Alfalfa leafcutter bees are apparently behaviourally hardwired to forage on alfalfa or other closely related legume plants. Placement of the leafcutter bee shelters and release of bees should have coincided with *E. angustifolia* flowering based on the onset of flowering observed in 2003 but the cold, wet spring delayed flowering by at least two weeks. In the meantime the ALBs foraged on alfalfa, yellow sweet clover and sow thistle and ignored *E. angustifolia* when it finally flowered. It would seem that *E. angustifolia* does not provide a great enough reward to overcome the strong attraction of ALB to legumes. The purple and pink colouration of *E. angustifolia* might also be less preferred by, or less visible to, ALBs. Osgood (1968) reported that although they can perceive purple and pink, ALBs receive the greatest stimulus from the colour yellow, so yellow flowers are more attractive to *M. rotundata*. Alfalfa leafcutter bees were shown to be attracted to only 21 of 209 species of flowers, concentrating on radially and bilaterally symmetrical flowers while ignoring radially asymmetrical flowers such as those of the Asteraceae (Small *et al*., 1997). Any of the few ALBs observed foraging on *E. angustifolia* were collecting pollen and not nectar. The palea of each floret may pose a physical barrier that prevents the female ALB from reaching inside the corolla tube to collect nectar. As *E. angustifolia* can only be grown as organic crops (no registered pesticides), then weeds will inevitably be present to compete for ALB pollination. It is concluded then that ALBs are not suitable as managed pollinators of *E. angustifolia* crops and introducing ALBs to an agricultural field will not result in an increase in *E. angustifolia* seed set but will only result in an increased seed set of weeds.

Chapter 5: GENERAL DISCUSSION AND CONCLUSION

5.1 Relevance of research conducted

Echinacea angustifolia and *E. purpurea* are crops of minor acreage in the prairie landscape but as specialty crops, have the potential to generate large profits for growers. Increasing acreages, replacing harvested crops and sales for profit are the main reasons why it is necessary to produce high quality *Echinacea* seed. To ensure that an adequate amount of seed is produced per year, the pollination ecologies of *E. angustifolia* and *E. purpurea* were investigated using both field and lab studies.

There have not been any previous studies on nectar production or nectary anatomy and ultrastructure in the genus *Echinacea*, and so this study is the first of its kind. Floret phenology and nectar-production dynamics were followed from the onset of nectar presentation in the indehiscent staminate phase (SPi) to the complete cessation of nectar presentation in the fourth day of the pistillate phase (PP4) in both *E. angustifolia* (field) and *E. purpurea* (growth chamber). Whorls of staminate (SP) and first-day pistillate (PP1) florets of both *E. angustifolia* and *E. purpurea* contained the greatest amount of nectar, and were thus highly attractive to insect visitors. Indeed, it was often observed that insect visitors to *Echinacea* inflorescences concentrated their foraging efforts on florets of these two whorls. Nectaries from several stages of disc floret development of both species of *Echinacea* were analyzed microscopically. It was determined that the most likely route of exit for secreted nectar was through modified stomata located randomly along the apical rim of both species. The ultrastructure of the nectary of *E. purpurea* was analyzed by transmission electron microscopy and this represents only the third ultrastructural study of nectaries in the family Asteraceae, which is surprising considering it is the largest family of plants and that many asteraceans are important to honey production. Notable features of nectary ultrastructure in *E. purpurea* were lobed nuclei, vascularization by phloem alone, evidence for an eccrine secretion mechanism with nectar following either an apoplastic

or symplastic route, and that specialized phloem cells occurred adjacent to the epidermis, which had a role in their ontogeny.

The literature suggests that *Echinacea* species must be cross-pollinated to set seed with insects implicated as pollen vectors, but it was unclear which insects were pollinating *E. angustifolia* in Saskatchewan. Evidence was presented to indicate that a limited amount of self-pollination may also occur in conjunction with cross pollination in both *E. angustifolia* and *E. purpurea*. Many insects were identified as visitors to *E. angustifolia* across two field seasons (2003, 2004) and these insects were evaluated based on their pollination efficiency, which was tested using several techniques.

Insects were completely excluded from one group of inflorescences and it was determined that in the absence of insect visitation, *E. angustifolia* produced achenes of low weight and very low germinability that would be useless to a commercial operation. A small amount of seed was set within cages that excluded insects, suggesting that other factors such as wind pollination may be contributing in small amounts to cross-pollination. The small amount of set seed in these trials may also indicate a breakdown in the sporophytic incompatibility system that functions to keep florets from self-pollinating. Insect visitation was therefore necessary to produce viable seed from a field of *E. angustifolia*. Small bodied insects, such as golden blister beetles (*Epicauta ferruginea*) and sweat bees (*Agapostemon* spp.), were capable of effecting cross-pollination to a lesser extent than the entire complement of insect pollinators on inflorescences within partial excluder cages. Results from partial excluder cages were not consistent across field sites or seasons, however, and may not have provided an accurate representation of pollination by small-bodied insects. Heavier achenes with greater germinability were produced by inflorescences where insects were allowed free access to florets. It was thus demonstrated conclusively that insect cross pollination is essential to the production of large amounts of viable seed with a good potential for germination.

A positive relationship was established between achene weights and their germination potential. Achenes that resulted from insect cross-pollination were twice as heavy on average as those produced by inflorescences excluded from insects, and these heavy achenes had better germination. This relationship between achene weight and

germination potential can help growers evaluate their seed for its germination potential before planting, by simply weighing a sub-sample of achenes. Through these methods of transect observations and achene weights and germinations, it became evident that there was a real possibility of a pollination deficit occurring in Saskatchewan on large agricultural plantings. Transect observations and single insect visit attempts at the Valley Road (VR) organic farm revealed that insects were not making frequent visits to *E. angustifolia* inflorescences. There were many insects within the confines of the field, and despite the thousands of available *E. angustifolia* inflorescences, most of these insects were visiting flowers of weedy plants. Germination of achenes from openly pollinated inflorescences at VR resulted in only 21% germination, suggesting that the scarce number of insects found visiting *E. angustifolia* were insufficient to effectively cross-pollinate the field. A similar situation occurred at the Prairie Plant Systems Inc. field site, where a low number of *E. angustifolia* plants created a pollen limitation situation where achene germination was also low. Pollen limitation did not occur at the Meewasin Valley Authority site, where there were many insects visiting inflorescences during both study years (2003, 2004), and achene weights and germinations were high for achenes from openly pollinated inflorescences.

Insect visitors to *E. angustifolia* inflorescences were identified using observations along a transect line on a set number of plants at various time intervals during the day. In this way it was hoped that all of the insect visitors to *E. angustifolia* could be recorded. Pollination of *E. angustifolia* was diurnal for the most part. There were no florets in the staminate phase during the night, suggesting that relatively few *E. angustifolia* pollen grains were available for insects to transfer. Any nocturnal visitors to *E. angustifolia* inflorescences may only contribute marginally to pollination by transferring residual pollen from the underside of reflexed PP1 stigmas. Late night transects and observations revealed only a few moths visiting inflorescences and their contributions to pollination may be negligible due to the paucity of pollen at night.

The pollination efficiency of frequent visitors was identified using pollen tube counts and shrivelled-style analysis following single insect visits to virgin inflorescences to directly evaluate the pollination potential of individual insects. Inflorescence visitors could then be identified as pollinators of *E. angustifolia* and ranked according to their

pollination efficiencies. Floral visitors such as bumble bees (*Bombus* spp.), butterflies (Nymphalidae, Pieridae, and Satyridae), honey bees (*Apis mellifera*), golden blister beetles (*Epicauta ferruginea*), and grasshopper bee flies (*Systoechus vulgaris*) were all identified as pollinators and together were responsible for the majority of *E. angustifolia* pollination at three field sites. Native leafcutter bees (*Megachile* spp.) visited *E. angustifolia* but were not efficient pollinators, whereas introduced leafcutter bees (*M. rotundata*) may have been efficient pollinators if they were consistent inflorescence visitors. The new technique of shrivelled-style analysis, used to more rapidly determine pollination efficiency, was modified from the recently developed measure of style persistence (Wagenius, 2004) to study *E. angustifolia* pollination. Observations of shrivelled versus persistent styles in an agricultural setting also has the potential to help growers identify if a pollination deficit is occurring in their fields so that they can remedy the situation by importing a managed pollinator.

Cross-pollination deficits seem likely when relying on native pollinators to cross-pollinate a field, based on evidence from the VR agricultural planting. Thus, a managed pollinator could be transported to the field to ensure that sufficient cross-pollination occurred. In Saskatchewan, there are two well developed managed insect pollinators: the European honey bee (*Apis mellifera*) and the alfalfa leafcutter bee (*Megachile rotundata*). With the goal in mind to reduce the reliance on honey bees for pollination duties, alfalfa leafcutter bees were released at two of the three *E. angustifolia* field sites. Initial behaviours of female bees on *E. angustifolia* inflorescences were promising as was the high number of pollen tubes started following an SIV by an ALB. Within a few days after field placement (2003), leafcutter bees were no longer seen foraging on *E. angustifolia* inflorescences. In 2004, it became clear that ALBs greatly preferred other flowers over *E. angustifolia*. Floral competition became an overwhelming factor with legume flowers, such as alfalfa (*Medicago sativum*) and yellow sweet clover (*Melilotus officinalis*), out competing *E. angustifolia* for ALB visits. Competition from flowers of weedy plant species will always be a factor in organic production of *E. angustifolia*, so *M. rotundata* may regularly find a more suitable flower on which to forage. The inflorescence of *E. angustifolia* was not attractive to the leafcutter bees for whatever reason, so it is highly unlikely that these bees can ever be adapted for managed

pollination of this or potentially any other asteracean crop. In regards to other managed pollinators, at the present time the only other widely available, managed pollinator on the Canadian prairies is the honey bee, *Apis mellifera*. Honey bees had excellent pollination efficiencies when they visited inflorescences of *E. angustifolia*, and presumably would be suitable as managed pollinators. Honey bees are used extensively in sunflower seed production and fields with honey bee colonies produced twice as much seed as fields isolated by distance from honey bees (Furgala, 1954). Nectar from *E. angustifolia* has a high carbohydrate concentration (approximately 61-63%) and sunflower cultivars producing nectar in the same percentage range as *E. angustifolia* (61% sugar concentration) attracted more honey bees and had greater seed yield than cultivars with lower nectar sugar concentrations (Burminstrove, 1965). Honey bee colonies could be placed in *E. angustifolia* fields to supplement the pollination activities of native insect pollinators with the added bonus of having "certified organic *Echinacea*" honey produced in hives. However, more research is necessary to determine whether a colony of honey bees can be supported on the nectar and pollen produced by a field of *E. angustifolia* and whether any of the reputed medicinal compounds are present in honey produced from *E. angustifolia* nectar.

5.2 Future research

This *Echinacea* project could progress into several other areas. A better understanding of the pollination ability of individual insects can be gained by capturing individual insects after a single insect visit and determining their pollen loads, identifying and documenting their pollinator behaviour, following insects in the field to determine fidelity to *Echinacea* and to understand the number of inflorescences visited by a single insect during the day. Individual insects could be captured and observed by light and scanning electron microscopy to see where on their bodies the majority of pollen grains of *E. angustifolia* was clustered. Gut contents or fecal samples from bee flies (*Systoechus vulgaris*) could be analyzed to truly identify whether or not they are consuming pollen. Observation of a single inflorescence in the field could indicate the entire diversity and numbers of visitors that an inflorescence receives over the course of a day or over the entirety of its flowering period. A shrivelled-style estimate of

pollination on a field level (as opposed to on an inflorescence level as utilized in this study), could be developed that would give *E. angustifolia* growers a method to assess whether sufficient pollination was occurring in their field. If insufficient pollination was occurring, many styles would remain persistently turgid in the crop and in this event, growers could take steps to increase insect numbers in the field to remedy the pollinator deficit. Further observations, including shrivelled-style analysis, at Valley Road and other agricultural sites would answer the question "Are current native insect levels sufficient to adequately pollinate the large numbers of inflorescences present in an agricultural field of *Echinacea angustifolia*?"

Determination of the pollinators of *E. purpurea* in Saskatchewan was only investigated at a moderate level by several observations made in 2005 in this study. A lack of *E. purpurea* within a suitable distance of Saskatoon and the accidental loss of the newly established *E. purpurea* field site behind the W.P. Thompson (Biology) Building between the 2003 and 2004 field seasons kept this portion of the study from being fully realized. A study on insect visitors to *E. purpurea* would still be interesting to undertake and this study could use the current knowledge of insect visitation to *E. angustifolia* as a comparison. A field study on the phenology of nectar production in disc florets of *E. purpurea* would be useful in order to compare field and growth chamber nectar dynamics.

While involved in pollination fieldwork it was observed that grasshoppers, which had reached severe economic thresholds in 2003, did not feed on leaves or stems of *E. angustifolia* plants. Further research uncovered that three compounds isolated from *E. angustifolia* had insecticidal effects: Echinacein (Jacobson, 1967), Echinolone (Jacobson, 1975), alkylamide (Soderlund and Knipple, 1995), which may explain why grasshoppers chose to feed on coloured marking tape before they would feed on *E. angustifolia* leaves. In a controlled trial, *Locusta migratoria* nymphs could not survive when offered only *E. angustifolia* plants as forage. Nymphs also chose wheat nearly exclusively over *E. angustifolia* plants in a two choice experiment. Third instar locusts in this choice experiment did however eat *E. angustifolia* plants when their wheat supply was exhausted. This study illuminates the potential of *E. angustifolia* plantings as border crops around high value crops that need protection from grasshopper feeding. An

E. angustifolia border may prevent the inward migration of newly hatched grasshopper nymphs from field margins into the interior of fields. As an added benefit, the long-lived inflorescences of *E. angustifolia* support the adult stages of the grasshopper bee fly (*Systoechus vulgaris*) and the golden blister beetle (*Epicauta ferruginea*) whose larvae are predators of grasshopper eggs.

LITERATURE CITED

Allen-Wardell, G., Bernhardt, P., Bitner, R., Burquez, A., Buchmann, J.C., Cox, P.A., Dalton, V., Feinsinger, P., Ingram, M., Inouye, D., Jones, C.E., Kennedy, K., Kevan, P., Koopowitz, H., Medellin, R., Medellin-Morales, S., Nabhan, G.P., Pavlik, B., Tepedino V., Torchio, P., Walker, S. 1998. The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. *Conservation Biology* **12**: 8-17.

Anderson MA, Cornish EC, Mau SL, Williams EG, Hoggart R, Atkinson A, Bonig I, Grego B, Simpson R, Roche R, Haley JD, Niall HD. Tregear GW, Coghlan JP, Crawford RJ, Clarke AE. 1986. Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. *Nature* **321**: 38-44.

Apsit VJ, Dixon PM. 2001. Genetic diversity and population structure in *Echinacea laevigata* (Boynton and Beadle) Blake, an endangered plant species. *Natural Areas Journal* **21**: 71-77.

Baker DA, Hall JL, Thorpe JR. 1978. A study of the extrafloral nectaries of *Ricinus communis***.** *New Phytologist* **81**: 129-137.

Baker HG, Baker I. 1983. A brief historical review of the chemistry of floral nectar. In: Bentley B, Thomas E, eds. *The biology of nectaries*. New York: Columbia University Press, 126-152.

Barl B. 1997. The potential for *Echinacea* production in Western Canada. In: *Proceedings of the Cropportunities IV Conference*, July 21-22, University of Saskatchewan, Saskatoon, Saskatchewan, p. 32-41.

Bauer R. 1998. The Echinacea story - the scientific development of an herbal immunostimulant. In: Prendergast HDV, Etkin NL, Harris DR, Haughton PJ, *Plants for food and medicine – modern treatment and traditional remedies*. The Royal Botanic Gardens: Kew, 317-332.

Beattie AJ, Turnbull C, Knox RB, Williams EG. 1984. Ant inhibition of pollen function: a possible reason why ant pollination is rare. *American Journal of Botany* **71**: 421-426.

Beardsell DV, Williams EG, Knox RB. 1989. The structure and histochemistry of the nectary and anther secretory tissue of the flowers of *Thryptomene calycina* (Lindl.) Stapf. (Myrtaceae). *Australian Journal of Botany* **37**: 65-80.

Belling J. 1921. On counting chromosomes in pollen-mother cells. *The American Naturalist* **55**: 573-574.

Belmonte E, Cardemil L, Kalin Arroyo MT. 1994. Floral nectary structure and nectar composition in *Eccremocarpus scaber* (Bignoniaceae), a hummingbird pollinated plant of central Chile. *American Journal of Botany* **81**: 493-503.

Binns SE, Baum BR, Arnason JT. 2002. A taxonomic revision of *Echinacea* (Asteraceae: Heliantheae). *Systematic Botany*: **27:** 610-632.

Block RJ, Durrum EL, Zweig G. 1958. *A manual of paper chromatography and paper electrophoresis*. Academic Press, New York. 2nd Ed.

Boch R. 1961. Honeybee activity on safflower (*Carthamus tinctorius* L.). *Canadian Journal of Plant Sciences* **41**: 559-562.

Bonnier G. 1879. Les nectaires - étude critique, anatomique et physiologique. *Annales de Sciences Naturelles Botanique* **8**: 5-212.

Bohart GE. 1972. Management of wild bees for the pollination of crops. *Annual Review of Entomology* **17**: 287-312.

Bratcher CB, Dole JM, Cole JC. 1993. Stratification improves seed germination of five native wildflower species. *HortScience* **28**: 899-901.

Brewbaker JL. 1957. Pollen cytology and self-incompatibility systems in plants. *Journal of Heredity* **48**: 271-277.

Brewbaker JL. 1967. The distribution and phylogenetic significance of bi nucleate and tri-nucleate pollen grains in the angiosperms. *American Journal of Botany* **54**: 1069-1083.

Buchmann SL, Nabhan GP. 1996. *The Forgotten Pollinators*. Washington D.C.: Shearwater Press.

Burminstrove AN. 1965. The melliferous value of some sunflower varieties. *20th International Apicultural Congress of Apimondia Bucharest*. Bucharest, Romania: Apimondia Publishing house, 320-323.

Búrquez A, Corbet SA. 1991. Do flowers reabsorb nectar? *Functional Ecology* **6**: 369-379.

Cane JH. 2001. Habitat fragmentation and native bees: a premature verdict? *Conservation Ecology* **5**: 3. Online as of 09/09/2005: http://www.consecol.org/vol5/iss1/art3.

Cane JH, Tepedino VJ. 2001. Causes and extent of declines among native North American invertebrate pollinators: detection, evidence and consequences. *Conservation Ecology* **5**: 1. Online as of 09/09/2005: http://www.consecol.org/vol5/iss1/art1.

Caspary JXR. 1848. De Nectariis. Commentationem botanicuam conscripsit. *Kayser Bucher Lexikon* **11/12**: 84.

Corbet SA. 1990. Pollination and the weather. *Israel Journal of Botany* **39**: 13-30.

Crane E. 1975. *Honey: A comprehensive survey*. London, England: William Heinemann Ltd.

Cruden RW, Hermann SM. 1983. Studying nectar? Some observations on the art. In: Bentley B, Thomas E, eds. *The biology of nectaries*. New York: Columbia University Press, 223-241.

Curry PS. 1984. Bumblebees of Saskatchewan (Hymenoptera: Apidae): a survey of their geographic distribution. *Saskatchewan Museum of Natural History Contribution*. **5**: 1-44.

Davis AR. 1992 *a*. Physiological and structural aspects of floral nectar secretion. Ph.D thesis, Australian National University, Canberra.

Davis AR. 1992 *b*. Evaluating honeybees as pollinators of virgin flowers of *Echium plantagineum* L. (Boraginaceae) by pollen tube fluorescence. *Journal of Apicultural Research* **31**: 83-95.

Davis AR. 1997. Pollination efficiency of insects. In: Shivanna KR, Sawhney VK, eds. *Pollen biotechnology for crop production and improvement*. New York: Cambridge University Press, 87-120.

Davis AR, Gunning BES. 1992. The modified stomata of the floral nectary of *Vicia faba* L. Development, anatomy and ultrastructure. *Protoplasma* **166**: 134-152.

Davis AR, Gunning BES. 1993. The modified stomata of the floral nectary of *Vicia faba* L. 3: Physiological aspects, including comparisons with foliar stomata. *Botanica Acta* **106**: 241-253.

Davis AR, Fowke LC, Sawhney VK, Low NH. 1996. Floral nectar secretion and ploidy in *Brassica rapa* and *Brassica napus* (Brassicaceae). II. Quantified variability of nectary structure and function in rapid-cycling lines. *Annals of Botany* **77**: 223-234.

Davis AR, Peterson RL, Shuel RW. 1986. Anatomy and vasculature of the floral nectaries of *Brassica napus* (Brassicaceae). *Canadian Journal of Botany* **64**: 2508-2516.

Davis AR, Peterson RL, Shuel RW. 1988. Vasculature and ultrastructure of the floral and stipular nectaries of *Vicia faba* (Leguminosae). *Canadian Journal of Botany* **66**: 1435-1448.

Davis AR, Pylatuik JD, Paradis JC, Low NH. 1998. Nectar-carbohydrate production and composition vary in relation to nectary anatomy and location within individual flowers of several species of Brassicaceae. *Planta* **205**: 305-318.

De Jong J, Kho YO. 1982. The shrivelling of pollinated pistils as an aid to rapid determination of Chrysanthemum pollen viability. *Euphytica* **31**: 519-521.

Deyrup MA. 1988. Pollen-feeding in *Peocilognathus punctipennis* (Diptera: Bombyliidae). *Florida Entomologist* **71**: 597-604.

Doebler SA. 2000. The rise and fall of the honeybee. *Bioscience* **50**: 738-740.

Drew MB. 1991. *The role of Tennessee coneflower, Echinacea tennesseensis, in its native habitat: the vegetation and a demographic analysis*. MSc thesis. University of Tennessee, Knoxville TN U.S.A.

Durkee LT. 1983*a*. Protein-containing cells in the nectary phloem of *Passiflora warmingii*. *American Journal of Botany* **70**: 1011-1018.

Durkee LT. 1983*b*. Ultrastructure of nectaries. In: Bentley B, Thomas E. eds. *The biology of nectaries.* Columbia University Press: New York, 1-29.

Durkee LT, Gaal DJ, Reisner WH. 1981. The floral and extra-floral nectaries of Passiflora. I. The floral nectary. *American Journal of Botany* **68:** 453-462.

Durkee LT, Haber MH, Dorn L, Remington A. 1999. Morphology, ultrastructure, and function of extrafloral nectaries in three species of Caesalpiniaceae. *Journal of the Iowa Academy of Science* **106**: 82-88.

Elleman CJ, Franklin-Tong V, Dickinson HG. 1992. Pollination in species with dry stigmas: the nature of the early stigmatic response and the pathway taken by pollen tubes. *New Phytologist* **121**: 413-424.

Evans EW, Smith CC, Gendron RP. 1989. Timing of reproduction in a prairie legume: seasonal impacts of insects consuming flowers and seeds. *Oecologia* **78**: 220-230.

Eriksson M. 1977. The ultrastructure of the nectary of red clover (*Trifolium pratense*). *Journal of Apicultural Research* **16**: 184-193.

Eymé J. 1963. Observations cytologiques sur les nectaires de trios Renonculaceés (*Helleborus foetidus* L, *H. niger* L. et *Nigella damascene* L.). *Le Botaniste* **46**: 137-179.

Eymé J. 1966. Infrastructure des cellules nectarigènes de *Diplotaxis erucoides* D.C., *Helleborus niger* L. et *H. foetidus* L. *Comptes rendus [Hebd. Seances] Acad. Sci. Ser. D. Nat. Sci.* **262**: 1629-1632.

Eymé J. 1967. Nouvelles observations sur l'infrastructure de tissus nectarigènes floraux. *Le Botaniste* **50**: 169-183.

Fahn A. 1979. *Secretory tissues in plants*. New York: Academic Press.

Fahn A, Benouaiche P. 1979. Ultrastructure, development and secretion in the nectary of banana flowers. *Annals of Botany* **44**: 85-93.

Fairey DT, Lefkovitch LP. 1991. Reproduction of *Megachile rotundata* Fab. foraging on *Trifolium* spp and *Brassica campestris*. The Sixth International Symposium on Pollination, Tilburg, the Netherlands, August 1990. *Acta Horticulturae* **288**: 185-189.

Figier J. 1971. Étude infrastructurale de la stipule de *Vicia faba* L. au niveau du nectaire. *Planta* **98**: 31-49.

Feghahati SMJ, Reese RN. 1994. Ethylene, light, and prechill-enhanced germination of *Echinacea angustifolia* seeds. *Journal of the American Society of Horticultural Science*. **119**: 853-858.

Free JB. 1964. The behaviour of honeybees on flowers (*Helianthus annuus* L.). *Journal of Applied Ecology* **1**: 19-27.

Free JB. 1993. *Insect pollination of crops, 2nd edn*. London: Harcourt Brace.

Free JB, Simpson J. 1964. The pollination requirements of sunflowers (*Helianthus annuus* L.). *Empirical Journal of Experimental Agriculture* **32**: 340-342.

Frei E. 1955. Die Innervierung der floralen Nektarien dikotyler Pflanzenfamilien. *Berichte der Schweizerischen Botanischen Gesellschaft* **65**: 60-114.

Freitas L, Paoli AAS. 1999. Structure and ultrastructure of the extrafloral nectaries of *Croton urucurana* Baill. (Euphorbiaceae). *Bol. Bot. Univ. São Paolo* **18**: 1-10.

Freund DE, Furgala B. 1982. Effect of pollination by insects on the seed set and yield of ten oilseed sunflower cultivars. *American Bee Journal* **122**: 648-652.

Frey-Wyssling A. 1955. The phloem supply to the nectaries. *Acta Botanica Neerlandica* **4**: 358-369.

Furgala B. 1954. Honey bees increase seed yields of cultivated sunflowers. *Gleanings in Bee Culture* **82**: 532-534.

Gaffal KP. Heimler W. and El-Gammal S. 1998. The floral nectary of *Digitalis purpurea* L., structure and nectar secretion. *Annals of Botany* **81**: 251-262.

Galetto L. 1995. Estudios sobre el nectar y los nectaries en *Hyaloseris rubicunda* y *Barnadesia odorata* (Asteraceae-Mutisieae). *Darwiniana* **33**: 127-133.

Galetto L, Bernardello G. 2003. Nectar sugar composition in angiosperms from Chaco and Patagonia (Argentina): An animal visitor's matter? *Plant Systematics and Evolution* **238**: 69-86.

Gillott C, Wist TJ, Wolfe J. 2003. Bee flies, blister beetles and the grasshopper connection. *Blue Jay* **61**: 214-217.

Goerzen DW. 2000. Alternative crops for alfalfa leafcutter bee pollination. *Spoke Program Research Reports, Agri-food Innovation Fund, Saskatchewan Agriculture*. **1997-2002**: 302-303.

Goodwillie C. 1999. Wind pollination and reproductive assurance in *Linanthus parviflorus* (Polemoniaceae), a self-incompatible annual. *American Journal of Botany* **86**: 948-954.

Gopinathan K, Varatharajan R. 1982. On the morphology, topography and the significance of stomata on floral nectaries of some Compositae. *Phytomorphology* **32:** 265-269.

Greenfield J, Davis JM. 2004. Narrow leaf purple coneflower (*Echinacea angustifolia* DC.). Medicinal Herb Production Guide, 1-5. Online as of 08/30/2005: http://www.naturalmedicinesofnc.org/Growers%20Guides/Ech.angust-gg.pdf

Gulyás S, Pesti J. 1966. Angaben zur Anatomie der Nektarien der Centaureae. *Acta Biologica Szeged* **12**: 17-23.

Gunning BES, Pate JS. 1969. Transfer cells: Plant cells with wall ingrowths, specialized in relation to short distance transport of solutes - their occurrence, structure, and development. *Protoplasma* **68**: 107-133.

Hadisoesilo S, Furgala B. 1986. The effect of cultivar, floral stage and time of day on the quantity and quality of nectar extracted from oilseed sunflower (*Helianthus annuus* L.) in Minnesota. *American Bee Journal* **122**: 648-652.

Harbage JF. 2001. Micropropagation of *Echinacea angustifolia*, *E. pallida*, and *E. purpurea* from stem and seed explants. *HortScience* **36**: 360-364.

Heil M, Rattke J, Boland W. 2005. Postsecretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. *Science* **308**: 560-563.

Hemmerly TE. 1976. *Life cycle strategy of a highly endemic cedar glade species: Echinacea tennesseensis (Compositae)*. PhD Thesis, Vanderbilt University Nashville, TN, U.S.A.

Heslop-Harrison Y, Shivanna KR. 1997. The receptive surface of the angiosperm stigma. *Annals of Botany* **41**: 1233-1258.

Hiscock SJ. 2000. Genetic control of self-incompatibility in *Senecio squalidus* L. (Asteraceae): a successful colonizing species. *Heredity* **85**: 10-19.

Hiscock SJ, Hoedemaekers K, Friedman WE, Dickenson HG. 2002. The stigma surface and pollen-stigma interactions in *Senecio squalidus* L. (Asteraceae) following cross (compatible) and self (incompatible) pollinations. *International Journal of Plant Science* **163**: 1-16.

Hiscock SJ, McInnis SM, Ralph DA, Henderson CA, Brennan AC. 2003. Sporophytic self-incompatibility in *Senecio squalidus* L. (Asteraceae) – the search for S. *Journal of Experimental Botany* **54**: 169-174.

Horner HT, Healy RA, Cervantes – Martinez T, Palmer RG. 2003. Floral nectary fine structure and development in *Glycine max* L. (Fabaceae). *International Journal of Plant Science* **164**: 675-690.

Inouye DW, Gill DE, Dudash MR, Fenster CB. 1994. A model and lexicon for pollen fate. *American Journal of Botany* **81**: 1517-1530.

Jacobsen M. 1967. The structure of Echinaceaein, the insecticidal component of American coneflower roots. *Journal of Organic Chemistry* **32**: 1646-1647.

Jacobsen M. 1975. Naturally occurring insect growth regulators III. Echinolone, a highly active juvenile hormone mimic from *Echinacea angustifolia* roots. *Lloydia* **38**: 473-476.

Johnson SD, Midgley JJ. 1997. Fly pollination of *Gorteria diffusa* (Asteraceae), and a possible mimetic function for dark spots on the capitulum. *American Journal of Botany* **84**: 429-436.

Kartashova NN. 1965. Stroeniei funcktsiya nektarnikov tsvetkadvudol'nykh rastenii. Istadel'stvo Tomskogo Universiteta, Tomsk.

Kearns CA, Inouye DW. 1993. *Techniques for pollination biologists*. Colorado U.S.A.: University Press.

Kearns CA, Inouye DW, Waser NM. 1998. Endangered mutualisms: the conservation of plant-pollinator interactions. *Annual Review Ecological Systematics* **29**: 83 112.

Kevan PG. 1997. Pollination biology and plant breeding systems. In: Shivanna KR, Sawhney VK, eds. *Pollen biotechnology for crop production and improvement*. Cambridge University Press, 59-83.

Kindscher K. 1989. Ethnobotany of purple coneflower (*Echinacea angustifolia*, Asteraceae) and other *Echinacea* species. *Economic Botany* **43**: 498-507.

Koteyava NK, Vassilyev AE, Tarlyn N, Franceschi VR. 2005. On mechanisms of nectar secretion. Abstract 10.3.4, XVII International Botanical Congress, Vienna, Austria. p. 162.

Kowyama Y, Tsuchiya T, Kukeda K. 2000. Sporophytic self-incompatibility in *Ipomoea trifida*, a close relative of sweet potato. *Annals of Botany* **85** (Supplement A): 191-196.

Kroll U. 1961. The influence of fertilization on the production of pyrethrins in the pyrethrum flower. *Pyrethrum Post* **6:** 19-21.

Kronestedt EC, Robards AW. 1987. Sugar secretion from the nectary of *Strelitzia*: an ultrastructural and physiological study. *Protoplasma* **137**: 168-182.

Kronestedt - Robards EC, Robards AW. 1991. Exocytosis in gland cells. In: Hawes CR, Coleman JOD, Evans DE, eds. *Endocytosis, exocytosis and vesicle traffic in plants*. Cambridge University Press, 199-232.

Kunin WE. 1993. Sex and the single mustard: population density and pollinator behaviour effects on seed-set. *Ecology* **74**: 2145-2160.

Ladd PG. 1994. Pollen presenters in the flowering plants – form and function. *Biological Journal of the Linnean Society* **115**: 165-195.

Langenberger MW, Davis AR. 2002. Temporal changes in floral nectar production, reabsorption, and composition associated with dichogamy in annual caraway (*Carum carvi*; Apiaceae). *American Journal of Botany* **89**: 1588-1598.

Langridge DF, Goodman RD. 1974. A study on pollination of sunflowers (*Helianthus annuus*). *Australian Journal of Experimental Agriculture and Animal Husbandry* **14**: 201-204.

Langridge DF, Goodman RD. 1981. Honey bee pollination of sunflower cultivars Hysun 30 and Sunfola. *Australian Journal of Experimental Agriculture and Animal Husbandry* **21**: 435-438.

Leuszler HK, Tepedino VJ, Alston DG. 1996. Reproductive biology of purple coneflower in southwestern North Dakota. *The Prairie Naturalist* **28**: 91-102.

Levin MD. 1983. Value of bee pollination to U.S. agriculture. *Bulletin of the Entomological Society of America* **29**: 50-51.

Lone Wolf Herb Resource. 2005. Online as of 08/30/2005: http://www.lonewolfherbdir.com/wolf_main.html.

Ma H, Xiao AJ, Cao R. 2002. Developmental and anatomic studies on the floral nectaries of *Tugarinovia mongolica*. *Acta Botanica Yunnanica* **24**: 638-644.

MacKenzie K, Rogers D, Javorek S. 1997. The alfalfa leafcutter bee, *Megachile rotundata* Fabr.: an alternative managed pollinator of lowbush blueberry. ISHS *Acta Horticulturae* **446**: Online as of 09/09/2005: http://www.actahort.org/books/446/446_11.htm

Mani MS, Saravanan JM. 1999. *Pollination ecology and evolution in Compositae (Asteraceae).* U.S.A: Science Publishers Inc.

Manitoba Agriculture, Food and Rural Initiatives. 2001. An overview of *Echinacea* production in Manitoba. Online as of 08/30/2005: http://www.gov.mb.ca/agriculture/crops/medicinal/bkq00s12.html

Martin FW. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* **34**: 125-128.

Masierowska ML, Stpiczynska M. 2005. Nectar resorption in flowers of *Sinapis alba* L., Brassicaceae and *Platanthera chlorantha* Custer (Rchb.), Orchidaceae. Abstract PO383. XVII International Botanical Congress, Vienna, Austria. p. 300.

McGregor RL. 1968. The taxonomy of the genus *Echinacea* (Compositae). *The University of Kansas Science Bulletin* **48**: 113-142.

McGregor SE. 1976. *Insect pollination of cultivated crops*. USDA Agricultural Handbook No. 496. Washington D.C.

McKenna M, Thomson JD. 1988. A technique for sampling and measuring small amounts of floral nectar. *Ecology* **69**: 1036-1037.

McKeown KA. 1999. A review of the taxonomy of the genus *Echinacea*. In: Janick, J, ed. *Perspectives on new crops and new uses*. ASHS Press, Alexandria, VA: 482-489.

Nepi M, Ciampolini F, Pacini E. 1996. Development and ultrastructure of *Cucurbita pepo* nectaries of male flowers. *Annals of Botany* **78**: 95-104.

Nepi M, Pacini E, Willemse MTM. 1996. Nectary biology of *Cucurbita pepo*: ecophysiological aspects. *Acta Botanica Neerlandica.* **45**: 41-54.

Nichol P, Hall JL. 1988. Characteristics of nectar secretion by the extrafloral nectaries of *Ricinus communis*. *Journal of Experimental Botany* **39**: 573-586.

Nicolson SW. 1995. Direct demonstration of nectar reabsorption in the flowers of *Grevillea robusta* (Proteaceae). *Functional Ecology* **9**: 584-588.

Osgood CE. 1968. Mechanisms of orientation in the leaf-cutter bee, *Megachile rotundata*. Ph.D thesis, Oregon State University, Corvallis.

Pacini E. 1996. Tapetum types in the Compositae: forms and function. In: Hind DJN, Beentje HJ, eds. *Proceedings of the International Compositae conference,* Royal Botanic Gardens, Kew, 21-28.

Pacini E, Nepi M, Vesprini JL. 2003. Nectar biodiversity: a short review. *Plant Systematics and Evolution*: **238** 7-21.

Parker FD, Frohlich DR. 1983. Hybrid sunflower pollination by a manageable composite specialist: the sunflower leafcutter bee. *Environmental Entomology* **12**: 576-581.

Parker FD, Frohlich DR. 1985. Studies on management of the sunflower leafcutter bee *Eumegachile pugnata* (Say) (Hymenoptera: Megachilidae). *Journal of Apicultural Research* **24**: 125-131.

Pate JS, Gunning BES. 1972. Transfer cells. *Annual Review of Plant Physiology*. **23**: 173-196.

Pederson MW, Lefevre CW, Wiebe HH. 1958. Absorption of C^{14} labelled sucrose by alfalfa nectaries. *Science* **127**: 758-759.

Percival MS. 1961. Types of nectar in angiosperms. *New Phytologist* **60**: 235-281.

Perrin A, Zandonella P. 1971. Presence d'invagination nucleaires dans les cellules de quelques nectaires floraux et hydathodes. *Planta* **96**: 136-144.

Porter B. 2003. *Echinacea* production in Saskatchewan. Saskatchewan Agriculture and Food product information document. Online as of 08/30/2005: http://www.agr.gov.sk.ca/docs/crops/special_crops/Production_Infomation/Echiacea.asp

Proctor M, Yeo P, Lack A. 1996. *The natural history of pollination*. London: Collins New Naturalist.

Radice S, Galati BG. 2003. Floral nectary ultrastructure of *Prunus persica* (L.) Batch cv. *Forastero* (Newcomer), an Argentine peach. *Plant Systematics and Evolution* **238**: 23-32.

Razem FA, Davis AR. 1999. Anatomical and ultrastructural changes of the floral nectaryof *Pisum sativum* L. during flower development. *Protoplasma* **206**: 57-72.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of cell biology* **17**: 208.

Richards AJ. 2001. Does low biodiversity resulting from modern agricultural practice affect crop pollination and yield? *Annals of Botany* **88**: 165-172.

Richards KW. 1984. Alfalfa leafcutter bee management in Western Canada. Agriculture Canada Publication 1495. Ottawa, Ontario.

Sammataro D, Erickson EH, Garment MB. 1985. Ultrastructure of the sunflower nectary. *Journal of Apicultural Research* **24**: 150-160.

Sanchez SM. 1977. The fine structure of the guard cells of *Helianthus annuus*. *American Journal of Botany* **64**: 814-824.

Sancho G, Otegui M. 2000. Vascularization and secretory tissues in florets of *Gochnatia polymorpha* (Asteraceae, Mutisieae): evolutionary considerations. *Phytomorphology* **50**: 172-179.

Saskatchewan, Agriculture, Food and Rural Revitalization Web page. 2002. Online as of 08/30/2005: http://www.agr.gov.sk.ca/docs/processing/nutraceuticals/Nutraceuticals.asp

Schnepf E, Deichgräber G. 1984. Electron microscopical studies of nectaries of some *Euphorbia* species. Akademie der Wissenschaften und der Literatur, Mainz. *Tropische und subtropische Pflanzenwelt* **45**: 55-93.

Schulthess BH, Giger E. Baumann TW. 1991. *Echinacea*: Anatomy, phytochemical pattern, and germination of the achene. *Planta Med*. **57**: 384-388.

Scott-Dupree CD, Winston ML. 1987. Wild bee pollinator diversity and abundance in orchard and uncultivated habitats in the Okanagan Valley, British Columbia. *Canadian Entomologist* **119**: 735-745.

Shuel RW. 1961. Influence of reproductive organs on secretion of sugars in flowers of *Streptosolen jamesonii* Miers. *Plant Physiology* **36**: 265-271.

Shuel RW. 1989. Improving honey production through plant breeding. *Bee World* **70**: 36-45.

Small E, Brookes B, Lefkovitch LP, Fairey DT. 1997. A preliminary analysis of the floral preference of the alfalfa leafcutter bee, *Megachile rotundata*. *Canadian Field-Naturalist* **111**: 445-453.

Soderlund DM, Knipple DC. 1995. Actions of insecticides on sodium channels: multiple target sites and site-specific resistance. In: Clark JM, ed. *Molecular action of insecticides on sodium channels*. American Chemical Society Symposium Series S91, 97-108.

Soroka JJ, Goerzen DW, Falk KC, Bett KE. 2001. Alfalfa leafcutter bee (Hymenoptera: Megachilidae) pollination of oilseed rape (*Brassica napus* L.) under isolation tents for hybrid seed production. *Canadian Journal of Plant Science* **81**: 199-204.

Souza-Silva M, Fontenello JCR, Martins RP. 2001. Seasonal abundance and species composition of flower visiting flies. *Neotropical Entomology* **30**: 351-359.

Stpiczynska M. 2003*a*. Nectar reabsorption in the spur of *Platanthera chlorantha* Custer (Rchb.) Orchidaceae: Structural and microautoradiographic study. *Plant Systematics and Evolution* **238**: 119-126.

Stpiczynska M. 2003*b*. Incorporation of (^{3}H) sucrose after the reabsorption of nectar from the spur of *Platanthera chlorantha* (Custer) Rchb. *Canadian Journal of Botany* **81**: 927-932.

Swan LA, Papp CS. 1972. *The Common Insects of North America*. New York: Harper and Row.

Tacina F. 1974. Observatii morfoanatomic comparative asupra glandelor nectarifere de la unele soiuri de floarea-soarelui. *Studii si Cercet Ari de Biologie*. **26**: 3-7.

Tacina F. 1979. Corelatia dintre ultrastructura celulelor nectarifere si secretia de nectar la floarea-soarelui. *Apicultura in Romania* **54**: 15-19.

Tasei JN, Delaude A. 1984. Efficacité pollinisatrice de *Megachile rotundata* F. (Hymenoptera: Megachilidae) utilisée sur luzerne (*Medicago sativa* L.). *Agronomie* **4**: 653-662.

Tepedino VJ, Parker FD. 1982. Interspecific differences in the relative importance of pollen and nectar to bee species foraging on sunflowers. *Environmental Entomology* **11**: 246-250.

Torres C, Galetto L. 2002. Are nectar sugar composition and corolla tube length related to the diversity of insects that visit Asteraceae flowers? *Plant Biology* **4**: 360 366.

Turgeon R, Medville R, Nixon KC. 2001. The evolution of minor vein phloem and phloem loading. *American Journal of Botany* **88**: 1331-1339.

Vassilyev AE. 1971. New data on the ultrastructure of the cells of the flower nectary. *Akad Nauk SSSR Botanical Journal* **56**: 1292-1306 (In Russian: English summary).

Vear F, Pham-Delegue M, Tourvielle de Labrouhe D, Marileau R, Loublier Y, le Métayer M, Douault P, Philippon JP. 1990. Genetical studies of nectar and pollen production in sunflower. *Agronomie* **10**: 219-231.

Vithanage HIMV, Knox RB. 1977. Development and cytochemistry of stigma surface response to self and foreign pollination in *Helianthus annuus*. *Phytomorpholgy* **27**: 168-179.

Vogel S. 1998. Remarkable nectaries: structure, ecology, organophyletic perspectives. IV. Miscellaneous cases. *Flora* **193**: 225-248.

Wagenius S. 2004. Style persistence, pollen limitation, and seed set in the common prairie plant *Echinacea angustifolia* (Asteraceae). *International Journal of Plant Science* **165**: 595-603.

Wagner, H. 1999. Phytomedicine in Germany. *Environmental Health Perspectives*. **107**: 779-781.

Walck JL, Hemmerly TE, Hidayati SN. 2001. The endangered purple coneflower, *Echinacea tennesseensis* (Asteraceae): its ecology and conservation. *Native Plants Journal* **3**: 54-64.

Warakomska Z, Kolasa Z. 2003. The flowering biology and apicultural value of coltsfoot (*Tussilago farfara* L.f. Asteraceae). *Annals Universitatis Mariae Curie-Sklodowska* **58**: 1-8.

Watanabe ME. 1994. Pollination worries rise as honey bees decline. *Science* **265**: 1170.

Weberling F. 1989. *Morphology of flowers and inflorescences*. Cambridge: Cambridge University Press.

Webster BD, Ross DM, Evans T. 1982. Nectar and the nectary of *Phaseolus vulgaris*. *Journal of the American Society for Horticultural Science* **107**: 497-503.

Westerkamp C. 1991. Honey bees are poor pollinators- why? *Plant Systematics and Evolution* **177**: 71-75.

Winston ML, Scott CD. 1984. The value of bee pollination to Canadian apiculture. *Canadian Beekeeper* **11**: 134.

Woltering E, de Vrije T, Harren F, Hoekstra F. 1997. Pollination and stigma wounding: same response, different signal? *Journal of Experimental Botany* **48:** 1027-1033.

Younus M, Chen W, Mirza M. 1997. Germination of *Echinacea*. Alberta Agriculture, Food and Rural Development handbook. CCN#97-GP-1.

Zandonella P. 1967. Stomates des nectaries floraux chez les Centrospermales. *Bulletin Societé Botanique du France* **114**: 11-20.

Zandonella P. 1970*a*. Infrastructure des cellules du tissu nectarigène floral de quelques Caryophyllaceae. C.R. Acad. Sci. Paris, Series D: 270: 1310-1313.

Zandonella P. 1970*b*. Infrastructure du tissu nectarigène floral de *Beta vulgaris* L.: le vacuome et la dégradation du cytoplasme dans les vacuoles. *Comptes Rendus Acad. Sc. Paris, Série D* **271**: 70-73.

Zimmermann JG. 1932. Uber die extrafloralen Nektarien der Angiospermen. *Beihefte zum Botanischen Centralblatt* **49**: 99-196.

Zimmermann MH, Ziegler H. 1975. List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmermann MH, Milburn JA, eds. *Encyclopedia of Plant Physiology: Transport in Plants I – Phloem Transport*. New York: Springer, 480-503.