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Male and female interaction in apple: Pollen tube growth, fruit set, fruit quality, and return bloom

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Date

MALE AND FEMALE INTERACTION IN APPLE: POLLEN TUBE GROWTH,
FRUIT SET, FRUIT QUALITY, AND RETURN BLOOM

A Thesis

Submitted to the Faculty

of

Purdue University

by

Khalil Rahman Jahed

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West Lafayette, Indiana

To my parents, Alhaj Abdul Jalil Wardak and Alhaj Fatemah Wardak, the very humble and frank but tremendously ingenious, cleverness and adventurous nation of my beloved country Afghanistan, and subsequently to the champions of horticulture.

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ABSTRACT

Jahed, Khalil Rahman. M.S., Purdue University, May 2015. Male and Female Interaction in Apple: Pollen Tubes Growth, Fruit Set, Fruit Quality and Return Bloom. Major Professor: Peter M Hirst.

In apple, adequate and appropriate pollination and fertilization is required for fruit set, fruit quality and subsequent fruit growth. Pollen source, pollen-style interaction and compatibility, and ample pollen tube growth are potentially highly influential factors on the fertilization and fruit setting process. Pollinizer is considered to be one of the influential factors and has a remarkable impact on fertilization. However, basic information on the level of pollinizer compatibility and its contribution to yield is lacking for many commercial apple cultivars. Hence, we conducted these experiments to compare pollinizers in terms of pollen tube growth, fruit set, fruit quality and return bloom. Honeycrisp, Gala, and Fuji cultivars were hand-pollinated by Crabapple, Red Delicious or Golden Delicious pollen. Pollen source had a significant influence on pollen tube growth and pollen tube enrichment in to the base of the style. Golden Delicious pollen had the highest and fastest growth followed by Red Delicious and Crabapple. Crabapple was not an effective pollinizer for Honeycrisp resulting in low fruit set, but both Red Delicious and Golden Delicious were adequate pollinizers of Honeycrisp apples. Pollen tube growth increased overtime after pollination and generally reached the base of the style 96 hours after pollination.

Fruit quality attributes and return bloom were generally not affected by pollen source. However, Crabapple pollen resulted in the lowest number of seeds per fruit in all cultivars. Seed number was positively correlated with Gala and Honeycrisp fruit fresh weight regardless of the pollen source. A significant positive correlation was found between pistil number and seed number indicating that reducing pistil number is an effective experimental tool to regulate seed number. The percent return boom was dramatically decreased with increasing individual fruit fresh weight. Likewise, percent return boom was reduced with increasing seed number per fruit. These results suggest that pollen source and seed number per fruit influence fruit set, fruit quality, and biennial bearing potential of Honeycrisp. This has real world implications for orchard design. Based on our findings, we recommend growers to do not plant Ralph-Shay or *Malus floribunda* Crabapples as pollinizers for Honeycrisp.

CHAPTER 1. LITERAURE REVIEW

1.1 The Biology of Pollination

1.1.1 Introduction

Pollination is the process in which pollen is transferred from the male reproductive parts of a plant (anthers) to female reproductive parts (stigma), which normally results in fertilization of the ovule (Raghavan, 2006). Pollination is one of the most important processes of fruit set, fruit growth, fruit quality, and development of seeded plants.

The mature pollen of seeded plants consists of a larger vegetative cell and a smaller generative cell (Land, 1907; Friedman, 1990; McCormick, 1993; Raghavan, 2006). Soon after pollination, the pollen hydrates and germinates on the stigmatic surface. The germination rate of pollen on the stigmatic surface increases with increasing temperature (Yoder *et al.*, 2009). Upon pollen germination on the stigmatic surface, the generative cell containing two sperm cells grow down the style where the larger vegetative cell provides food and creates an easier pathway for the sperm cells.

One of the two delivered sperm cells fertilize the egg, resulting of the embryo sac which is the beginning of the sporophyte; while the second sperm cell fuses with the two polar nuclei, leading to the formation of endosperm that surrounds and nurtures the developing embryo. This process is called double fertilization (Berger *et al.*, 2008; Drews and Koltunow, 2011; Dresselhaus and Snell, 2014).

Apple (*Malus x domestica* Borkh) expresses a self-incompatible system, leading to a reduction in self-fertilization and self-fruitfulness by arresting growth of the self-pollen tubes within the style (Broothaerts *et al.*, 2002). Fruit set upon self-pollination is reported to be 0% (Yoder *et al.*, 2009). For satisfactory cropping, at least two cross-compatible cultivars are required in an orchard. Kobel *et al.* (1939) reported these phenomena a multi-allelic gametophytic called S-locus. Information on the S-genotype of different cultivars could be used to improve cross-pollination and better design commercial orchards (Broothaerts *et al.*, 2002).

During meiosis, a large number of both male and female gametophytes are produced, but only a small proportion become fertilized (Stephenson 1981; Rigney 1995). However, some autotetraploid cultivars show self-fertility where the pollen and pistil alleles are compatible, but they grow significantly slower than compatible cross-pollinated cultivars (Adachi *et al.* 2009).

1.1.2 Plants reproductive systems

1.1.2.1 Male gametophyte growth, development, and functions

In flowering plants, male gametophyte development requires the formation of the stamen, consisting the differentiation of anther from single - achesporial cells (Scott *et al.*, 2004 & Hong, 2005). Generally, most plant organs derive from meristems, the undifferentiated and complex population of cells; whereas anther unusually derives from single-achesporial cells. Adaxial and abaxial polarity are the key stages in this process. Cell types are specified in the former and radially symmetrical microsporangia constituted in the later, respectively (Scott *et al.*, 2004).

The floral meristem of *Arabidopsis* is composed of three histogenic layers of cells with separate lineages: layer 1 is the epidermis, layer 2 is the sub-epidermis and layer 3 is the core. Stamen primordia are initiated from layer 2 where layer 3 contributes vasculature and sometimes to the connective tissues (Jenik and Irish, 2000). During anther primordium development, cells of the layer 2 undergo a complex series of divisions leading to the formation of four radially symmetrical microsporangia, and comprising of tissues where they finally will be linked to the filament (Scott *et al.*, 2004).

Formation of male gametophytes, also called pollen grains or microgametophytes, occurred in two distinct sequential phases: microsporogenesis and microgametogenesis. In microsporogenesis, the division of a diploid sporophytic cell results in the tapeta initial and the sporogenous initial called the mother cell. Microsporogenesis concludes when the initial sporogenous cell undergoes meiosis, resulting in a tetrad haploid cell that is released by the reaction of the callase enzyme which is produced by the tapetum layer of the anther (Scott *et al.*, 2004; Michael *et al.*, 2009). During microgametogenesis, these uninucleate microspores undergoes a two stage asymmetric mitotic division. The first mitotic division is known as Pollen Mitosis I (PMI), ensuing in a pollen grain consisting of a larger vegetative cell and a smaller generative cell. The generative cell, comprising of a condensed nuclear chromatin, is enclosed entirely within the vegetative cell that mostly nurtures the generative cell. The second mitotic division of the generative cell, called Pollen Mitosis II (PMII), gives two sperm cells, but occurs differently in different plants. Most plant families release pollen grains in a bicellular state, having both vegetative and generative cell. In these plants, the second mitotic division occurs while the pollen tube grows through the female pistil (McCormick, 1993; Michael *et al.*, 2009).

The mature pollen is released when the anther dehisces and pollination has occurred (Hong, 2005). Other plant families shed tricellular pollen, the second mitotic division occurs prior to anthesis (McCormick, 1993).

The vegetative cell does not undergo the second mitotic division, which is closely associated with generative cells and sperm cells. Numerous studies have concluded that the adjacent surfaces of vegetative cells to generative cells have more nuclear pores than the opposite surface, determining close communication between vegetative and generative cells (McCormick, 1993).

In many plants, it is difficult to identify all the important components of male gametophytic meiosis presumably due to its synchronous division within an anther which is the cytoplasmic connection between the mother cells (McCormick, 1993). However, the functional specialization and microspore and pollen grain simplistic isolation are considered to be the key factors of the evolutionary success of flowering plants (Honys & Twell, 2004). Meanwhile, meiotic mutation can help delineate some of these important constituents including mutations that affect entry into meiosis, chromosome synapsis, recombination, spindle formation, stamen identity, promoting anther dehiscence, and regulating anther cell division and differentiation (McCormick, 1993; Hong, 2005). A series of expressed genes control floral structure. Based on microarray datasets, a total of 5000-7000 genes are expressed in mature pollen whereas this number up to 14000 throughout male gametophyte development (Twell *et al.*, 2006). For instance, the ABC class genes and SEP genes control floral organ identities and continue to be expressed during stamen development (Jack, 2001; Hong, 2005).

Upon meiosis completion, pollen cell wall synthesis begins. The mature pollen cell wall is composed of two layers; an inner pectocellulosic intine and an outer sporopollenin-based exine, a highly degradation-resistant substance. The exine itself is composed of two layers, the inner nexine and the outer sexine, where the latter is very complex and provides most of the species-specific variation in pollen wall (McCormick, 1993; Scott *et al.*, 2004). Microsporocytes and tapetal cells share many developmental pathways during pollen wall formation where it is believed that the tapetal likely only provides nutrition for pollen wall formation. Additionally, tapetal contributes to a lipid-rich exine layer in many species (Scott *et al.*, 2004). The ephemeral callose wall layers form first followed by the primexine (a precursor of the sexine), nexine, and finally the inner intine. Primexine is apparently an accumulation of sporopollenin, the main structural component of pollen wall and is mainly composed of polysaccharides. Soon after the development of the pollen grain, anther dehiscence occurs which begins with the degeneration of the middle layer and tapetum that finally results in the release of mature pollen.

1.1.2.2 Female gametophyte growth, development and functions

Angiosperms are heterosporous and produce two types of spores which then develop into two types of unisexual gametophytes: megaspore and microspore. These unisexual gametophytes then undergo two developmental phases: the microspore undergoes microsporogenesis followed by microgametogenesis. The diploid microspore undergoes meiosis and gives rise to haploid microspores during microsporogenesis. These then develop into male gametophytes during microgametogenesis. Megaspores

undergo megasporogenesis followed by megagametogenesis, where the diploid megaspore undergoes meiosis during megasporogenesis and gives rise to haploid megaspores. Subsequently the megaspore develops into female gametophytes during megagametogenesis (Drews and Yadegari, 2002; Yadegari and Drews, 2004; Drews and Koltunow, 2011).

The complex structures of ovules are developed from the placenta as a group of meristematic cells (Angenent and Colombo, 1996), which are the precursors of seeds (Colombo *et al.*, 2008). The completely developed female reproductive structure of the plant is composed of three fundamental elements: the funiculus which attaches the ovule to the placenta; the chalaza, which forms integuments; and the nucellus, which is covered by integument where the megaspore mother cell differentiates to form the embryo sac (Colombo *et al.*, 2008), and nurtures the developing embryo (Pallardy, 2010). In some studies, the embryo sac is considered a fourth constituent of the female gametophyte (Shi and Yang, 2011). The development of the placenta and ovules vary among species. In *Petunia* and rice, placenta and ovules arise directly from the inner part of the floral meristem, whereas in *Arabidopsis*, both placenta and ovules develop from the inner ovary wall. The ovule development process has been characterized by proximal-distal symmetry in the early developmental stages and by an adaxial-abaxial polarity, similar to the male gametophyte, at the time of integument differentiation and elongation. The correct switch from proximal-distal symmetry into adaxial-abaxial polarity is an important step. In *Arabidopsis*, this switch has been determined by the initiation of the outer integument on the abaxial side and expression of *Inner No Outer (INO)*, a gene

essential for outer integument development (Villanueva, *et al.*, 1999; Balasubramanian, and Schneitz, 2000).

The integument, a protective wall of the ovule that surrounds the nucellus and eventually develops into the seed coat, gives originates from the chalaza. Some studies have proposed the integument as an analogous structure to leaves by sharing some identical morphological, developmental and genetic features (Shi and Yang, 2011). Most basal angiosperms have two integuments and several genes including *AINTEGUMENTA* (*ANT*), and *WUSCHEL* (*WUS*) (Colombo *et al.*, 2008), *BELLI* (*BEL1*) and *INNER NO OUTER* (*INO*) (Shi and Yang, 2011). These genes have essential roles in the initiation of the integument of *Arabidopsis*. The closest region of the ovule to the placenta will develop into funiculus that connects ovule to placenta.

The structure of the mature female gametophyte has been described in many flowering plants. The female gametophytes, also called embryo sac or megagametophyte, are composed of seven cells (or eight nuclei), embedded within the ovary: three antipodal cells, one central cell (containing two polar nuclei), two synergid cells, and one egg cell (Gifford and Foster, 1989; Angenent and Colombo, 1996; Drews and Yadegari, 2002; Yadegari and Drews, 2004; Drews and Koltunow, 2011; Hamamura *et al.*, 2012). The egg cell and the two polar nuclei which are the target of delivered sperm cells for fertilization, are formed close to each other. The surrounding cell wall of the egg cell, two polar nuclei, and synergid cell is absent or discontinuous, but they are directly connected throughout their plasma membranes. The absence of cell walls facilitates direct access of sperm cells to the egg cell and two polar nuclei (Punwani and Drews, 2008; Drews and Koltunow, 2011).

1.1.3 Pollen germination, pollen tube growth and fertilization

Shed pollen has been dehydrated prior to anthesis, which provides a metabolically quiescent state. This avoids environmental stresses which are encountered during pollen shedding, and might be a prerequisite for pollen viability and subsequent germination (Taylor and Hepler, 1997). Upon pollen shedding from the anther and deposition on the stigmatic surface, the desiccated pollen rehydrates in two phases. During the initial phase, putative signals are exchanged between pollen and stigma. In the second phase, the inner pollen wall (intine) introverts in the colpial zone (aperture where pollen tube will emerge), and the formation of the pollen coat containing stigmatic papilla is completed (Doughty *et al.*, 1993).

Soon after pollen rehydration, pollen germinates on the stigmatic surface and pollen tubes grow quickly down the style. The pollen tube forms channels through which the sperms cells transfer to reach the ovary and fertilize the egg (Cai *et al.*, 2015). Pollen tubes can be schematically divided into two main regions: the non-growing area called the shank, and the growing points that are the domed apices. The microtubules are organized along the longitudinal axis in the former and are uncertain in the later (Raudaskoski *et al.*, 2001; Lovy-Wheeler *et al.*, 2005). Recent live-cell imaging studies proposed three specific steps of sperm liberation after pollen tube discharge (Hamamura *et al.*, 2012). During the first step, pollen tubes penetrate the transmitting tract of the style. The pollen tubes then emerge from the transmitting tract and grow along the placenta toward the ovule. Upon reaching an ovule, the pollen tube grows along the surface of the ovule's funiculus through the micropyle and then sperm cells are liberated from the pollen tube (Kandasamy *et al.*, 1994; Yadegari and Drews, 2004).

The sperm cells then contact the synergid cells and cease growth. One of the synergid cells degenerates and undergoes cell death. Soon after this degeneration, pollen tubes rupture and release their constituents including the two immotile sperm cells (Rotman *et al.*, 2003; Sandaklie-Nikilova *et al.*, 2007; Drews and Koltunow, 2011; Hamamura *et al.*, 2012; Dresselhaus and Snell, 2014). Molecular genetic analysis determined that the synergid cell does not deteriorate until the pollen tube arrives (Kessler and Grossniklaus, 2011). In the second step, released sperm cells are maintained in the female gametophyte for a period of time, approximately 7.4 minutes (Hamamura *et al.*, 2011). These sperm cells are delivered to the apical edge of the degenerated synergid cell facing the apical edge of the egg cell and the central cell (Hamamura *et al.*, 2012; Dresselhaus and Franklin-Tong, 2013). In the third step, one of the two delivered sperm cells fertilizes the egg, which is the beginning of the sporophyte while the second sperm fuses with the two polar nuclei, leading to the formation of the endosperm, which surrounds and nurtures the developing embryo. This process is called double fertilization (Berger *et al.*, 2008; Drews and Koltunow, 2011; Dresselhaus and Snell, 2014). Each of the sperm cells is capable of fertilizing either female gamete, showing that each sperm cell has equivalent function (Ingouff *et al.*, 2009; Hamamura *et al.*, 2011).

Many megazoan genes/proteins are considered to be directly involved in sperm – egg plasma interaction. The hydrophobic tetraspanin family member CD9; IZUMO1, a plasma membrane protein (Dresselhaus and Snell, 2014), and EC1 (EGG CELL 1), which accumulating storage vesicles of the egg cell (Spunck *et al.*, 2012), are distinguished as essential proteins for gamete interaction. Additionally, HAP2 (HAPLESS 2)/GCS1 (GENERATIVE CELL SPECIFIC 1) is reported as the only

essential gene encoding sperm proteins required for fertilization. This gene is also essential for gamete fusion after membrane adhesion in the ovary (Spunck *et al.*, 2012; Dresselhaus and Snell, 2014). GCS1 mutations prevent fertilization presumably because the *gcs1* mutant sperm cells are not recognized by the female gametes (Berger *et al.*, 2008).

1.1.3.1 Factors affecting pollination

Environmental factors including high/low temperature stresses dramatically reduces percent pollen germination, pollen tube growth and subsequently fertilization (Snider and Oosterhuis, 2011; Pereira *et al.*, 2014; Huang *et al.*, 2014; Gao *et al.*, 2014; Das *et al.*, 2014). In many species, the highly specialized meiotic and mitotic cell division including haploid gamete formation are highly-sensitive to temperature stress (Pereira *et al.*, 2014). In a study with tomato, plants were exposed to heat stress. Meiosis and pollen grain development were reported to be the most heat-sensitive phases followed by pollen germination and pollen tube growth down the style, which could lead to productivity losses up to 70% (Snider and Oosterhuis, 2011; Pereira *et al.*, 2014).

Heat-stress, reduces seed development and seed yield in *Arabidopsis* (Huang *et al.*, 2014), disrupts pollen grain integrity, and reduces pollen viability in grapevine apparently due to cell wall fragility (Pereira *et al.*, 2014). It reduces pollen viability and pollen tube length, leads to poor anthesis and reduces the total number of pollen germinating on the stigmatic surface. The pollen protein concentration is reduced (Das *et al.*, 2014) eventually preventing pollen-pistil interactions of rice (Snider and Oosterhuis, 2011).

On the other hand, low temperature-stress negatively affects the fertilization process. It inhibits pollen tube growth, and reduces the induction of enzymes which are essential for pollen tube growth in pear (Gao *et al.*, 2014). The optimum temperature for pollen germination of most angiosperms is reported to be around 20° C (Hedhly *et al.*, 2004) and 27°C was reported to be the best for walnut pollen germination (Mert, 2009).

1.1.4 Cross and self-pollination

1.1.4.1 Self-incompatibility mechanism in apples

Almost all apple cultivars are either self- incompatible, or semi-incompatible. Cross-pollination is required to set fruit in marketable quantities (Garratt *et al.*, 2013; Matsumoto, 2014). For commercial production, at least two cross-compatible cultivars with synchronous flowering are required in an orchard (Goldway *et al.*, 2012; Garratt *et al.*, 2013). The most known phenomenon behind this is sharing the same alleles, named the S-locus (haplotype) between male and female parents. This is called S-RNase-mediated gametophytic self-incompatibility (GSI). For example, ‘Topred’ shared the S9-RNase allele with ‘Jonathan’ (Goldway *et al.*, 2012; Meng *et al.*, 2014).

This GSI system is a genetically controlled mechanism enabling floral styles to reject self-pollen in Rosaceae (Wu *et al.*, 2013). Two major determinations of self-incompatibility have been reported: female-specificity or pistil S-determination, and male-specificity or pollen S-determination. Female-specificity determinations of self-incompatibility are those genes that are primarily expressed with a high level of sequence polymorphisms in the pistils. RNase is reported to be critical for the incompatibility response (Wu *et al.*, 2013; Meng *et al.*, 2014). The same S-genotype is reported to

prevent the transmission of S-RNase genes in incompatible cultivars; therefore, it is determined as pistil-S determinants that interact with pollen-S when pollen tubes grow down the style (Heng *et al.*, 2008). However, in male-specificity determination, other genes such as SLFL (SLF) and SFB are tightly linked with S-RNases in pollen. The S-RNases in the pistil, present in the extracellular matrix of the transmitting tissue, recognize self-pollen, degrade pollen RNA, and eventually block pollen tubes penetrating through the transmitting tract of the style (Ortega *et al.*, 2013) and excessive S-RNases cause pollen tube death (Meng *et al.*, 2014). In contrast, in successful cross-pollination, the pollen S-determinants will inhibit S-RNases and pistil S-RNases will not recognize non-self-pollen, and subsequently pollen tubes grow rapidly through the style and will reach the ovary (Wu *et al.*, 2013).

Beside fruit set, significantly influence of cross-pollination on fruit quality and development has been reported. Apples and pears generally produce 10 ovules, leading to developed seeds after fertilization that contribute to the fruit's size – the higher the seed number, the larger the fruit will be (Goldway *et al.*, 2012). Meanwhile, an increase in the concentration of almond kernel-amygdalin was reported when the trees were crossed with a productive pollinizer (Sánchez-Pérez *et al.*, 2012). Additionally, anthocyanin concentration, the main pigment in fruit coloration that possesses strong antioxidant activity and are potent inhibitors of lipid peroxidation, is assumed to increase with cross-pollination resulting of good colored fruits (Matsumoto, 2014).

1.1.4.2 The roles of pollinators in pollination

The role and efficacy of pollinators in fruit set and fruit quality has been widely studied. Pollinators visit plant flowers for gaining food, usually nectar, returning with pollen attached to their body and subsequently pollinate other flowers (Hepburn and Radloff, 2011). Most angiosperms, around 78% temperate zone and 94% in tropical crops (Ollerton *et al.*, 2011), which overall are more than 70% of land-species (Hepburn and Radloff, 2011), and 43.5% of world's leading food crops are considered to rely on animal-pollination (Klein *et al.*, 2007). The annual gross benefit of animal-pollinated crops was reported to be around € 153 billion (US \$172.6 billion), representing 9.5% of the value of agricultural products used by humans in 2005 (Gallai *et al.*, 2009). A total of \$4.1 billion animal-pollinated crops was estimated annually in the US (Prescott-Allen, 1990).

Apple is one of the most valuable crops globally, with a total gross income of US \$64 billion across 93 countries in 2010. However, for marketable quantities of production, fruit set requires pollen transfer from a different cultivar. Apple pollen is sticky and heavy, hence insects such as honey bees and hoverfly are required to transfer the pollen from one flower to another, and their activity during pollination is essential in the orchard (Garratt *et al.*, 2014).

Bees, particularly honeybees are reported as a predominant apple pollinator. Honeybees have the potential of working for long period of time and are able to adapt to different climates. Plants pollinated by insects are called entomophilous, and the pollination process called entomophily (Hepburn and Radloff, 2011). Pollinators significantly influence apple fruit set, total yield at harvesting time and seed number. The

greater seed number observed with pollinator treatments resulted in fruit with improved size, shape and marketability (Garratt *et al.*, 2014). However, the relative values of the pollinators depend on how much pollen they remove from anthers and how much they deposit on stigmatic surfaces. An insect that removes and delivers more pollen grains is usually considered to be the better pollinator than ones that remove and deliver fewer (Goodell and Thomson, 1996). In 2014 Garratt and his colleagues reported that pollinators added over £36 million (US \$54.3 million) to the output of apples across the UK (Garratt *et al.*, 2014) and increased other crops yields 18-71% depending on the crop (Bartomeus *et al.*, 2014). On the other hand, landscape complexity, crop type, and agriculture intensification has impacted pollinator richness and visitation in the field. However, the higher richness did not increase total yield but the higher visitation increased it significantly (Bartomeus *et al.*, 2014).

1.2 Agricultural aspects of pollination

1.2.1 Orchard design and management

Apple orchard designing is a complex process and planning decisions should be made carefully before orchards are established. Orchardists should consider both biological and economic factors in determining the preferred orchard system (Hester and Cacho, 2003). Barritt (1987) defines the orchard system as the assimilation of all horticultural factors influencing establishment and maintenance of the planting. Several striking decisions should be made prior to orchard establishment to ensure the productivity and marketability of the orchard including choosing cultivars (scion), rootstock, density of the trees, pollinizer, and training and pruning system (Hester and

Cacho, 2003). Cultivar compatibility is a very crucial factor during orchard design because of its economic implications. Therefore, research-based information on cultivar compatibility and marketability should be considered. Apple cultivars are considered either self- incompatible, semi-incompatible, or compatible. The majority of cultivars require cross-pollination to set fruits in marketable quantities (Garratt *et al.*, 2013; Matsumoto, 2014).

To design an orchard, at least two cross-compatible cultivars should be selected. The basis for selection should be obvious criteria such as synchronous flowering, compatibility, market demand, and date of maturity, but also less obvious effects including possible metaxenia effects should be taken into consideration (Bodor *et al.*, 2008; Goldway *et al.*, 2012; Garratt *et al.*, 2013). Additionally, apple pollen is sticky and heavy, and insects such as honey bees, and hoverfly are required to transfer the pollen from one flower to another, and their activity during pollination is essential in the orchard (Garratt *et al.*, 2014). Bees, particularly honeybees are reported being the predominant pollinators for apples, which have the potential of working for long period of time and are able to adapt to different climates.

Several environmental factors affect fruit quality which should be deliberated on during the commencement of orchard design. Light distribution and interception is one of the most important factors determining orchard success. (Wünsche and Lakso, 2000). Tree planting system (orchard design), and tree canopy are also reported as critical factors for orchard design and management. The Y-trellis system was determined as an efficient and highly productive system for apple orchards (Robinson *et al.*, 1991), and tended to produce more fruiting spurs during the following year (Hampson *et al.*, 2004).

1.2.2 Xenia and Metaxenia in apples

The terms Xenia and Metaxenia are taken from Greek word, *xenos*, meaning “foreigner” or “guest”. In Greek the word xenia means “hospitality” and the prefix meta- means “beyond, behind and after”. Xenia was first coined by Wilhelm Focke (1881), describing direct pollen source effects only on maternal plant tissue that is, on seed coat, pericarp, and attending structure (Denney, 1992; Bodor *et al.*, 2008). These effects contrasted with those associated with hybridization revealed in the embryo. However, similar interpretations have been used by Bradley (1739) in apples, which predates the discovery of the fertilization mechanism. On the other hand, the term Metaxenia used by Swingle in 1926, describes the phenomena of the paternal-pollen influence upon the surrounding maternal tissues of the zygote (Nebel, 1936). In other words, the effects of pollen provider expressed on the fruit of pollinated cultivar (Bodor *et al.*, 2008). Later on xenia was defined as the direct effects of pollen on the size, shape, color, developmental timing, and chemical compositions of seeds and fruits. Such differences might simultaneously be found in embryo, endosperm, and maternal tissues in some species; therefore, xenia was considered covering metaxenia (Denney, 1992).

Soon after the phenomenon was described, Nebel and Trump (1932) reported Xenia and Metaxenia in apple. They crossed McIntosh with Yellow Bellflower and Red Astrachan. The harvested fruits from the two crosses were characterized with significant differences in the quality, seed number, fruit and seed shape and subsequently fruit weight (Nebel and Trump, 1932). Xenia has not only been used in genetic and physiological studies but also in plant breeding and crop production (Denney, 1992). The simplest hypothesis behind this phenomenon is that the father expressively affects zygote

formation in higher plants, assisting liberating growth hormones that cause the growth of outside parts of the embryo and endosperm of seeds. Likewise, Swingle (1926) argued that the father is considered to speed the development of fruit tissues leading to early maturity (Nebel, 1936).

Kumar *et al.* (2003) conducted an experiment on three scab resistant apple cultivars ‘Co-op 12’, ‘Redfree’, and ‘Liberty’ using the pollen of three scab-susceptible apple cultivars ‘Tydeman’s Early’, ‘Stark Spur Golden Delicious’, and ‘Golden Spur Delicious’. Effects of pollen source on fruit set, seed number, and fruit physical and chemical characteristics were studied. Significant metaxenic effects on fruit set, seed number, and other aspects were reported (Kumar *et al.*, 2003). A similar experiment was conducted by Bodor *et al.* (2008) on three disease-resistant apple cultivars as pollen producers (‘Baujade’, ‘Relinda’, ‘Rewena’) crossed with other marketable cultivars. They also reported significant metaxenic differences on fruit size, and fruit flesh firmness. (Bodor *et al.*, 2008).

Xenia has not only been proposed in apples but its use is widespread in other crops such as cross-pollination to increase corn total yield (Weingartner *et al.*, 2002), raising porosity and reduced seed weight in tomato (Piotto *et al.*, 2013), increasing percentage of berry set, seed number per berry, and the quality of grape berries (Sabir, 2014) and subsequently increasing yield of; pecan nuts, pistachio nuts, and avocado (Robbertse *et al.*, 1996; Sedgley and Griffin, 1989). Usman *et al.* (2013) reported xenia and metaxenia in guava. They proposed that pollen parent enhanced various fruit quality aspects including fruit diameter, soluble solid concentration (SSC), titratable acidity, ascorbic

acid and total and non-reduced sugar composition (Usman *et al.*, 2013). Therefore, xenia and metaxenia can be critical determinants of fruit quality and marketability.

1.2.3 Fruit Quality

Quality is often defined as the degree of product excellence and its suitability and acceptability to consumers for a particular use. Quality is a human construct and usually encompasses many properties and characteristics including sensory properties (appearance, texture, taste, and aroma), nutritive values, chemical constituents, mechanical properties and functional properties (Abbott, 1999). The component attributes of quality vary with context and have different acceptability criteria among consumers. However, for grades and standards of the products, the definition, attributes, and customer's perceptions of quality are formalized and institutionalized. Normally instrumental measurements are preferred over sensory measures for research and commercial applications because it reduces the variability among individuals, is more precise, and would provide common language among researchers (Abbott, 1999; Shewfelt, 1999).

Many factors are reported to affect fruit set and fruit quality. Pollen density on stigmatic surface was considered to be one of the most striking factors influencing fruit quality attributes. Higher pollen density on the stigmatic surface improved fruit set, fruit quality and seed viability of pears (Zhang *et al.*, 2010). Higher pollen density on the stigmatic surface was strongly positively correlated with pollen tube growth, fruit growth rate and endogenous gibberellin concentration that initially improves pollen tube growth down the style (Zhang *et al.*, 2010). A similar study has been conducted to determine the

effect of repeated pollination on seed set and fruit shape. Repeated pollination from cross-compatible cultivars contributed to high fruit quality and increasing seed production in apples (Matsumoto *et al.*, 2012). Irrigation system, watering regimes and frequency can influence fruit quality and coloration (Fallahi *et al.*, 2010). Rootstocks and harvesting time affects fruit quality and nutritional values (Remorini *et al.*, 2008). Some of the important fruit quality attributes are as follows.

1.2.3.1 Soluble Solids Concentration (SSC)

Apple taste is primarily related to the amount of sugar and acid and their balance in the fruit tissues. Glucose, fructose, sucrose, and sorbitol are the primary carbohydrates in apple, which increase at fruit approach maturity. Fructose is considered as the main sugar of mature apples of most cultivars (Jackson, 2003). Sugar and acid content change over time and sugar level increases 2-3 weeks prior to commercial harvest time which increases fruit sweetness. Several different techniques have been used to measure quality attributes. Some of them are oriented to detect physical aspects of quality (i.e. firmness, seed number and weight, fruit fresh weight, color, size/shape). Others are determined by detecting chemical compositions, such as sugars, acids and starch (Valero *et al.*, 2004). The easiest and cheapest method to measure soluble solids concentration is to drop juice on the prism of a refractometer and read the soluble solids concentration as a percentage.

Tuan and Chung-Ruey (2013), reported that pollen source affected SSC of 'Long Red B' apples. They proposed 'Black' and 'Thyto' as the best pollinizers for 'Long Red B' apples, resulting in higher fruit weight, fruit diameter, fruit length, flesh thickness, and soluble solids concentration. Similarly, pollen density on the stigmatic surface (Zhang et

al., 2010), and repeated pollination (Matsumoto *et al.*, 2012), were considered the most crucial factors influencing apples soluble solids concentration.

1.2.3.2 Starch as a Maturity Index

Starch is the main carbohydrate of plant storage organs. Starch accumulates in the immature fruits of apple and hydrolyses as fruit ripen to increase sweetness. Apple starch was first identified by Potter, Hassid, and Joslyn in 1949, when they used two different methods to analyze amylose content. Starch concentration in young fruitlets declines during the first 30 days after anthesis and increases again until it reaches a maximum level at 110-130 days after anthesis. It declines to a very low level 2-3 weeks before harvest as starch is hydrolyzed into soluble sugars (glucose and fructose) (Jackson, 2003). A similar study on Fuji and Royal Gala supported the idea that starch concentration decreases until 30 days after anthesis, then increases reaching a maximum level at 100 days after anthesis (Brookfield *et al.*, 1997). The study also reported the highest starch level in the outer cortex and lowest in the core and the degradation rate was also lower in core. Starch is also widely used as a maturity index for determining the appropriate harvest time of apple. It has also been reported that apple fruit abscission occurs at a fixed starch level and is suggested to have a close linkage with fruit natural ripening and senescence processes (Jackson, 2003).

1.2.3.3 Fruit Color

Fruit color and shape influence fruit appearance, marketability, and customer acceptability. The most rapid increase in fruit color occurs one to two weeks before commercial harvest (Iglesias *et al.*, 2008; Iglesias *et al.*, 2012). Apple red coloration is not related to fruit maturity or internal quality attributes. The quality attributes were similar across the strains of Gala (Iglesias *et al.*, 2008) and Fuji (Iglesias *et al.*, 2012), while fruit color intensity and type were different at harvest time. The main pigment responsible for apple red color is cyanidin-3-galactoside (cyanidin), belonging to the red pigment family named anthocyanin and is regulated by light (Talos *et al.*, 2006), temperature (Honda *et al.*, 2014), cultivar (Iglesias *et al.*, 2008), and maturity time (Iglesias *et al.*, 2012). Several agricultural practices increase apple red coloration including bagging, cooling by sprinkler irrigation to reduce fruit temperature in Delicious apple, and using the reflective film to increase light intensity into the tree canopy, however all these practices are expensive. A most common, easier and cheaper method is to plant new higher-coloration cultivars (Iglesias *et al.*, 2008). Growers are now tending to plant cultivars exhibiting high-productivity, high quality, good color and most importantly high consumer appeal. These factors result in higher economic return (i.e. Honeycrisp, Fuji and Gala) (Iglesias *et al.*, 2012).

1.2.4 Return bloom

1.2.4.1 Flower bud induction and differentiation

Flower bud development begins with the transformation of a vegetative apex to a reproductive structure (Ferree and Warrington, 2003). The first detectable change in bud

development after induction of flowering is increasing DNA and RNA synthase. Flower bud initiation typically occurs in mid-to-late spring but can extend to early autumn in certain cultivars, in certain years, and/or in certain areas where the growing season is long (Buban and Faust, 1982). It is generally accepted that apple flowering is autonomous, where the flower initiation is led by internal developmental signals (Wilkie *et al.*, 2008).

In the first stage of flower differentiation, several morphological changes occur, the first of which is the flat apical meristem becomes domed and the pith meristem develops. (Hanke *et al.*, 2007). As a result, the central reproductive part of the apical meristem differentiates into the king flower and later four lateral flowers, sepals, petals, anthers, and pistils are produced in an orderly sequence (Jackson, 2003; Hanke *et al.*, 2007). It is generally accepted that the mitotic division in the apical meristem increases prior to flower bud differentiation (Gifford and Corson 1971). Nucleic acids play a significant role in cell division and their synthesis increases during flower induction. However, different zones might synthesize differently in the same growing apex (Teltscherova and Pleskotova, 1973).

The vegetative apical meristem structure tends to be similar to the reproductive growing point in most respects. The major distinction between the reproductive growing point and vegetative apical meristems are a shortening of plastochron, an increase in mitotic activity in certain meristematic tissues, an increased RNA content of nucleoli in meristematic cells, and an increase of growing point (Gifford and Corson 1971).

1.2.4.2 Flower bud formation

Fruiting and non-fruited terminal spur buds typically have similar apices in the early part of the growing season. Later on, vegetative buds receive signals to change into flowering buds following a sequence of events that are considered to be the progressive transformation of the vegetative growing point such as: generalized increase in mitotic division in the entire meristematic apex, changing the histological structure of the apex, and subsequently development of the apical meristem into flower primordium (Buban and Faust, 1982). This histological transformation is followed by morphological differentiation of the bud. After bud differentiation, all constituent parts of the flowering bud are recognizable: bud scales, transition leaves, true leaves, and bracts. The shortened axis of the bud terminates in king flower primordia whereas the primordia of lateral buds initiate in the axils of the bracts.

King flowers develop faster than lateral flowers which explains the phenomenon of later blooming in lateral buds. Lateral meristems typically do not develop until the terminal flowers have initiated sepals which usually continues throughout the autumn until the commencement of dormancy. When dormancy ends, flower formation is completed and anthesis ensues (Foster *et al.*, 2003). The expression of flowering genes and formation of gametes through meiosis are the final event in flower formation (Koutinas *et al.*, 2010).

1.2.4.3 Sites of flower bud formation

Traditionally, apples bear fruits terminally on short (less than 5 cm) bearing shoots called spurs and/or terminally or laterally in some cultivars on axillary buds of one year

old elongated shoots (Buban and Faust, 1982; Ferree and Warrington, 2003; Hanke *et al.*, 2007; Wilkie *et al.*, 2008; Koutinas *et al.*, 2010). Leaves on fruiting spurs can initially account for 60% of the total leaf area of the tree (Koutinas *et al.*, 2010). Apple flower buds are mixed and comprise primordia of both vegetative and reproductive organs.

1.2.4.4 Factors affecting flowering

Several endogenous and exogenous factors influence apple flowering. Nutrition, flower-inducing hormones, cultivars, rootstocks, crop load, seed number, tree vegetative growth, plant age, and environmental conditions are the major factors considered to affect return bloom in apples (Buban and Faust, 1982; McLaughlin and Greene, 1991; Hirst and Ferree, 1995, 1996; Hanke *et al.*, 2007; Koutinas *et al.*, 2010). Carbon: Nitrogen ratio is reported to be the most predominantly factor influencing flowering. High C/N ratio promotes flowering whereas excessive nitrogen fertilization inhibits flowering (Hanke *et al.*, 2007).

Different hormones are reported as regulatory factors of flowering. Auxins are considered to be promoters of flowering. Cytokinins (Ramírez *et al.*, 2004), and ethylene (Hanke *et al.*, 2007), are associated with promotion of apple flowering, whereas the effects of abscisic acid are still unknown. Of all currently known hormones, gibberellins appear to be the most strongly associated with flowering. GAs are known to inhibit apple flower bud formation, and are closely related to alternate bearing (Goldschmidt *et al.*, 1997; Tu, 2000). However GA₄, appears to promote apple flowering (Looney *et al.*, 1985). Biennial bearing cultivars have been reported to produce more hormones, particularly GAs, than more annual cultivars (Hoad, 1977).

Temperature is the most influential environmental factors on flower bud formation. Flowering was stimulated by lower temperatures applied 4-5 weeks before full bloom, and reduced when the temperature increased from 17 to 24° C seven weeks prior to harvest (Tromp, 1976). A similar experiment was conducted by Zhu *et al.* (1997), who reported that temperature had a tremendous influence on flower bud formation in apple. The most beneficial range of temperature in their study was 13 - 20° C throughout the growing season (Zhu *et al.*, 1997). Blossom density, crop load and thinning effects on return bloom were studied on Honeycrisp biennial bearing apples. Unthinned trees displayed extreme biennial bearing with typically no fruits in the 'off year' (Embree *et al.*, 2007). The proportion of flowering spurs was influenced by flower density, and was indirectly affected by rootstock via shoot growth effects (Hirst and Ferree, 1995a).

Pollen source is considered to be one of the most important factors affecting fruit set, fruit quality and subsequently orchard design. The direct effect of pollen source on return bloom in apple is still unclear. However, since pollen source increases seed set in apple, and seed set influences flowering, therefore pollen may have an indirect effect on flowering. We therefore, conducted these experiments to determine the impact of pollen source on pollen tube growth, fruit set, fruit quality and subsequently return bloom in apple.

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CHAPTER 2. EXPERIMENT ONE AND TWO: EFFECT OF POLLEN SOURCE ON POLLEN TUBE GROWTH, FRUIT SET, FRUIT QUALITY AND RETURN BLOOM IN APPLE CULTIVARS DIFFERING IN BIENNIAL BEARING POTENTIAL

2.1 Abstract

In agriculture, pollination is a vital prerequisite for crop production and adequate, compatible and viable pollen is one of the crucial elements and has a remarkable impact on fertilization. Pollen germination, pollen tube growth and pollen-style interaction are the most important factors for successful fertilization, fruit set and productivity in apples. However, these processes are not clearly understood therefore efficient selection of effective pollinizers for commercial orchards is not possible. Hence, we conducted this experiment to compare pollinizers in terms of pollen tube growth, fruit set, fruit quality and return bloom. Honeycrisp, Gala, and Fuji cultivars were hand-pollinated by Crabapple, Red Delicious and Golden Delicious pollen.

Pollen source had a significant influence on pollen germination on the stigmatic surface, number of pollen tubes penetrating the stigma, pollen tube growth down the style, and pollen enrichment to the base of the style. Golden Delicious pollen grew fastest followed by that of Red Delicious and Crabapple. Crabapple was not an effective pollinizer for Honeycrisp and resulted in low fruit set, but was an effective pollinizer for both Gala and Fuji. Fruit quality attributes and return bloom were generally not affected by pollen source. However, fewer seeds were apparent when crabapple pollen was used

as a pollinizer in all cultivars. Seed number was positively correlated with fruit fresh weight in Gala and Honeycrisp regardless of the pollen source. Fruit fresh weight, and seed number had no significant influence on return bloom. These results suggest that pollen source has a tremendous impact on pollen tube growth, fruit set and subsequently are important factors to be considered during orchard design.

2.2 Introduction

Pollination is one of the most important processes for fruit set, fruit growth, fruit quality, and reproduction of seeded plants. Pollen grains are produced in the anther and after maturation are released to the surrounding environment to deliver sperm cells to the ovule. The first step of pollination is adhesion of pollen grains (which are transported by bees) to the papilla cells of the stigmatic surface (Dresselhaus and Franklin-Tong, 2013; Selinski and Scheibe, 2014). The deposited pollen then hydrates and germinates with pollen tubes growing down the style.

Pollen source and temperature have a tremendous influence on the rate of pollen tube growth. Petropoulou and Alston (1998), and Jackson (2003) proposed that the percent germination of pollen on the stigmatic surface of apples and pears depends on the pollen donor and environmental temperature at the time of pollination. They reported that ‘Spartan’ pollen had a higher germination percentage than that of ‘Cox’ at 8-10° C and ‘Idared’ at 14-16° C. A linear correlation between pollen germination on the stigmatic surface of ‘Golden Delicious’ and temperature was reported from 13 to 29° C for ‘Manchurian’ crabapples and ‘Golden Delicious’ (Yoder et al. 2009) and also from 6 to 33.5° C (Jefferies and Brain, 1984).

Almost all apple cultivars are either self- incompatible, or semi-compatible, and require cross-pollination to set fruit in marketable quantities (Garratt *et al.*, 2013; Matsumoto, 2014). For commercial production, at least two cross-compatible cultivars with synchronous flowering are required in an orchard (Goldway *et al.*, 2012; Garratt *et al.*, 2013). The distance of pollinizer from the main cultivars is an important consideration. Matsumoto *et al.* (2008) reported a significant decline in fruit set with

increasing distance between pollinizer and main cultivars. They suggested pollinizers should be planted not more than 10 meters from the cultivars (Matsumoto *et al.*, 2008).

Besides fruit set, cross-pollination has been reported to significantly influence fruit quality and development. Apples generally produce 10 ovules, leading to seed set after fertilization. There is a positive relationship between seed number and fruit size (Goldway *et al.*, 2012). It is generally accepted that a threshold of at least 6 - 7 ovules must be fertilized otherwise fruit are likely to be misshapen and small (Delaplane and Mayer, 2000). Pollen density on the stigmatic surface was considered to be the most important element influencing fruit quality attributes. Higher pollen density on the stigmatic surface improved fruit set, fruit quality and seed viability of pears. A strong positive correlation of higher pollen density on stigmatic surface was reported with pollen tube growth, fruit growth rate and endogenous gibberellin concentrations that initially improve pollen tube growth down the style (Zhang *et al.*, 2010). A similar study was conducted to determine the effect of repeated pollination on seed set and fruit shape. Repeated pollination from cross-compatible cultivars was effective in increasing seed production in apples (Matsumoto *et al.*, 2012).

Pollen source is considered to be one of the most influential factors affecting fruit set, fruit quality and subsequently orchard design. The direct effect of pollen source on return bloom in apple is still unclear. However, it is generally accepted that pollen significantly increases seed set in apple, which could influence return bloom in the following year. Meanwhile, pollen tube growth down the style has been widely studied but pollen source effects on pollen tube growth is not well understood. We therefore,

conducted this experiment to determine the impact of pollen source on pollen tube growth, fruit quality, and subsequently return bloom in apple.

2.3 Materials and Methods

2.3.1 General

2.3.1.1 Plant materials

Experiments were conducted in 2013 and repeated in 2014 at the Purdue University Meigs Research Farm, Lafayette, Indiana, USA. Three commercial apple cultivars: Honeycrisp/M.9 planted in 2010 (very biennial bearing), Fuji/ M.9 planted in 2001 (somewhat biennial bearing), and Gala/ M.9 planted in 2001 (annual bearing), were hand pollinated using three pollen sources: Crabapples, Red Delicious and Golden Delicious in both 2013 and 2014 years. ‘Ralph Shay’ crabapple was found to be a poor pollinizer of Honeycrip in 2013, so a different crabapple (*Malus floribunda*) was used in 2014.

2.3.1.2 Design of the experiment

The experiment layout was designed as a completely randomized design (CRD) where two uniform adjacent trees were selected for each cultivar in late April of 2013. The same trees were used in 2014. Flowering spurs were randomly selected and tagged prior to flowering in the same tree for both experiments.

2.3.2 Specific

2.3.2.1 Experiment1: Effect of Pollen Source on Pollen Tube Growth in Apple cultivars differing in Biennial Bearing Potential

Selected trees were netted in late-April, prior to flower opening, to avoid cross-pollination by bees (Figure 2.1). At the tight cluster stage of floral development, a total of 60 flowers were randomly selected on each cultivar, distributed between the two selected trees. Of the 60 flowers, 20 were randomly assigned to each of the three pollinizer treatments. At late pink (popcorn) stage (just before the flower completely opened), all anthers were removed to prevent self-pollination and flowers were hand-pollinated using a small brush. Only king flowers were pollinated for uniformity of the experiment and all lateral flowers as well as all non-tagged flowers on the tested trees were either manually removed or dropped off. Pollen used for the experiment was collected from orchards in south Indiana in 2013 and from branches placed in the greenhouse to force flower opening (2014).



Figure 2.1: Trees are netted to exclude pollinators and prevent contamination.

2.3.2.1.1 Pollen viability test

Pollen was tested in the laboratory in petri dishes prior to pollination to determine viability (Figure 2.2). Following the method used by Yoder *et al.* (2009), pollen was placed on a medium of 1 % agarose, 10 % sucrose and 10 ppm boric acid at room temperature for 24 hours. Percent germination was visually observed under a light microscope. All pollen used in these experiments had a high percent germination (more than 80 %).

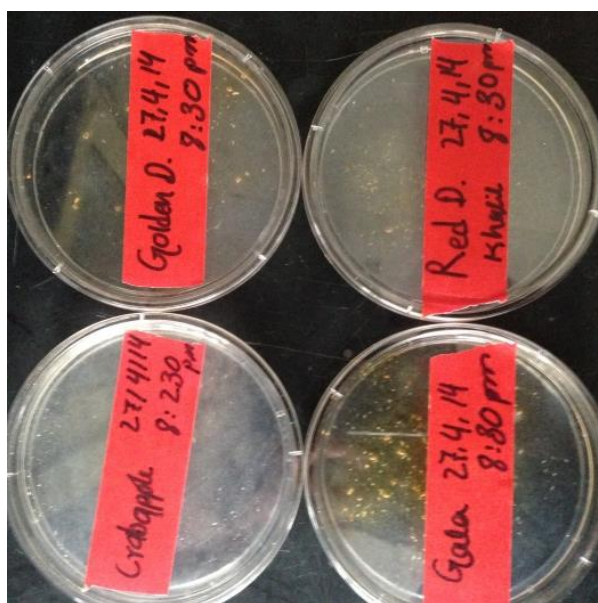


Figure 2.2: Cultivated pollen in petri dishes on artificial medium of apples

2.3.2.1.2 Pollen tube growth and microscopy examination

The method of Yoder *et al.* (2009) was modified to evaluate pollen germination on the stigmatic surface, number of pollen tubes penetrating the stigma, the longest pollen tubes growing the style, and number of pollen tubes that reached the base of the style.

The method was modified as five hand-pollinated flowers were collected from the trees at one, two, three, and four days after pollination (DAP) from each treatment.

The flowers were placed in a solution of 5% sodium sulfate in 40 ml and 80 ml labeled glass beakers, boiled for 15 minutes on PC-420D Corning Hot Plate Stirrer, and then subsequently refrigerated in 20 ml labeled glass bottles in the same solution until the time of microscopic examination. Later, five pistils from each flower were detached from the ovary, rinsed with distilled water, and then placed in a water-soluble solution of 0.01% Aniline Blue stain in 0.067 M K_2HPO_4 on microscope slides. Detached pistils were squashed between two microscope slides. The pistils were then incubated in the dark at room temperature for 24 hours, and the length of each pollen tube was measured by viewing it in epi-fluorescence mode through a blue filter (350/50 460/50) using a 10x 0.45 NA objective. The position of each pistil's end was located based on xy coordinates provided by an encoded, motorized xy stage (Nikon Ti-S-ER) on a Nikon Ti-E microscope. Length was defined as the linear distance between each end point. Images of pistils were acquired using a 10x 0.45 NA objective and Nikon DS-Ri1 color camera on a Nikon 90i epi-fluorescence microscope using blue (350/50 460/50) and red (560/40 630/60) filter sets. Individual images of each pistil were manually assembled into a montage.

Collected data comprised rating of pollen tube growth on stigmatic surface (0 % to 100 % of visible pollen tubes germinated on the stigmatic surface), number of visible pollen tubes penetrating the stigma, average length of the longest pollen tube growing down the style, percent of the maximum growth of pollen tubes in the style, and

subsequently number of visible pollen tubes that reached the base of the style (Figure 2.3).

The percent pollen germination on the stigmatic surface was visually rated on a scale of 0 to 10 where 0 = no visible germinated pollen tubes were detected on the stigmatic surface, 1 = 1 % to 10 % of the stigmatic surface was covered by germinated-pollen tubes, 2 = 11 % to 20 % of the stigmatic surface was covered by germinated-pollen tubes, 3 = 21 % to 30 % of the stigmatic surface was covered by germinated-pollen tubes, 4 = 31 % to 40 % of the stigmatic surface was covered by germinated-pollen tubes, 5 = 41 % to 50 % of the stigmatic surface was covered by germinated-pollen tubes, 6 = 51 % to 60 % of the stigmatic surface was covered by germinated-pollen tubes, 7 = 61 % to 70 % of the stigmatic surface was covered by germinated-pollen tubes, 8 = 71 % to 80 % of the stigmatic surface was covered by germinated-pollen tubes, 9 = 81 % to 90 % of the stigmatic surface was covered by germinated-pollen tubes, and finally 10 = 91 % to 100 % of the stigmatic surface was covered by germinated-pollen tubes.. The number of pollen tubes that penetrated the stigma and reached the base of style were counted and the longest pollen tubes were measured using the previously indicated software. Maximum growth of pollen tubes down the style was calculated as the total length of the longest tubes divided by the total length of the style.

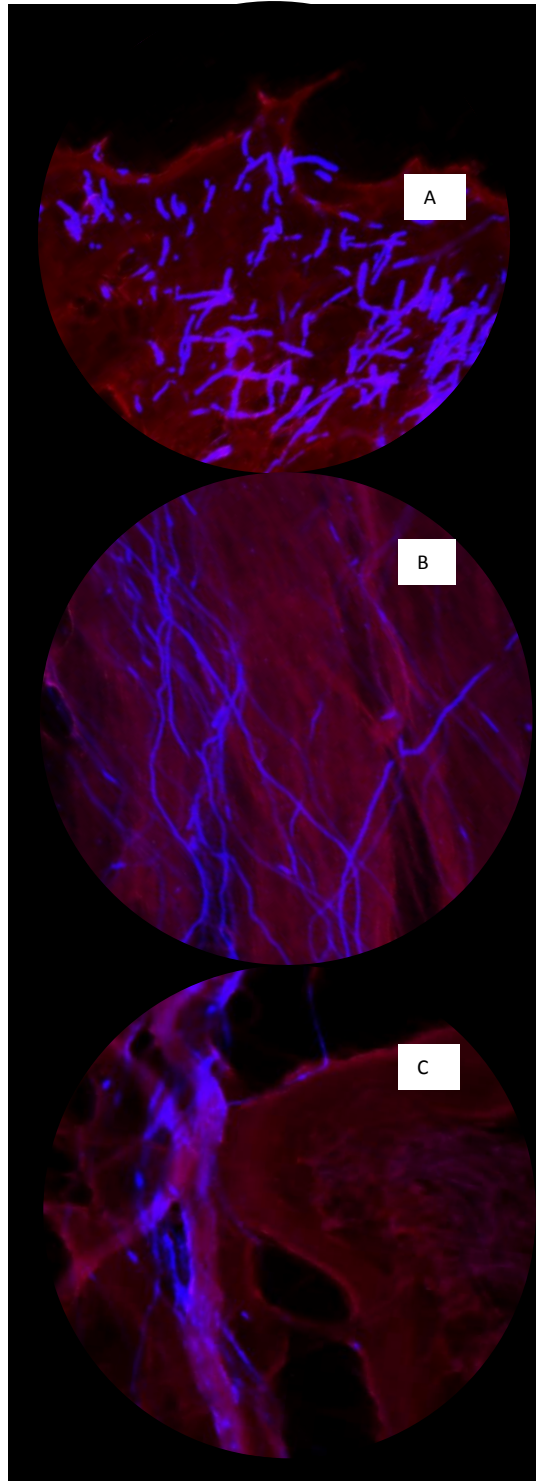


Figure 2.3: (A) pollen tubes germinated on the stigmatic surface of Fuji, (B) pollen tubes growing the style of Fuji, (C) pollen tubes that reached the base of the style of Fuji.

2.3.2.2 Experiment 2: Effect of Pollen Source on Fruit Set, Fruit Quality, and Return Bloom in Apple cultivars differing in Biennial Bearing Potential

The same methods as in experiment 1 were applied except a total of 90 flowers in each cultivar, 30 pollinated by each pollen source, were randomly selected on the trees. Pollen used for the experiment was collected from orchards in southern Indiana in 2013 and from branches forced in the greenhouse in 2014.

2.3.2.2.1 Fruit set

Selected flowers were rated on the treated trees every other week starting from the second week after pollination until one week before harvest.

2.3.2.2.2 Quality aspects measurement

Fruits were harvested during the normal commercial harvesting period for each cultivar. Harvest measurements included fruit fresh weight using *Mettler Toledo College Model: B3002 DeltaRange Scale. B3002DR*; Soluble Solids Concentration (SSC) using *Atago 3810 Digital Handheld Pocket Refractometer PAL-1*; total seed number per fruit; total seed fresh weight per fruit; and starch pattern index using the methods of *Reid et al.* (1982) where half fruit were dipped for 30 seconds in an iodine solution and rated on a 1-6 scale where 1 = a very dark-black color of the stained fruit, indicating higher starch content and 6 = very little staining.

2.3.2.2.3 Buds dissection to determine return bloom

Bourse buds on tagged spurs, which would be expected to produce flowers for the following year's crop, were collected at the time of leaf abscission. Buds were placed in a 5% acetic acid-based FAA (Formalin-Acetic-Alcohol) solution containing 50% ethyl alcohol, 5% glacial acetic acid, 10% formaldehyde and 35% distilled water. Buds were then dissected under a light microscope to determine reproductive or vegetative status (Figure 2.4).

2.3.2.3 Statistical analysis

Both experiments were conducted and analyzed as completely randomized design (CRD). Statistical analyses included analysis of variance, Tukey multiple range test, and regression analysis. Logistic regression analysis was used for the binary data of flowering, using Statistical Analysis Systems Software for PC (SAS 9.4). Means and standard errors are reported, whereas for the binary data, Chi-square analysis was performed and percent flowering reported.

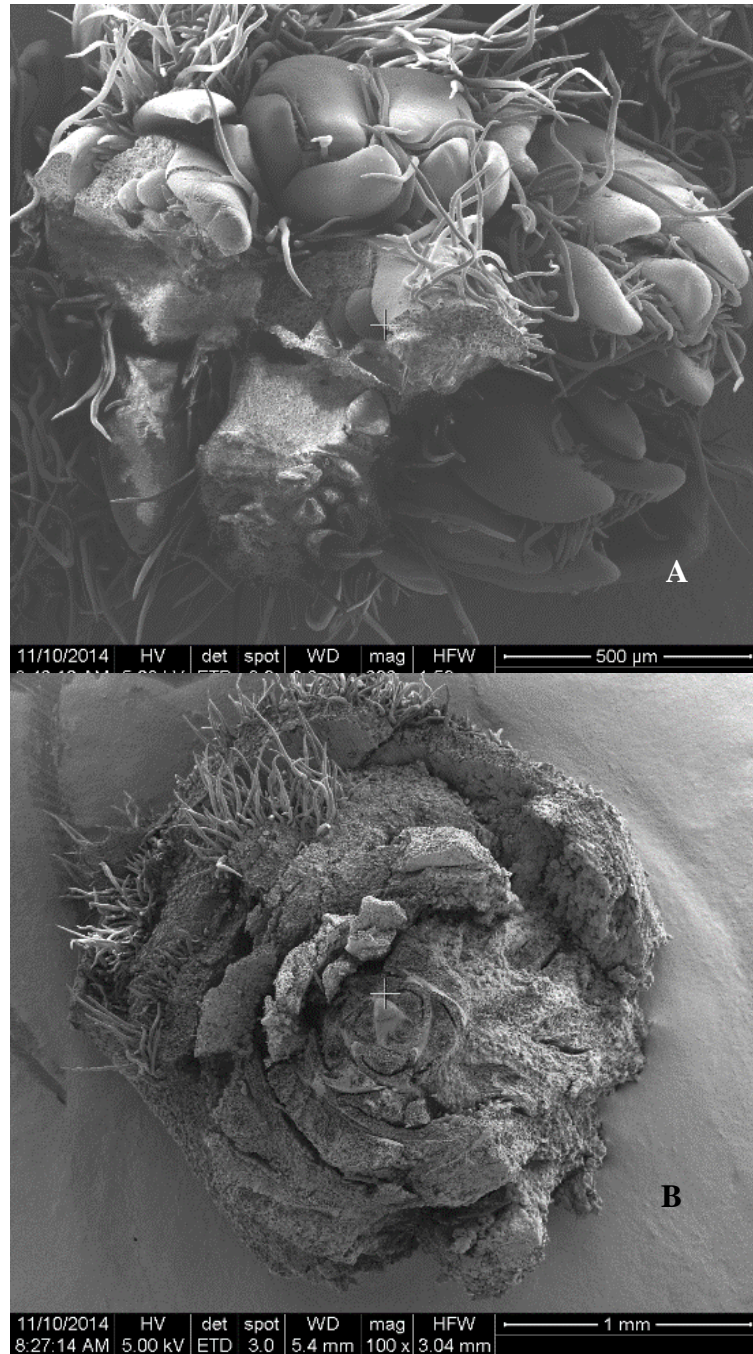


Figure 2.4: (A) Reproductive bud (B) Vegetative bud, images were acquired using Scanning electron microscope.

2.4 Results

2.4.1 Experiment 1. Effect of Pollen Source on Pollen Tube Growth in Apple cultivars differing in Biennial Bearing Potential

Pollen germination percentage on the stigmatic surface increased with time after pollination in all cultivars regardless of the year (Tables 2.1 and 2.2). Less Crabapple pollen germinated on the stigmatic surface of Honeycrisp compared to Gala and Fuji, but more crabapple pollen germinated on Fuji stigmatic surfaces in 2013 and on Gala stigmatic surface in 2014. The degree of pollen germination on stigmatic surfaces depended on both male and female cultivar. For instance, Golden Delicious pollen germination was higher followed by Red Delicious and then Crabapples on all cultivars in 2013, whereas pollen from Red Delicious had a higher germination rate on Honeycrisp followed by Golden Delicious in 2014.

A strong male / female interaction was found for both years except the third sampling day of 2013 and the first sampling day of 2014. For instance, the visible germinated pollen of Golden Delicious and Red Delicious was greater than Crabapples on Honeycrisp stigmas on all sampling dates in 2013. However, crabapple was similar or higher than the other two pollinizers when applied to Gala and Fuji (Table 2.1). Similar trends were observed in 2014 (Table 2.2). Pollen germination was similarly low with crabapple on Honeycrisp, but it performed better on the other cultivars in both years (Tables 2.1, 2.2). Golden Delicious was superior to other pollinizers in 2013 on both Honeycrisp and Fuji, but was similar to Red Delicious on Gala (Table 2.1). In 2014, Golden Delicious performed similarly to Red Delicious in terms of pollen germination through the stigma (Table 2.2).

Table 2.1: Effects of pollen source on pollen germination on stigmatic surface and number of pollen tubes penetrating the stigma of ‘Honeycrisp’, ‘Gala’ and ‘Fuji’ (2013)^Z

Male	Rating of visible pollen tube growth on stigmatic surface (0-10) ^Y				Number of visible pollen tubes penetrating the stigma			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Honeycrisp								
Crabapple	3.6±0.5 b	4.5±0.5 b	1.8±0.4 b	1.5±0.4 c	11.6±1.0 b	6.1±1.1 b	1.6±0.9 b	5.7±1.2 c
Red Delicious	6.1±0.5 a	4.5±0.5 b	2.3±0.4 b	4.6±0.4 b	18.2±1.0 a	4.6±1.1 b	3.6±0.9 b	10.3±1.2 b
Golden Delicious	6.2±0.5 a	7.1±0.5 a	5.4±0.4 a	7.7±0.4 a	18.2±1.0 a	15.6±1.1 a	13.6±0.9 a	15.0±1.2 a
Significance	***	***	***	***	***	***	***	***
Gala ^W								
Crabapple	5.6±0.5 a	2.1±0.3 b	5.1±0.5 a	3.3±0.6 b	11.2±1.4 a	10.5±1.1 b	12.5±0.9 b	8.7±1.4b
Red Delicious	3.7±0.5 ab	2.8±0.3 b	5.5±0.5 a	7.1±0.6 a	6.0±1.3 b	9.3±1.1 b	16.8±0.9 a	16.2±1.4a
Golden Delicious	2.5±0.5 b	7.1±0.3 a	6.8±0.6 a	5.2±0.6 ab	9.3±1.3 ab	15.7±1.1 a	19.8±0.9 a	16.8±1.4a
Significance	***	***	NS	***	**	***	***	***
Fuji								
Crabapple	5.5±0.5 a	4.4±0.5 b	6.0±0.6 a	5.4±0.5 a	7.2±1.5 a	7.7±1.2 a	5.8±1.3 b	7.0±1.0 a
Red Delicious	2.4±0.7b	5.5±0.5 ab	6.3±0.6 a	3.6±0.5 b	4.0±1.8 a	10.0±1.3 a	12.2±1.3 a	2.2±1.0 b
Golden Delicious	5.1±0.5 a	6.5±0.5 a	7.2±0.6 a	3.6±0.6 b	8.2±1.4 a	8.7±1.3 a	13.4±1.3 a	6.0±1.2 a
Significance	***	**	NS	**	NS	NS	***	***
Male	NS	***	***	***	*	***	***	***
Female	**	***	**	NS	***	**	***	***
Male x Female	***	**	NS	***	***	***	***	***

^ZMean and standard deviation of 25 observations in each cell respectively.

^YRating of visible pollen tubes germinated on the stigmatic surface scaled 0 to 10 where 0 = no pollen tubes visible on the stigmatic surface; 1 = 1% to 10% stigmatic surface covered; 2 = 11% to 20%; 3 = 21% - 30%; 4 = 31% to 40%; 5 = 41% to 50%; 6 = 51% to 60%; 7 = 61% to 70%; 8 = 71% to 80%; 9 = 81% to 90% and 10 = 91% to 100% stigmatic surface was covered by pollen tubes.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

^WData of pollen tube germinated on the stigmatic surface of Gala were transformed by the square root of Y'

Table 2.2: Effects of pollen source on pollen germination on stigmatic surface and number of pollen tubes penetrating the stigma of ‘Honeycrisp’, ‘Gala’ and ‘Fuji’ (2014)^Z

Male	Rating of visible pollen tubes growth on stigmatic surface (0-10) ^{Y W}				Number of visible pollen tubes penetrating through the stigma			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Honeycrisp								
Crabapple	0.6±0.1 a	0.7±0.5 b	0.8±0.2 b	0.7±0.2 b	2.6±0.7 a	4.8±1.7 a	8.2±1.5 b	3.0±1.9 b
Red Delicious	0.4±0.1 a	1.5±0.5 a	1.9±0.2 a	2.1±0.2 a	1.0±0.6 ab	8.6±1.7 a	11.5±1.5 ab	14.9±1.9 a
Golden Delicious	0.6±0.1 a	2.4±0.5 a	1.2±0.1 a	1.4±0.2 a	0.0±0.6 b	8.5±1.7 a	14.6±1.5 a	14.0±1.8 a
Significance	NS	**	***	***	**	NS	**	***
Gala								
Crabapple	0.6±0.1 a	1.0±0.4 a	1.1±0.1 a	3.0±0.3 a	6.4±1.2 a	9.7±1.3 b	11.6±1.3 b	17.6±1.3 a
Red Delicious	0.7±0.1 a	1.6±0.4 a	1.3±0.1 a	0.8±0.3 b	4.1±1.2 ab	15.2±1.3 a	16.8±1.3 a	9.4±1.3 b
Golden Delicious	0.5±0.1 a	2.6±0.4 a	1.6±0.1 a	1.0±0.3 b	0.1±1.2 b	11.9±1.3 ab	13.6±1.3 ab	13.6±1.3 ab
Significance	NS	NS	NS	***	***	**	**	***
Fuji								
Crabapple	0.6±0.1 a	2.4±0.2 a	1.0±0.4 b	1.0±0.2 a	7.8±1.4 a	17.0±1.2 a	12.0±1.4 a	9.6±1.6 a
Red Delicious	0.6±0.1 a	1.2±0.2 a	1.1±0.4 b	2.5±0.2 a	6.1±1.4 a	16.0±1.2 a	14.7±1.4 a	14.8±1.6 a
Golden Delicious	0.5±0.1 a	1.3±0.2 a	4.3±0.4 a	1.3±0.2 a	0.0±1.4 b	13.9±1.3 a	14.3±1.4 a	11.2±1.6 a
Significance	NS	NS	***	NS	***	NS	NS	NS
Male	NS	*	***	*	***	*	**	*
Female	NS	NS	***	NS	***	***	NS	NS
Male x Female	NS	***	***	***	NS	NS	NS	***

^ZMean and standard deviation of 25 observations in each cell respectively.

^YRating of visible pollen tubes germinated on the stigmatic surface scaled 0 to 10 where 0 = no pollen tubes visible on the stigmatic surface; 1 = 1% to 10% stigmatic surface covered; 2 = 11% to 20%; 3 = 21% - 30%; 4 = 31% to 40%; 5 = 41% to 50%; 6 = 51% to 60%; 7 = 61% to 70%; 8 = 71% to 80%; 9 = 81% to 90% and 10 = 91% to 100% stigmatic surface was covered by pollen tubes.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

^WData of pollen tube germinated on the stigmatic surface were transformed by the ‘Y^{*25}’.

Table 2.3: Effects of pollen source on pollen tube growth down the style of ‘Honeycrisp’, ‘Gala’ and ‘Fuji’ (2013).^Z

Male	Average length of the longest pollen tubes (mm)				Average length of the style (mm)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Honeycrisp								
Crabapple	2.9±0.4 b	7.5±1.0 b	1.1±1.0 b	9.5±0.7 b	12.7±0.3 a	14.6±0.4 a	13.5±0.4 a	12.6±0.3 ab
Red Delicious	7.2±0.4 a	8.2±1.0 ab	9.7±1.0 a	10.8±0.7 ab	13.4±0.3 a	12.5±0.4 b	12.4±0.4 a	12.0±0.3 b
Golden Delicious	5.8±0.4 a	11.3±1.0 a	11.1±1.0 a	13.2±0.8 a	10.9±0.3 b	13.7±0.4 ab	13.8±0.4 a	13.7±0.3 a
Significance	***	**	***	***	***	***	NS	***
Gala								
Crabapple	4.7±0.5 a	4.2±0.5 b	9.7±0.6 b	6.1±0.9 b	12.2±0.4 a	13.3±0.4 a	13.6±0.4 a	11.7±0.5 a
Red Delicious	2.9±0.5 b	4.9±0.5 b	11.5±0.6 b	10.6±0.8 a	12.2±0.4 a	12.3±0.4 ab	11.6±0.4 b	11.2±0.5 a
Golden Delicious	3.2±0.5 ab	10.3±0.5 a	14.1±0.7 a	11.3±0.9 a	11.8±0.4 a	11.9±0.4 b	14.2±0.4 a	11.9±0.5 a
Significance	**	***	***	***	NS	*	***	NS
Fuji								
Crabapple	1.8±0.6 b	3.8±0.8 a	4.1±0.8 b	7.0±1.0 a	11.3±1.1 a	12.8±0.3 a	11.2±0.4 a	10.3±0.5 a
Red Delicious	1.1±0.7 b	5.0±0.9 a	10.0±0.8 a	4.3±1.0 a	14.0±1.3 a	11.7±0.3 b	11.5±0.4 a	11.5±0.5 a
Golden Delicious	3.9±0.5 a	5.6±0.8 a	8.0±0.8 a	5.7±1.1 a	11.6±1.0 a	11.8±0.3 b	10.5±0.4 a	11.4±0.5 a
Significance	***	NS	***	NS	NS	*	NS	NS
Male	*	***	***	**	*	***	**	*
Female	***	***	***	***	NS	***	***	***
Male x Female	***	NS	***	***	NS	NS	***	NS

^ZMean and standard deviation of 25 observations in each cell respectively.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 2.4: Effects of pollen source on pollen tube growth down the style of ‘Honeycrisp’ and ‘Gala’ and ‘Fuji’ (2014).^Z

Male	Average length of the longest pollen tubes (mm)				Average length of the style (mm)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
	Honeycrisp							
Crabapple	1.0±0.2 a	1.5±0.5 b	3.1±0.6 b	4.9±0.7 b	10.8±0.4 a	11.1±0.3 a	10.6±0.3 b	12.4±0.3 a
Red Delicious	0.1±0.2 b	3.2±0.5 a	4.0±0.6 a	10.6±0.7a	8.6±0.4 b	11.0±0.3 a	11.7±0.3 a	11.5±0.3 ab
Golden Delicious	0.0±0.2 b	3.5±0.5 a	5.1±0.6 a	9.7±0.7 a	9.2±0.4 b	10.6±0.3 a	9.2±0.3 c	10.9±0.3 b
Significance	***	**	*	***	***	NS	***	**
	Gala							
Crabapple	2.7±0.3 a	7.1±0.6 a	10.3±0.7 a	11.9±0.8 a	10.9±0.3 a	11.1±0.3 ab	11.1±0.4 a	12.0±0.4 a
Red Delicious	0.5±0.3 b	4.0±0.6 b	9.8±0.7 a	9.9±0.8 ab	10.5±0.3 a	12.1±0.3 a	11.6±0.4 a	11.3±0.4 a
Golden Delicious	0.1±0.3 b	6.6±0.6 a	10.0±0.7 a	8.8±0.8 b	9.0±0.3 b	10.2±0.3 b	10.9±0.4 a	10.8±0.4 a
Significance	***	**	NS	**	***	***	NS	NS
	Fuji							
Crabapple	2.6±0.3 a	7.9±0.6 a	9.5±0.8 ab	7.4±0.9 b	11.3±0.4 a	11.0±0.3 a	11.4±0.3 ab	9.9±0.4 b
Red Delicious	0.5±0.3 b	5.3±0.6 b	10.2±0.8 a	10.7±0.9 a	9.7±0.5 b	10.9±0.3 a	12.2±0.3 a	12.1±0.4 a
Golden Delicious	0.0±0.3 b	7.0±0.6 ab	7.0±0.8 b	5.3±0.9 b	9.4±0.5 b	10.8±0.3 a	10.6±0.3 b	9.3±0.4 b
Significance	***	**	**	***	*	NS	***	***
Male	***	**	NS	***	***	**	***	***
Female	**	***	***	**	NS	NS	**	***
Male x Female	**	***	**	***	NS	*	NS	***

^ZMean and standard deviation of 25 observations in each cell respectively.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

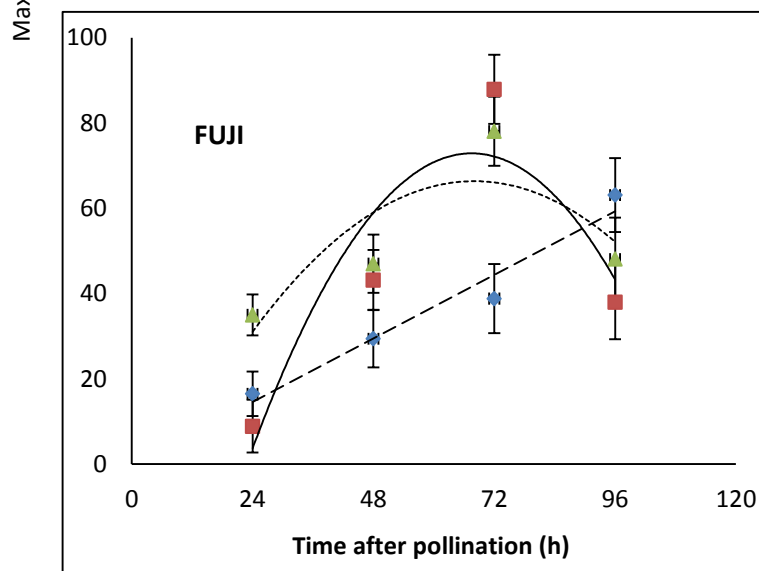
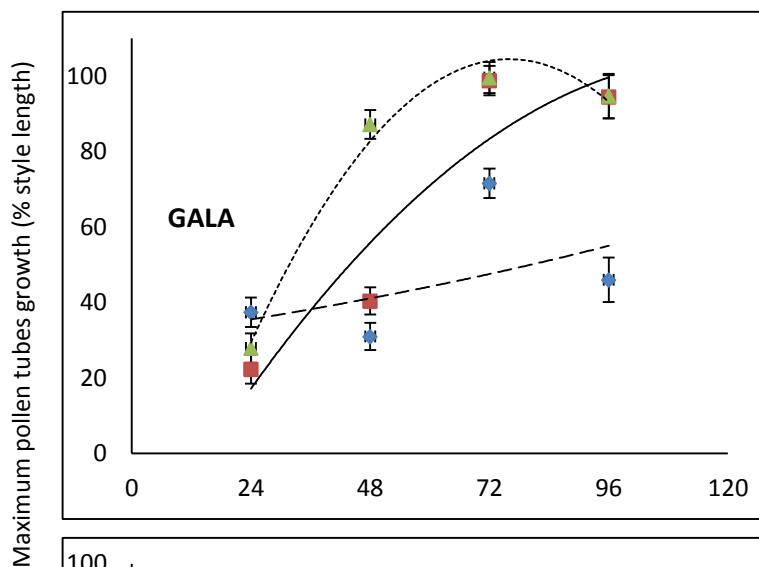
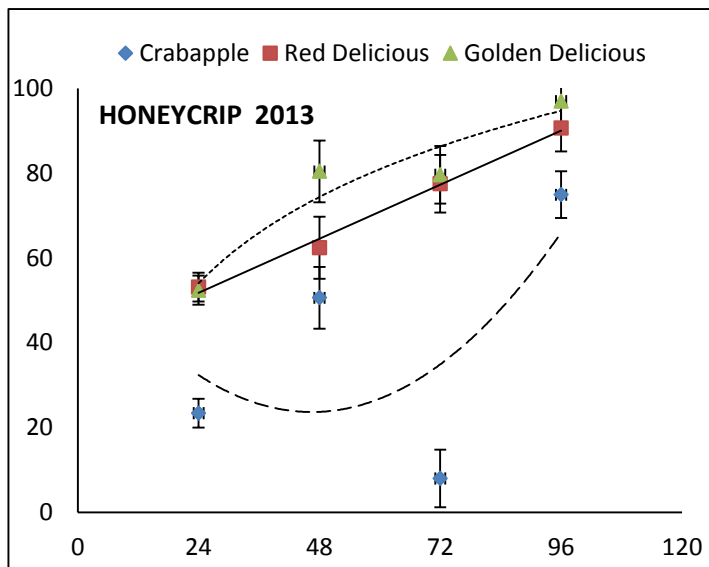


Figure 2.5: Effect of pollen source on pollen tubes growth of ‘Honeycrisp’, ‘Fuji’ and ‘Gala’ apple in 2013. In Honeycrisp style, Crabapple pollen tubes grew at $y = 0.0172x^2 - 1.6015x + 60.875$, $R^2 = 0.3882$; Red Delicious: $y = 0.5317x + 39$, $R^2 = 0.9919^{**}$; Golden Delicious, $29.355\ln(x) - 39.264$, $R^2 = 0.9133^{**}$. In Gala style, Crabapple pollen tubes grew at $y = 30.702e^{0.0061x}$, $R^2 = 0.2739$; Red Delicious, $y = -0.0097x^2 + 2.3125x - 32.75$, $R^2 = 0.8805^*$; Golden Delicious, $y = -0.0279x^2 + 4.2304x - 56.1$, $R^2 = 0.9869^{**}$. In Fuji style, Crabapple pollen tubes grew at $y = 0.6217x - 0.35$, $R^2 = 0.9575^{**}$; Red Delicious, $y = -0.0365x^2 + 4.9354x - 93.75$, $R^2 = 0.8275^*$; Golden Delicious, $y = -0.0182x^2 + 2.4808x - 18.05$, $R^2 = 0.6817$.

Comparable results were found for pollen tubes growing down the style of treated cultivars in both 2013 and 2014 years (Table 2.3, 2.4). Pollen tube growth increased with time after pollination. There was an interreaction between female genotype and pollinizer for length of the longest pollen tube. Crabapple pollen grew slowly within Honeycrisp styles in both years (Tables 2.3, 2.4). In 2013, Golden Delicious and Red Delicious tended to have the longest pollen tubes in Honeycrisp and Gala whereas there was no difference among pollinizers in Fuji styles. In 2014 Crabapple tended to have the longest pollen tubes within Gala and Fuji, but Golden Delicious and Red Delicious were superior in Honeycrisp (Figures 2.5, 2.6).

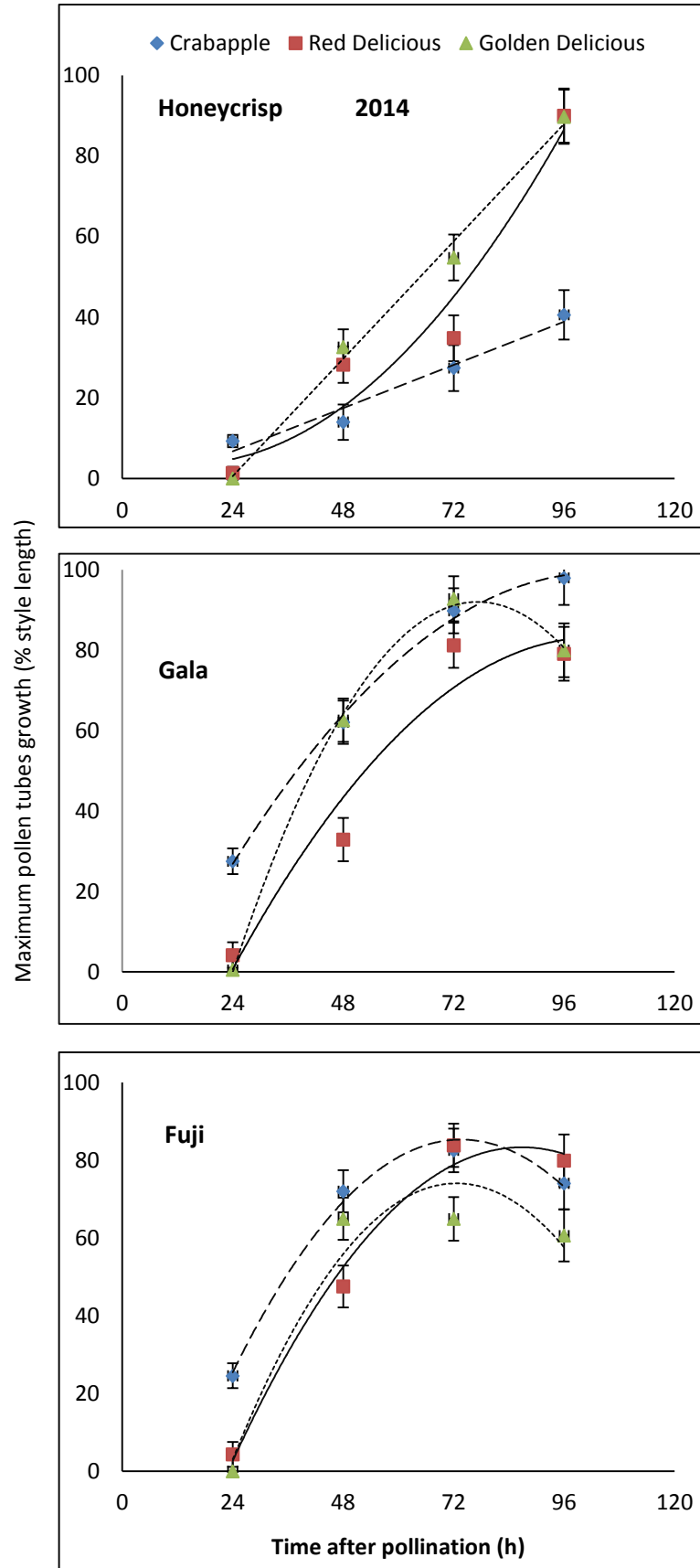


Figure 2.6: Effect of pollen source on pollen tubes growth of ‘Honeycrisp’, ‘Fuji’ and ‘Gala’ apple in 2014. In Honeycrisp style, Crabapple pollen tubes grew at $y = 0.4471x - 4$, $R^2 = 0.9632^{**}$; Red Delicious, $y = 0.0123x^2 - 0.3442x + 6$, $R^2 = 0.94^{**}$; Golden Delicious, $y = 1.2138x - 28.55$, $R^2 = 0.99^{**}$. In Gala style, Crabapple pollen tubes grew at $y = -0.0115x + 2.3717x - 23.45$, $R^2 = 0.99^{**}$; Red Delicious, $y = -0.0134x^2 + 2.7417x - 57.4$, $R^2 = 0.94^*$; Golden Delicious, $y = -0.0325x^2 + 5.02x - 101.82$, $R^2 = 0.99^{**}$. In Fuji style, Crabapple pollen tubes grew at $y = -0.024x^2 + 3.579x - 46.5$, $R^2 = 0.99^{**}$; Red Delicious, $y = -0.02x^2 + 3.549x - 70.77$, $R^2 = 0.98^{**}$; Golden Delicious, $y = -0.03x^2 + 4.3617x - 84.35$, $R^2 = 0.939^{**}$.

While both pollen germination on the stigmatic surface and pollen tube growth within the style are important, the most critical consideration is the number of pollen tubes reaching the base of the style and fertilizing the egg cell. Pollen tubes that reached the base of the style linearly increased overtime upon pollination (Table 2.5). Very few (2013) and no (2014) crabapple pollen tubes reached the base of Honeycrisp styles (Table 2.5). In 2013 Red Delicious and Golden Delicious pollen performed similarly in all cultivars in terms of number of pollen tubes reaching the base of the style. However in 2014 Red Delicious was superior for both Honeycrisp and Fuji. In 2013 fewer pollen tubes of Crabapple were reached the base of Honeycrisp style, but none was reached in 2014.

The number of pollen tubes that reached the base of styles depended on pollen source, cultivar and their interaction. Generally pollen tube migration through the style

took approximately four days after pollination to reach the base of the style, which implies the time period required for fruit set.

Table 2.5: Effects of pollen source on pollen tubes that reached the base of the style of ‘Honeycrisp’, ‘Gala’ and ‘Fuji’ (2013) and (2014).^Z

Male	Number of pollen tubes that reached the base of style (2013)				Number of pollen tubes that reached the base of style (2014)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
	Honeycrisp							
Crabapple	0.0±0.0 a	0.0±0.0 b	0.0±0.0 b	1.3±0.9 b	0.0±0.0 a	0.0±0.0 a	0.0±0.0 b	0.0±0.0 c
Red Delicious	0.0±0.0 a	0.7±0.6 b	2.0±0.4 a	4.5±0.9 a	0.0±0.0 a	0.0±0.0 a	0.0±0.1 b	3.6±0.4 a
Golden Delicious	0.0±0.0 a	4.0±0.6 a	3.2±0.4 a	6.9±0.9 a	0.0±0.0 a	0.0±0.0 a	0.2±0.1 a	1.4±0.4 b
Significance	NS	***	***	***	NS	NS	**	***
	Gala							
Crabapple	0.3±0.1 a	0.0±0.6 b	1.4±0.9 b	1.4±1.1 b	0.0±0.0 a	0.2±0.1 a	5.5±0.8 a	5.4±0.7 a
Red Delicious	0.0±0.1 a	0.0±0.6 b	7.5±0.9 a	7.1±1.0 a	0.0±0.0 a	0.0±0.1 a	1.6±0.8 b	2.7±0.7 b
Golden Delicious	0.0±0.1 a	4.3±0.6 a	8.2±0.9 a	6.0±1.1 a	0.0±0.0 a	0.0±0.1 a	3.1±0.8 ab	3.6±0.7 ab
Significance	NS	***	***	***	NS	NS	***	**
	Fuji							
Crabapple	0.1±0.1 a	0.0±0.2 a	0.5±1.2 b	2.6±0.4 a	0.0±0.0 a	0.3±0.1 a	2.4±0.6 a	2.3±0.7 b
Red Delicious	0.0±0.1 a	0.2±0.2 a	4.4±1.2 b	0.4±0.4 b	0.0±0.0 a	0.0±0.1 a	1.6±0.6a	5.5±0.7 a
Golden Delicious	0.2±0.1 a	0.5±0.2 a	10.0±1.2 a	0.2±0.5 b	0.0±0.0 a	0.0±0.1 a	3.2±0.6a	1.7±0.7b
Significance	NS	NS	***	***	NS	NS	NS	***
Male	NS	***	***	***	NS	*	**	**
Female	NS	**	***	***	NS	NS	***	***
Male x Female	NS	***	**	***	NS	NS	**	***

^ZMean and standard deviation of 25 observations in each cell respectively.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

2.4.2 Experiment 2. Effect of Pollen Source on Fruit Set, Fruit Quality, and Return Bloom in Apple cultivars differing in Biennial Bearing Potential

2.4.2.1 Fruit Set

As previously indicated, fruit set was rated every two weeks. Overall, most fruits dropped within two weeks after pollination. Fewer fruit were set in Honeycrisp apple pollinated by crabapple regardless of the particular crabapple species used (Ralph-Shay in 2013 and *Malus floribunda* in 2014) (Table 2.6, 2.7). In 2013, Red Delicious pollen resulted in the highest fruit set followed by Golden Delicious and crabapple in all cultivars, but in 2014 all pollinizers performed similarly with all cultivars with the exception of crabapple on Honeycrisp which resulted in very low fruit set (Table 2.6, 2.7).

Table 2.6: Number of fruit set (Maximum 30/combination (Female x Male)), 2013

Female	Male		
	Crabapple	Golden Delicious	Red Delicious
Honeycrisp	7	18	20
Gala	15	19	26
Fuji	7	15	20

* Crabapple used was Ralph Shay

Table 2.7: Number of fruit set (maximum 30/combination (female x male)), 2014

Female	Male		
	Crabapple	Golden Delicious	Red Delicious
Honeycrisp	2	17	16
Gala	23	23	25
Fuji	17	24	19

* Crabapple used was *Malus floribunda*

2.4.2.2 Fruit Quality

2.4.2.2.1 Fruit Fresh Weight

Fruit fresh weight was not affected by pollen source across the treatments regardless of the experimental years except in Fuji in 2013 where crabapple pollen resulted in smaller fruit (Table 2.8, 2.9). There was neither male, female main effects nor their interaction associated with fruit fresh weight for both years.

2.4.2.2.2 Soluble Solids Concentration (SSC)

Soluble Solids Concentration (SSC) of the fruit was not significantly affected by pollen source for all the combinations regardless of the year (Table 2.8, 2.9). This was presumably due to the strong male x female interaction in both years, but pollinizer had indistinct effects within an individual female parent (male female interaction plots are in the appendix B).

2.4.2.2.3 Starch Index

Starch index was constant in all the treatments and pollen source had no influential impact on starch except for Gala in 2013, where fruit pollinated by crabapples were slightly less ripe as indicated by a lower starch rating (Table 2.8, 2.9). Neither male nor female and their interaction influenced fruit starch index in either experimental year.

2.4.2.2.4 Seed Number

Significant differences were determined in seed number among the treatments. Seed number was affected by male female and interaction and individual pollen donor had different attribution. Overall, seed number was lower in all cultivars when pollinated by crabapples in 2013. Similar trends were evident in 2014 although were only significant for Gala.

Table 2.8: Effects of pollen source on fruit quality of ‘Honeycrisp’, ‘Gala’ and ‘Fuji’ (2013)^Z

Male	Fruit weight (g)	Starch	SSC	Seed number	Seed weight (g)	Return bloom (%) ^Y
Honeycrisp						
Crabapple	376.7±26.3 a	5.7±0.1 a	15.7±0.3 a	3.7±0.8 b	0.3±0.1 b	0
Red Delicious	360.1±15.5 a	5.9±0.1 a	15.3±0.2 a	8.0 ±0.5 a	0.6±0.1 a	70
Golden Delicious	355.7±16.4 a	5.8±0.1 a	15.4±0.2 a	8.3±0.5 a	0.6±0.1 a	53
Significance	NS	NS	NS	***	***	NS
Gala						
Crabapple	180.8±10.3 a	5.0±0.2 b	15.3±0.3 a	2.6±0.6 b	0.2±0.1 b	100
Red Delicious	197.4±7.8 a	5.7±0.1 a	15.5±0.2 a	5.7±0.5 a	0.4±0.1 a	100
Golden Delicious	186.4±9.1 a	5.8±0.2 a	15.5±0.3 a	6.1±0.6 a	0.4±0.1 a	96
Significance	NS	**	NS	***	***	NS
Fuji						
Crabapple	249.6±22.7 b	6.0±0.1 a	18.7±0.6 a	3.0±0.9 b	0.2±0.1 b	63
Red Delicious	310.9±14.3 a	6.0±0.1 a	18.3±0.4 a	5.4±0.6 ab	0.3±0.1 b	75
Golden Delicious	311.8±16.5 a	5.9±0.1 a	17.1±0.5 a	7.3±0.7 a	0.5±0.1 a	80
Significance	*	NS	NS	**	***	NS
Male	NS	NS	***	***	***	NS
Female	NS	NS	*	***	***	NS
Male x Female	NS	NS	***	***	**	NS

^ZMeans and standard error are represented in each cell.

^YReturn bloom is calculated as the number of flowering bud divided by the total number of buds times 100.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

2.4.2.2.5 Seed Fresh Weight

Seed fresh weight generally followed similar trends as those for seed number. All cultivars had lower seed fresh weight when crabapples were used as the pollinizer in 2013, although once again this was only significant for Gala in 2014 (Table 2.8, 2.9).

2.4.2.2.6 Seed Number Affecting Fruit Fresh Weight

The seed number per fruit was significantly correlated with fruit fresh weight of Honeycrisp apple in 2014 regardless of the pollinizer (Figure 2.7). Gala fruit fresh weight was similarly positively related to seed number per fruit regardless of pollinizer or year of experiment (Figure 2.8). However, there was no relationship between seed number per fruit and fruit fresh weight of Fuji apple in either year.

2.4.2.2.7 Return Bloom

Pollen source and cultivar had no influence on return bloom in either year (Table 2.8, 2.9). The effects of other related components (i.e. fruit fresh weight, seed number, and seed weight), did not have any significant impact on return bloom.

Table 2.9: Effects of pollen source on fruit quality of ‘Honeycrisp’, ‘Gala’ and ‘Fuji’ (2014)^Z

Male	Fruit weight (g)	Starch	SSC	Seed number	Seed weight (g)	Return bloom (%) ^Y
Honeycrisp						
Crabapple	324.5±48.0 a	5.0±0.4 a	13.5±0.6 a	4.5±1.5 a	0.3±0.1 a	100
Red Delicious	355.7±16.5 a	5.8±0.1 a	13.5±0.2 a	7.6±0.5 a	0.5±0.1 a	65
Golden Delicious	307.7±17.0 a	5.8±0.1 a	13.8±0.2 a	6.4±0.5 a	0.4±0.1 a	67
Significance	NS	NS	NS	NS	NS	NS
Gala						
Crabapple	165.5±6.3 a	5.5±0.2 a	16.0±0.1 a	6.7±0.6 b	0.4±0.1 b	100
Red Delicious	168.7±6.3 a	5.4±0.2 a	15.9±0.1 a	8.6±0.6 a	0.5±0.1 a	100
Golden Delicious	161.5±6.1 a	5.8±0.2 a	16.0±0.1 a	8.0±0.6 a	0.5±0.1 a	96
Significance	NS	NS	NS	*	*	NS
Fuji						
Crabapple	212.0±12.4 a	5.2±0.2 a	17.5±0.3 a	5.2±0.9 a	0.3±0.1 a	89
Red Delicious	210.5±10.4 a	5.4±0.2 a	17.2±0.3 a	7.2±0.8 a	0.4±0.1 a	96
Golden Delicious	205.5±11.7 a	5.1±0.2 a	17.5±0.3 a	7.1±0.9 a	0.5±0.1 a	85
Significance	NS	NS	NS	NS	NS	NS
Male	NS	NS	**	*	NS	NS
Female	NS	NS	NS	***	***	*
Male x Female	NS	NS	**	***	***	NS

^ZMeans and standard error are represented in each cell.

^YReturn bloom is calculated as the number of flowering bud divided by the total number of buds times 100.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

2.5 Discussion

Yoder et al (2009) reported that pollen tube growth down the style depends on temperature and takes approximately 24 up to 96 hours depending on the temperature. Namikawa (1923) reported 48 hours were required for pollen tube growth down the entire style and fertilize the egg, whereas Williams (1970) reported 5 to 7 days and Albuquerque Junior *et al.* (2010) reported 120 hours. However, our results revealed that it takes a maximum of 96 hours for pollen tubes to reach the base of the style under field conditions although pollen source had a significantly influence. For instance, crabapple pollen tubes had the slowest growth down Honeycrisp styles in both years but grew the fastest in Gala styles in 2014. Similarly, Golden Delicious pollen tubes grew the fastest in Honeycrisp and Gala styles in 2013 but in 2014 was only intermediate in Honeycrisp and slowest in Fuji styles (Table 2.5).

Petropoulou and Alston (1998), and Jackson (2003) proposed that apple pollen tube germination rate on the stigmatic surface depends on the pollen donor and environmental temperatures at the time of pollination. Petropoulou and Alston (1998) reported that ‘Spartan’ pollen had a higher germination percentage than that of ‘Cox’s Orange Pippin’ and ‘Idared’ regardless of the temperature, but the difference among pollinizers was more pronounced at 8-10° C and 14-16° C, respectively. Similarly, Albuquerque Junior *et al.* (2010) studied pollen donor effects on pollen germination capacity when they crossed 34 apple cultivars. They found that Condessa had the higher germination capacity while Princesa was considered as the best pollinizer in terms of having the highest number of anthers/flower, having the greatest pollen grains/anther, and subsequently having the greatest pollen germination capacity. These results coincide with our findings and support

the above proposed statements that the pollen source had a significant influence on pollen germination on the stigmatic surface as well as the growth down the style. For example, crabapple pollen had a lower germination rate on Honeycrisp stigmatic surfaces compared with on the stigmas of Gala and Fuji in both years. Crabapple pollen had higher germination percentage on Fuji stigmatic surfaces in 2013 and on Gala stigmas in 2014. The visualized pollen that germinated on the stigmatic surface of each cultivar was significantly different for each individual pollen source. For instance, Golden Delicious pollen germination was highest on Honeycrisp and Gala in 2013 but lowest on Gala in 2014.

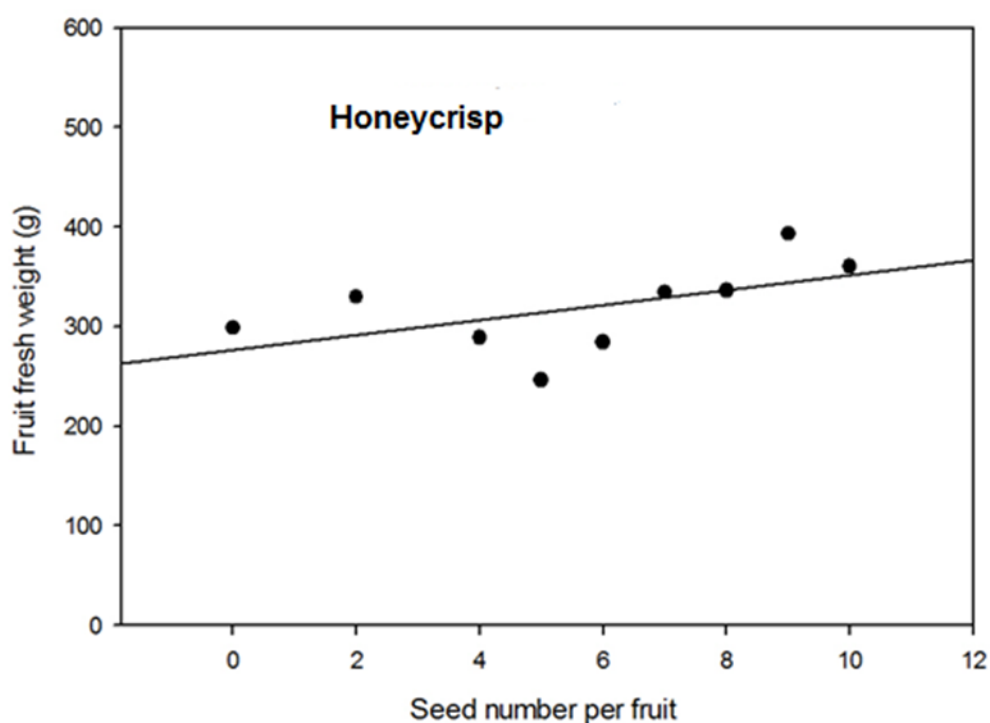


Figure 2.7: Effects of seed number per fruit on Honeycrisp apple fruit fresh weight in 2014. $y = 13.838x + 236.29$, $R^2 = 0.1918$, $P = 0.0085$

Likewise, several studies reported that semi- and cross-incompatibilities have become more prevalent as more inter-related cultivars are grown. These kind of results could become critical considerations when planning orchards in the future. For instance, Alston (1996) showed that even though ‘Cox’s Orange Pippin’ and ‘Idared’ are completely cross-compatible, a much lower percentage of fruit set was recorded following hybridization between their progenies. This was suggested to be a result of sharing the same alleles from their ancestors, creating semi-incompatibility. Analogous results were found in our experiment where crabapple pollen tubes had the lowest germination and slowest growth in ‘Honeycrisp’ stigmas and styles and comparatively fewer pollen tubes reaching the base of the style regardless of the experimental year (for both the Ralph-Shay Crabapple in 2013 and *Malus floribunda* Crabapple in 2014). The presumptuous reason might be a semi-incompatibility of ‘Ralph-Shay’ and ‘*Malus floribunda*’ Crabapples with Honeycrisp, which resulted in fewer fruit set (Table 2.6, 2.7). Additionally, Delaplane and Mayer (2000) reported that overall closely-related apples cultivars, (for example, McIntosh, Early McIntosh, Cortland, and Macoun) don’t cross-pollinate each other well. However, our results did not support this statement. We found good pollen tube growth rates of Red Delicious (grandparent) and Golden Delicious (parent) in Gala styles in both 2013 and 2014 years.

Bessho *et al.* (2009) found that 5 out of 19 Crabapple cultivars performed as suitable and compatible pollinizers for two commercial Fuji and Tsugaru cultivars. They evaluated crabapples for their suitability as pollinizers for these two commercial cultivars in terms of bloom time, pollen compatibility, seed number, and productivity. They selected the following potential pollinizers: *M. baccata* 79091 and Sentinel for early

blooming cultivars, and *M. x atrosanguinea* 20004522, Red Bud and Snowdrift for mid-blooming cultivars. Among these cultivars, Sentinel was reported to produce fewer seed per fruit when it was crossed with Fuji. Meanwhile, Das *et al* (2011) reported that ‘Manchurian’ crabapple was found to be a very effective pollinizer for spur type ‘Oregon Spur’ apple in terms of fruit set and having higher blossom density among the combinations tested. Our results were consistent with these findings. For example, the percent fruit set of Fuji was comparatively lower in combinations crossed with ‘Ralph-Shay’ Crabapple in 2013, and relatively higher when pollinated by *M. floribunda* in 2014. However in contrast with earlier findings with crabapple (Delaplane and Mayer 2002), and Gala and Fuji (Bashir *et al.*, 2010) who found these cultivars to be effective pollinizers for commercial orchards, our results disputed this assertion. For example, crabapple pollen tubes grew the slowest in Honeycrisp styles and fewer pollen tubes reached the base of the styles. This resulted in fewer seeds per fruit as well as fewer or no Honeycrisp fruits set in combinations with either ‘Ralph-Shay’ Crabapple or *M. floribunda*. Likewise, Fuji apple, as previously indicated, produced fewer seed per fruit in combination with *M. floribunda* as compared to the other pollen donors.

Denne (1963), Keulemans *et al.* (1996), Volz *et al.* (1996), and Bashir *et al.* (2010) reported that fruit size, fruit weight, and fruit growth rate were positively linearly correlated with seed number per fruit. We also found such relationships with Gala apples with all the pollinizer combinations in both experimental years, and with Honeycrisp with all pollinizer combinations 2014. We also found that all cultivars had a lower seed number per fruit when pollinated by crabapples although fruit weight was not affected by pollinizer at all. Similar results were reported by Volz *et al.* (1996) when they evaluated

the influence of pollen source on fruit set, fruit final size, and fruit mineral concentrations.

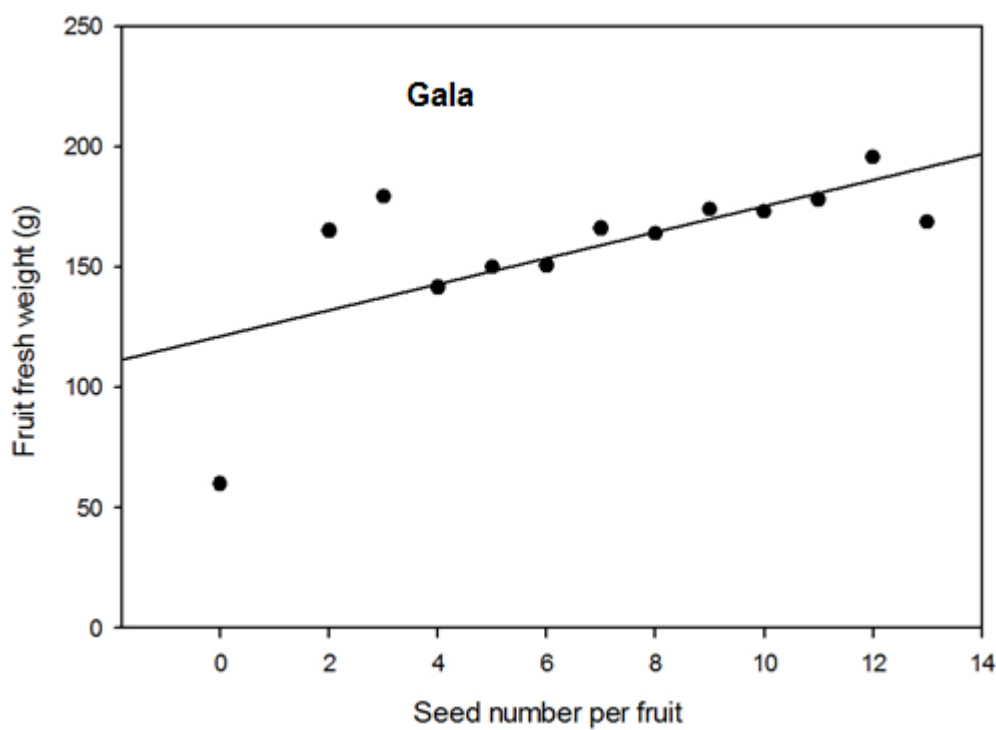


Figure 2.7: Effects of seed number per fruit on Gala apple fruit fresh weight at $y = 4.6412x + 129.13$, $R^2 = 0.1956$, and $P\text{-value} = 0.0001$

There are few studies examining the effect of pollen source on return bloom.

However, many fruit parameters such as seed number per fruit, fruit weight, fruit size, and shoot length have been extensively studied. Chan and Cain (1967), and Neilsen (1998) reported that a linear negative correlation between seed number per fruit and return bloom. When fruits remained until harvest time return bloom declined significantly with increasing seed number per fruit. However, in our experiment, neither the pollen source nor the seed number per fruit affected return bloom. Other fruit constituents such as fruit fresh weight and seed fresh weight did not have a significant effect on return boom.

2.6 Conclusion

Pollen source had a significant influence on pollen germination on the stigmatic surface, pollen tube growth down the style, and enrichment of pollen tubes to the base of the style. All these aspects of pollen germination and pollen tube growth increased with time after pollination under field conditions. Pollen tubes enhancement into the base of the style was affected by pollen donor, but overall 96 hours were required to reach the base of the style. Crabapple was not an effective pollinizer for Honeycrisp apple and very few fruit were set.

On average, Red Delicious pollen resulted in high percent fruit set followed by Golden Delicious and Crabapple, respectively. Fruit quality attributes and return bloom were generally not affected by pollen source. However, fewer seeds with less seed fresh weight were found when crabapple was used as a pollinizer in all cultivars. Seed number had significantly positive relationship with Gala and Honeycrisp fruit fresh weight regardless the pollen source, but there was no such relationship with 'Fuji'. Fruit fresh weight, and seed number had no significant influence on return bloom.

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CHAPTER 3. EXPERIMENT THREE: EFFECT OF POLLEN SOURCE AND SEED NUMBER ON FRUIT SET, FRUIT QUALITY AND RETURN BLOOM OF HONEYCRISP APPLE

3.1 Abstract

Pollen source, seed set and subsequent seed development are highly influential factors on the fertilization and fruit setting process. Pollinizer genotype in particular can have a remarkable impact on fertilization. However, there has been little information published on the most effective and compatible pollinizers for particular commercial cultivars. This study was conducted to determine the effect of three pollen sources, crabapple, Gala and Red Delicious, on Honeycrisp fruit set, fruit quality and subsequent return bloom. In addition, we studied the effect of seed number, manipulated by removing 0, 1, 2, 3 or 4 pistils from treated flowers, on these same aspects of Honeycrisp production.

There was no effect of pollen source on fruit fresh weight, soluble solids concentration and starch index. Seed number per fruit and seed fresh weight per fruit were significantly influenced by pollen source. When crabapple was used as a pollinizer, fruit contained fewer seeds and lower seed fresh weight compared to Red Delicious and Gala pollinizers. Pistil number had a significant positive linear relationship with seed number, although there was considerable variation. Fruit fresh weight was increased linearly with seed number. Pollen source had no influence on return boom regardless of

the year. Return boom was negatively related to fruit fresh weight. Likewise, percent return boom was negatively related to seed number per fruit. These results suggest that pollen source and seed number per fruit influence fruit set, fruit quality, biennial bearing potential of Honeycrisp, and therefore should be factors that are considered in the orchard design process. Based on our findings, we recommend growers to do not plant Ralph-Shay and *Malus floribunda* Crabapples as pollinizers for Honeycrisp.

3.2 Introduction

Honeycrisp pollination is commonly considered to be effectively pollinated by any diploid cultivars that have synchronous flowering (Cline and Gardner, 2005). Pollinizer trees should be reliable annual producers of flowers and be precocious. Honeycrisp is a valuable cultivar but has the potential to be extremely biennial in its bearing habit (Luby and Bedford, 1992; Robinson *et al.*, 2009). Both high and low crop load situations affects the quality of the fruit, tree growth, fruit size, fruit color, storage disorders and subsequent return boom (Robinson and Watkins, 2003).

Honeycrisp apple has a strong tendency for biennial bearing where it bears heavy crops in one year followed by light crops in the following year. Biennial bearing in apple is influenced by several factors, most importantly crop load, although the ratio between carbohydrates and nitrogen, and endogenous hormonal activity may also play a role (Jonkers, 1979).

A number of studies have investigated the causes and best practices for managing biennial bearing. Robinson *et al.* (2009) reported a consistent negative linear relationship between crop load and return bloom, although this relationship depended on the age of the tree. The magnitude of the negative relationship declined as tree aged. Similar results were reported by Wright *et al.* (2006), who showed that the time of thinning is crucial for return bloom. Nichols *et al.* (2008) studied the relationship between vegetative growth and return bloom. They found that increasing the severity of spur pruning increased shoot number and shoot growth and decreased biennial bearing.

A linear positive correlation between seed number per fruit and fruit size, fruit weight, and fruit growth rate, has been repeatedly reported (Denne, 1963; Keulemans *et*

al., 1996; Volz *et al.*, 1996; and Bashir *et al.*, 2010). Fruit weight and seed number per fruit was linearly increased using different pollen source Keulemans *et al.* (1996), and Bashir *et al.* (2010).

It has been repeatedly reported that seed number per fruit has a linear negative relationship with return bloom in apples. Chan and Cain (1967), Jonkers (1979) and Neilsen (1998) all showed a strong correlation between the seed content of the fruit in one year and the quantity of flowering in the next year.

Pollen source, seed number per fruit and their combination can be considered to be among the most influential factors on fruit set, fruit quality, and return bloom, and should be considered during orchard design. Several studies reported pollen source effect on fruit quality ((Nebel and Trump, 1932; Nebel, 1936; Kumar *et al.*, 2003). However, few studies have been conducted on the direct effect of pollen source on both fruit quality and return bloom in apple. However, since pollination significantly increases seed set in apple, it seems logical to suggest that factors influencing pollination may also influence return bloom in the following year. We therefore conducted an experiment to determine the impact of pollen source and seed number per fruit on fruit set, fruit quality, and subsequent return bloom in apple to enable growers to better design their orchards in terms of choosing commercially compatible combinations of cultivars

3.3 Materials and Methods

The experiment was conducted in 2013 and repeated in 2014 at the Purdue University Meigs Research Farm, Lafayette, Indiana, USA. Pollen of three cultivars, crabapple, Red Delicious and Gala was evaluated on Honeycrisp/M.9 trees planted in 2010. ‘Ralph Shay’ crabapple was used in 2013 but was found to be a poor pollinizer of Honeycrisp, so a different crabapple (*Malus floribunda*) was used in 2014. Selected trees were netted in late-April, prior to flower opening, to avoid cross-pollination by bees.

3.3.1 Pollen viability test

Pollen was tested in the laboratory in petri dishes prior to pollination to determine viability (Figure 2.2). Following the method used by Yoder *et al.* (2009), pollen was placed on a medium of 1 % agarose, 10 % sucrose and 10 ppm boric acid at room temperature for 24 hours. Percent germination was visually observed under a light microscope. All pollen used in these experiments had a high percent germination (more than 80 %).

3.3.2 Pistils removal

Overall, apple flower consists a total of 5 pistils individually connected into a locule each encompasses two ovules (Anvari and Stösser, 1981 cited by Pratt, 1988). An apple ovary contains 10 ovules which results in a theoretical maximum of 10 seeds per fruit. To investigate the effect of seed numbers on fruit development and return bloom we manipulated seed numbers by removing 0, 1, 2, 3 or 4 pistils from treated flowers with four single flower replications for each pollinizer (Figure 3.1). Pistils were removed using

small scissors prior to (2103) and after (2014) manual pollination treatments were applied.



Figure 3.1: Flower pistil's removal prior to pollination using Fiskars 4 inch detail scissors.

3.3.3 Design of the experiment

The experimental layout was designed as a split-split-plot design (Tables 3.1, 3.2). Six uniform adjacent trees were selected each as a whole plot (block), removal of pistils was a sub-plot, and pollen source was a sub-sub plot. Flowering spurs were selected on each treated tree and randomly assigned to treatments using different colored tags.

Selected trees were netted in late-April before flower opening to avoid cross-pollination.

A total of 60 flowers were tagged on each tree (block), 20 to be pollinated by each pollen source were randomly selected when the flowering buds were at the tight-cluster stage. At the late-pink stage (just before flower completely opened), all anthers were removed to prevent the possibility self-pollination and flowers were hand-pollinated

using a number 2 brush. Only king flowers were pollinated for uniformity of the experiment and all the lateral flowers as well as all non-tagged flowers on the trees were either manually removed or dropped off. Pollen used for the experiment was collected from orchards in southern Indiana in 2013 and from branches placed in the greenhouse to force flower bud opening in 2014.

3.3.4 Fruit set

Selected flowers were rated on the treated trees every other week starting from the second week after pollination until one week before harvest.

3.3.5 Quality attributes measurement

Fruits were harvested during the normal commercial harvesting period for each cultivar. Harvest measurements included fruit fresh weight using *Mettler Toledo College Model: B3002 DeltaRange Scale. B3002DR*; Soluble Solids Concentration (SSC) using *Atago 3810 Digital Handheld Pocket Refractometer PAL-1*; total seed number per fruit; total seed fresh weight per fruit; and starch pattern index using the methods of *Reid et al.* (1982) where half fruit were dipped for 30 seconds in an iodine solution and rated on a 1-6 scale where 1 = a very dark-black color of the stained fruit, indicating higher starch content and 6 = very little staining.

3.3.6 Buds dissection to determine return bloom

Bourse buds on tagged spurs, which would be expected to produce flowers for the following year's crop, were collected at the time of leaf abscission. Buds were placed in a 5% acetic acid-based FAA (Formalin-Acetic-Alcohol) solution containing 50% ethyl alcohol, 5% glacial acetic acid, 10% formaldehyde and 35% distilled water. Buds were then dissected under a light microscope to determine reproductive or vegetative status (Figure 2.4).

3.3.7 Statistical analysis

Experiment was conducted and analyzed as split-split-plot design, as indicated previously. Statistical analyses included analysis of variance, Tukey multiple range test, and regression analysis. Logistic regression analysis was used for the binary data of flowering, using Statistical Analysis Systems Software for PC (SAS 9.4). Means and standard errors are reported, whereas for the binary data, Chi-square analysis was performed and percent flowering reported.

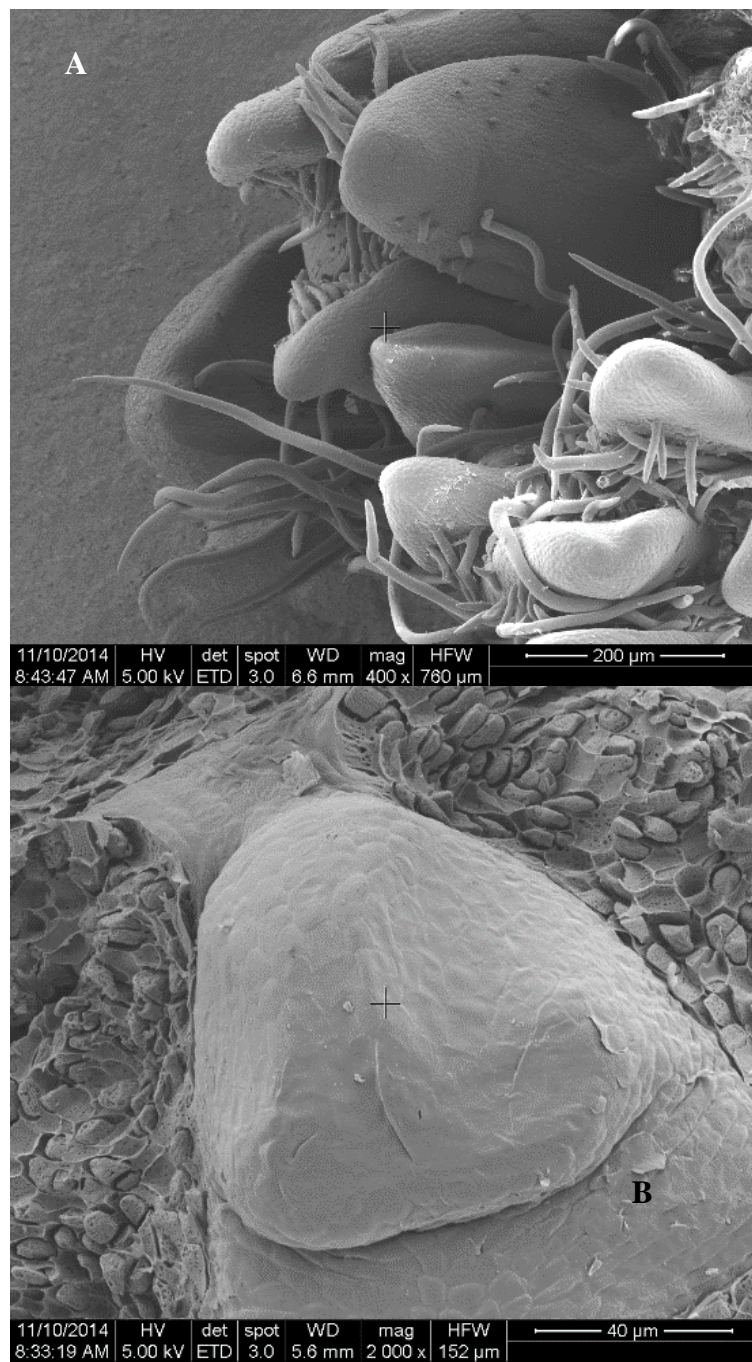


Figure 3.2: (A) Reproductive bud (B) Vegetative bud, images were acquired using Scanning electron microscope.

3.4 Results

3.4.1 Fruit Set

Overall, most fruit drop occurred within two weeks after pollination. Crabapple pollen resulted in the fewest fruit set regardless of the pollinizer species used (Ralph-Shay in 2013 and *Malus floribunda* in 2014) (Table 3.1, 3.2). Red Delicious pollen resulted in the highest percent fruit set followed by Gala and Crabapple. Pollen source clearly had a significant influence on fruit set.

3.4.2 Fruit Fresh Weight

Fruit fresh weight was not affected by pollen source across the treatments regardless of the experimental years except when four pistils remained in flowers during 2014 (Tables 3.3, 3.4).

3.4.3 Soluble Solids Concentration

Fruit soluble solids concentration was not significantly affected by pollen source in either year except when two pistils remained in flowers during 2013 (Tables 3.5, 3.6).

3.4.4 Starch Index

Starch index was not affected by pollinizer or pistil number in either year (Tables 3.7, 3.8).

Table 3.1. Number of fruit set (maximum 4/pistil/tree) on Honeycrisp in 2013 when pollinated by one of three pollinizers and where pistil number was manipulated manually.

	Tree (Block)																																							
	1					2					3					4					5					6					Means									
	Pistil					Pistil					Pistil					Pistil					Pistil					Pistil					Pistil									
Male	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Crabapple	0	0	1	0	0	0	0	2	1	2	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	1
Red Delicious	2	3	2	2	3	2	1	3	3	4	2	2	3	4	3	1	2	3	4	2	3	3	2	2	4	1	2	2	2	1	2	2	3	3	3	2	2	3	3	3
Gala	3	1	2	4	3	0	3	1	2	2	1	0	3	2	2	2	2	3	1	3	1	3	3	4	3	0	1	0	2	1	1	2	2	3	2	1	2	2	3	2

Table 3.2. Number of fruit set (maximum 4/pistil/tree) on Honeycrisp in 2014 when pollinated by one of three pollinizers and where pistil number was manipulated manually.

	Tree (Block)																																							
	1					2					3					4					5					6					Means									
	Pistil					Pistil					Pistil					Pistil					Pistil					Pistil					Pistil									
Male	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Crabapple	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Red Delicious	2	3	3	2	3	1	4	1	4	3	1	3	3	2	2	2	1	2	2	3	3	2	3	2	1	3	2	3	2	3	2	2	3	2	3	3	3	3	3	3
Gala	2	2	2	2	3	0	2	1	2	2	2	2	4	3	2	2	0	2	3	3	2	1	2	1	3	3	0	3	4	1	3	1	3	2	2	3	3	3	3	3

Table 3.3: Effect of pollen source and pistil number on fruit fresh weight of Honeycrisp apples in 2013. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	375.6±56.1 a	NA ^Z	295.8±54.1 a	377.6±52.6 a	330.9±46.9 a
Red Delicious	383.4±23.9 a	372.3±22.1 a	398.8±24.2 a	386.7±18.1 a	357.9±22.7 a
Gala	317.7±30.0 a	353.3±25.2 a	351.5±27.1 a	383.1±19.2 a	383.5±25.1 a
Significance	NS	NS	NS	NS	NS

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.4: Effect of pollen source and pistil number on fruit fresh weight of Honeycrisp apples in 2014. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	NA ^Z	NA	NA	NA	273.5±65.8 a
Red Delicious	275.3±28.9 a	297.5±20.8 a	296.9±21.7 a	344.0±21.0 a	247.1±23.3 a
Gala	187.0±40.9 a	339.9±25.5 a	278.9±20.9 a	250.6±21.0 b	275.4±24.1 a
Significance	NS	NS	NS	***	NS

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.5: Effect of pollen source and pistil number on soluble solids concentration of Honeycrisp apples in 2013. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	14.3±0.9 a	NA ^Z	15.3±0.9 a	14.9±0.6 a	15.1±0.7 a
Red Delicious	15.0±0.4 a	15.8±0.3 a	15.3±0.4 a	14.9±0.2 a	14.9±0.3 a
Gala	14.2±0.5 a	14.4±0.4 b	14.7±0.5 a	15.1±0.2 a	14.3±0.4 a
Significance	NS	**	NS	NS	NS

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.6: Effect of pollen source and pistil number on soluble solids concentration of Honeycrisp apples in 2014. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	NA ^Z	NA	NA	NA	14.5±1.1 a
Red Delicious	13.3±0.7 a	13.6±0.3 a	13.1±0.3 A	14.1±0.4 a	13.1±0.4 a
Gala	12.1±0.9 a	13.6±0.4 a	13.6±0.3 a	13.8±0.4 a	13.5±0.5 a
Significance	NS	NS	NS	NS	NS

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.7: Effect of pollen source and pistil number on starch pattern index of Honeycrisp apples in 2013. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	6.0±0.3 a	NA ^z	5.7±0.3 a	5.5±0.3 a	5.5±0.2 a
Red Delicious	5.7±0.1 a	5.7±0.1 a	5.8±0.1 a	5.9±0.1 a	5.9±0.1 a
Gala	5.9±0.2 a	5.9±0.1 a	5.8±0.1 a	5.7±0.1 a	5.8±0.1 a
Significance	Ns	Ns	NS	NS	NS

^zNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.8: Effect of pollen source and pistil number on starch pattern index of Honeycrisp apples in 2014. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	NA ^z	NA	NA	NA	6.0±0.3 a
Red Delicious	5.6±0.3 a	5.9±0.1 a	5.9±0.1 a	5.6±0.2 a	5.8±0.1 a
Gala	5.2±0.4 a	5.8±0.1 a	5.9±0.1 a	5.7±0.2 a	5.9±0.1 a
Significance	NS	NS	NS	NS	NS

^zNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

3.4.5 Seed Number

Pollen source had a significantly influence on seed number per fruit in 2013 when three, four and five pistils remained in flowers but pollinizer had no effect when one or two pistils remained (Table 3.9). Seed numbers were lowest when crabapple was the pollinizer but Red Delicious and Gala performed similarly as pollinizers. There was no effect of the treatments on seed number in 2014, although there were a number of missing plots due to poor fruit set with crabapple as the pollinizer (Table 3.10).

3.4.6 Seed Fresh Weight

Seed fresh weight per fruit followed similar trends as for seed number per fruit, where the lowest values were found when crabapples were used as pollinizer in 2013, and no differences were apparent in 2014 (Table 3.11, 3.12).

3.4.7 Seed Number Affecting Fruit Fresh Weight

There was a significant relationship between fruit fresh weight and seed number in 2014 regardless of pollen source. Similar trends were found in 2013, was not statistically significant. Fruit fresh weight increased with increasing seed number per fruit (Figure 3.3).

Table 3.9: Effect of pollen source and pistil number on seed number per fruit of Honeycrisp apples in 2013. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	2.0±1.5 a	NA ^Z	1.7±1.0 c	0.5±1.8 b	1.5±0.9 b
Red Delicious	5.4±0.6 a	6.6±0.7 a	7.7±0.4 a	8.1±0.6 a	8.6±0.4 a
Gala	4.3±0.8 a	7.1±0.9 a	5.1±0.5 b	6.6±0.7 a	8.2±0.5 a
Significance	NS	NS	***	***	***

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.10: Effect of pollen source and pistil number on seed number per fruit of Honeycrisp apples in 2014. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	NA ^Z	NA	NA	NA	3.0±2.3 a
Red Delicious	3.8±0.8 a	4.7±0.5 a	5.0±0.5 a	6.8±0.6 a	5.3±0.8 a
Gala	1.0±1.1 a	4.9±0.6 a	4.3±0.5 a	5.2±0.6 a	5.8±0.8 a
Significance	NS	NS	NS	NS	NS

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.11: Effect of pollen source and pistil number on seed fresh weight of Honeycrisp apples in 2013. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	0.2±0.1 a	NA ^Z	0.1±0.1 b	0.1±0.1 b	0.1±0.1 b
Red Delicious	0.4±0.1 a	0.5±0.1 a	0.6±0.1 a	0.6±0.1 a	0.6±0.1 a
Gala	0.3±0.1 a	0.5±0.1 a	0.5±0.1 a	0.5±0.1 a	0.6±0.1 a
Significance	NS	NS	***	***	***

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

Table 3.12: Effect of pollen source and pistil number on seed fresh weight of Honeycrisp apples in 2014. Means and standard deviations are reported for each treatment combination (n=25).

Male	Pistil number				
	1	2	3	4	5
Crabapple	NA ^Z	NA	NA	NA	0.2±0.1 a
Red Delicious	0.3±0.5 a	0.3±0.1 a	0.4±0.1 a	0.5±0.1 a	0.3±0.1 a
Gala	0.1±0.1 a	0.3±0.1 a	0.3±0.1 a	0.4±0.1 a	0.4±0.1 a
Significance	NS	NS	NS	NS	NS

^ZNo fruit set.

NS, *, **, *** Non-significant; and significant at $P = 0.05, 0.01, 0.001$, respectively.

3.4.8 The correlation between pistil number and seed number

In our study, we found that pistil number is highly correlated with seed number (Figure 3.4), but is highly variable (Table 3.13), regardless of the combinations and years. Overall, seed number was linearly positively related to pistil number. However, the variability within each pistil number refutes the suggestion that each pistil is only connected to two ovules and support the results of Pratt (1988) and Jackson (2003) showing that the base of pistils are fused at the base. For example, when there was only one pistil, based on the hypothesis that each pistil is only connected to two ovules, this should be resulted in a maximum of two seeds, but we found a maximum of 10 seeds for a single pistil.

Table 3.13: Correlation between pistil number and seed number

Seed Number	Pistil Number				
	1	2	3	4	5
Mean	5	7	6	7	8
Minimum	1	0	1	0	0
Maximum	10	11	10	12	12

3.4.9 Return Bloom

Return bloom was not influenced by pollen source in either year (data not presented). Seed number had a significantly negative impact on return bloom in 2014, but not in 2013, regardless the pollen donor (Figure 3.5). Individual fruit fresh weight per spur also negatively influenced return boom (Figure 3.6).

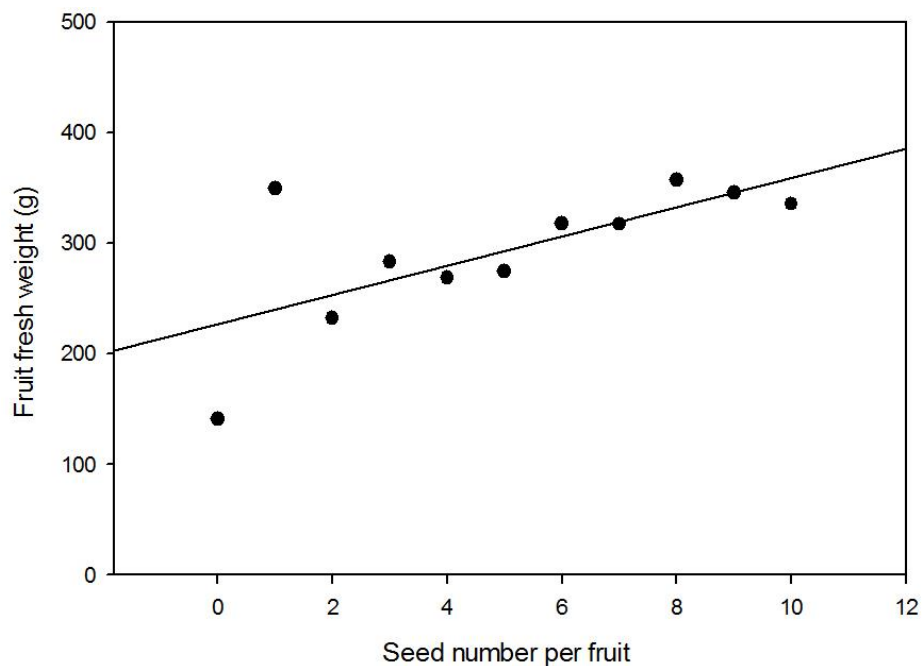


Figure 3.3: Effect of seed number on fruit fresh weight at: $y = 19.49847x + 188.46988$, $R^2 = 0.3206$, $P < 0.0001$

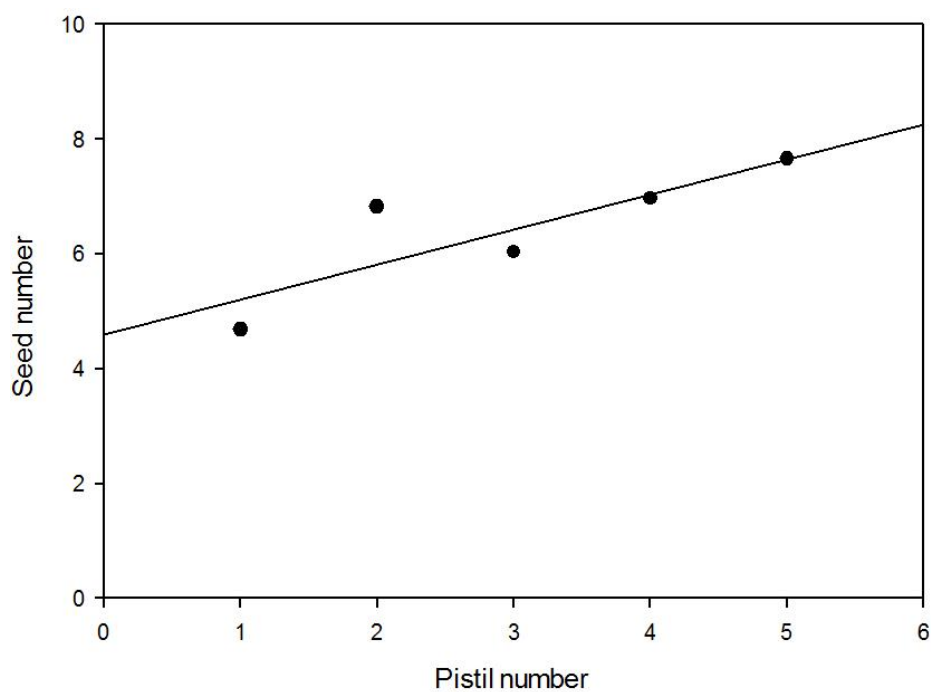


Figure 3.4: Pistil number is highly correlated with seed number at $y = 0.60904x + 4.60716$, $R^2 = 0.7188$, $P = 0.0007$

3.5 Discussion

Hartman and Howlett (1954), and Degrandi-Hoffman *et al.* (1987) reported that pollinizer is among the most decisive factors which influence fruit set of apple. Unsatisfactory fruit-set is often due to the lack of suitable pollinizers, lack of flowering synchrony between pollinizers and cultivars, inadequate number of pollinators and several environmental factors (Hartman and Howlett, 1954; Degrandi-Hoffman *et al.*, 1987). Our results confirmed some of these findings. For example, we found that crabapple was a poor pollinizer for Honeycrisp regardless the species ('Ralph Shay' crabapple used in 2013 and *Malus floribunda* in 2014). 'Ralph Shay' crabapple pollen resulted in less than 10% fruit set in 2013 whereas fruit set with *Malus floribunda* was less than 2% in 2014. This was not due to pollen viability since testing showed the pollen to be highly viable. Red Delicious pollen resulted in the highest percent fruit set in both years followed by Gala (Tables 3.1, 3.2).

Tuan and Chung-Ruey (2013) reported the influence of pollen source on fruit set, seed set, seed number, and subsequent fruit quality. Their findings showed that fruit set percentage of 'Long Red B' was significantly higher when 'Black' and 'Thyto' cultivars were used as pollinizers. Similarly, our findings showed that crabapple pollen performed poorly as a pollinizer for Honeycrisp compared with Red Delicious and Gala as pollinizers. Tuan and Chung-Ruey (2013) also reported higher seed number, fruit weight, fruit diameter, fruit length, and soluble solids concentration as a result of using 'Black' and 'Thyto' pollinizers. In our study, pollinizer also affected seed number but had no effect on fruit quality attributes such as fruit weight and SSC.

Das *et al.* (2011) proposed that ‘Manchurian’ crabapple performed well as a pollinizer for ‘Oregon Spur’ apple in terms of fruit set. However our results clearly demonstrated that the two crabapple genotypes we used were not effective pollinizers of Honeycrisp apple. Crabapple (Delaplane and Mayer 2002), Gala and Fuji (Bashir *et al.*, 2010) were deemed to be suitable pollinizers for commercial orchards. Our results partially agree in that Gala was a good pollinizer for Honeycrisp orchard but crabapple was not. Milutinovic and Milutinovic (1999) also stated the tremendous impact of genotype on success as pollinizers.

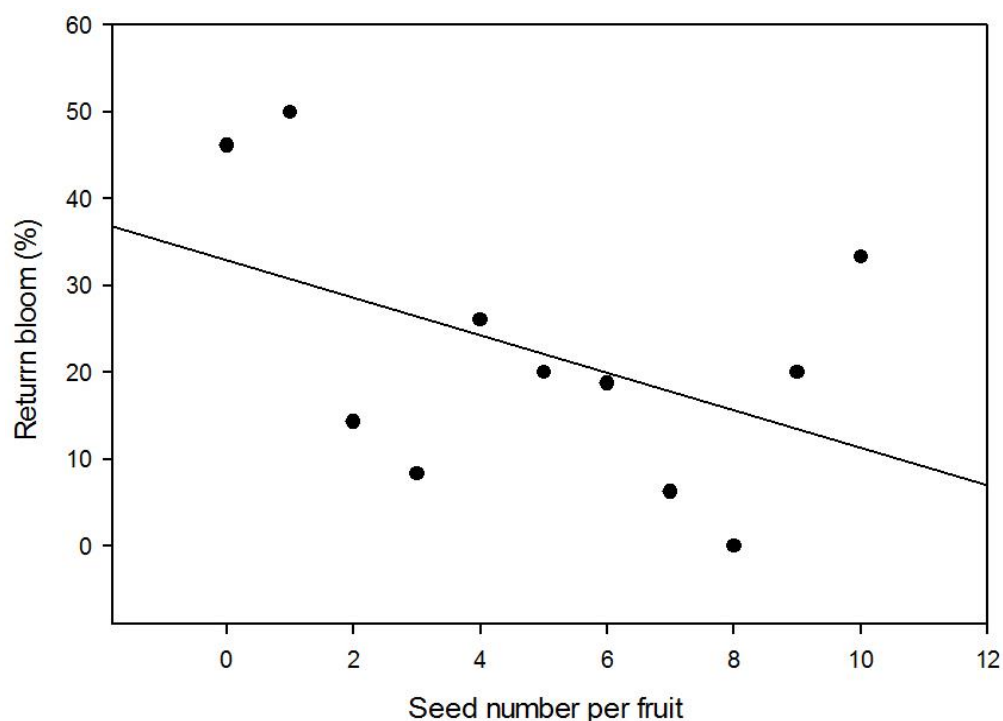


Figure 3.5: Effect of seed number on return bloom at: $y = -0.0217x + 0.3295$, $R^2 = 0.206$, $P = 0.0400$

A positive linear correlation between fruit weight and seed number was found by Keulemans *et al.* (1996), and Bashir *et al.* (2010). Denne (1963), and Volz *et al.* (1996) also reported that fruit size, fruit weight, and fruit growth rate were positively correlated

with seed number per fruit. Our results in 2014 are in agreement with the earlier study, although we found no such relationship in 2013.

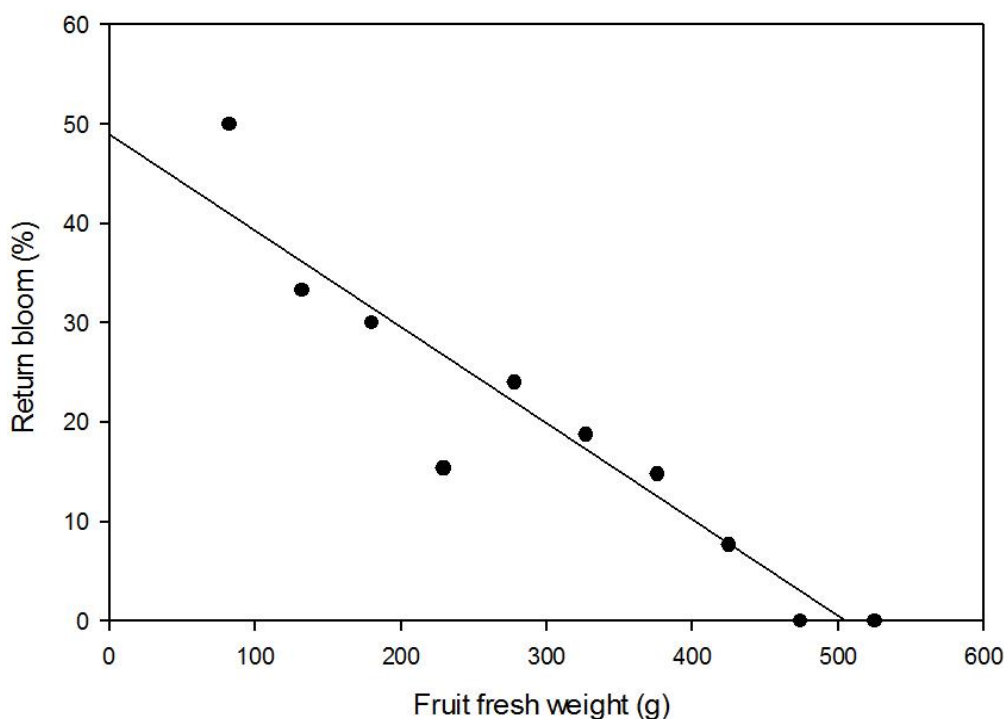


Figure 3.6: Effect of fruit fresh weight on return bloom at: $y = -0.00097x + 0.48922$, $R^2 = 0.9019$ and $P < 0.0001$

In apple, the flower comprises a total of 5 pistils (Pratt, 1988), each functioning to deliver the two sperm cells to fertilize the egg and endosperm cells (Rotman et al., 2003; Sandaklie-Nikilova *et al.*, 2007; Drews and Koltunow, 2011; Hamamura *et al.*, 2012; Dresselhaus and Snell, 2014). It is also postulated that each pistil transmitting tissue is separated and is connected with one locule, each consisting of a maximum of two to four ovules (Anvari and Stösser, 1981 cited by Pratt, 1988). However, Pratt (1988) and Jackson (2003) reported that pistils are fused at the base and a maximum of 10 seeds are produced per fruit. Our findings agree with Pratt (1988) and Jackson (2003) report that pistil are fused. Although pistil number was linearly correlated with seed number (Figure

3.4), it was highly variable (Table 3.13), regardless of the combinations and years. For example, with only one pistil a maximum of 10 seeds were counted in some fruit strongly suggesting pistils are fused.

Several studies have reported a negative influence of seed number on return bloom in apples. Chan and Cain (1967), Jonkers, (1979) and Neilsen (1998) reported return bloom was negatively affected by seed content of the fruit. Our results agree with these reports. Seed number was negatively correlated with return bloom in 2014 regardless of the pollen source. However, seed number only accounted for 20% of the observed variation in return bloom. Similar findings were reported by Fulford (1965), and Dennis and Neilsen (1999) as the seed effect was hypothesized to be a result of exporting gibberellins that inhibit flowering (Fulford, 1965 cited by Jonders, 1979; and Dennis and Neilsen, 1999).

Negative linear correlations between fruit load and return bloom has been reported. For example, Embree *et al.* (2007), and Robinson *et al.* (2009) proposed a negative effect of crop load on return bloom. Similar results were reported by Wright *et al.* (2006), who observed that the time of thinning was crucial for return bloom. Meland (2009) also reported the effect of thinning time on return bloom of Elstar apple. However, the effect of individual fruit weight per spur on return bloom has received little attention. Our results showed that a significant negative correlation between individual fruit weight per spur and the percentage of return bloom was evident in 2014 (Figure 3.6). Therefore, it is not only total fruit load but individual fruit weight per spur has a significant influence on return boom.

3.6 Conclusion

A significant influence of pollen source was detected on fruit set. This was particularly evident when crabapples pollen was used as a pollinizer for Honeycrisp. Fruit fresh weight, soluble solids concentration and starch pattern index were unaffected by pollen source. Seed number per fruit and seed fresh weight per fruit were significantly influenced by pollen source, but only in 2013. Fruit pollinated with crabapples pollen produced fewer seeds per fruit as well as lower seed fresh weight compared with those pollinated by Red Delicious or Gala pollen. Pistil number was positively related to seed number, but was highly variable.

Seed number per fruit was positively related to fruit fresh weight. However, no differences were found in other fruit quality attributes, such as SSC and starch pattern index. Pollen source did not influence return boom in either year. The percentage of return boom was negatively related to fruit fresh weight per spur and seed number per fruit.

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CHAPTER 4. OVERALL CONCLUSION

Pollen source had a significant influence on pollen germination on the stigmatic surface, pollen tube growth down the style, and enrichment of pollen tubes into the ovary. The rating of germinated pollen tubes on the stigmatic surface, number of pollen tubes penetrating the stigma, pollen tube growth down the style, and pollen enrichment to the base of the style increased over time following pollination. Pollen tubes enhancement into the base of the style was affected by pollen donor, but overall 96 hours was required to reach the base of the style after pollination. Golden Delicious and Red Delicious pollen had the higher germination rates on the stigmatic surface, had higher numbers of pollen tubes penetrating the stigma, had faster pollen tube growth down the style, and had the most pollen tubes that reaching the base of the style. Crabapple was the lowest in terms of pollen performance across all cultivars. Pollen tubes grew slowest and fewer pollen tubes reached the base of Honeycrisp styles compared with those of Gala and Fuji. Crabapple was not an effective pollinizer for Honeycrisp apple.

Fruit quality attributes and return bloom were generally not affected by pollen source. However, fewer seeds with less seed fresh weight were found when crabapple pollen was used as a pollinizer in all cultivars. Seed number had significant positive correlations with Gala and Honeycrisp fruit fresh weight regardless the pollen source. A

significantly positive correlation was found between pistil number and seed number, but was highly variable.

Fruit fresh weight and seed number per fruit had significantly negative linear correlations with return bloom when pistils were removed. However, this correlation was not significant when we only tested the pollen source effect on return bloom, but a trend was still found with huge sampling variation.

APPENDICE

Appendix A Experiment one interaction plots

Experiment 1: Male and female interaction plots of pollen growth rate, 2013

1. Rating of visible pollen germinated on the stigmatic surface – days 1, 2, 3, 4;

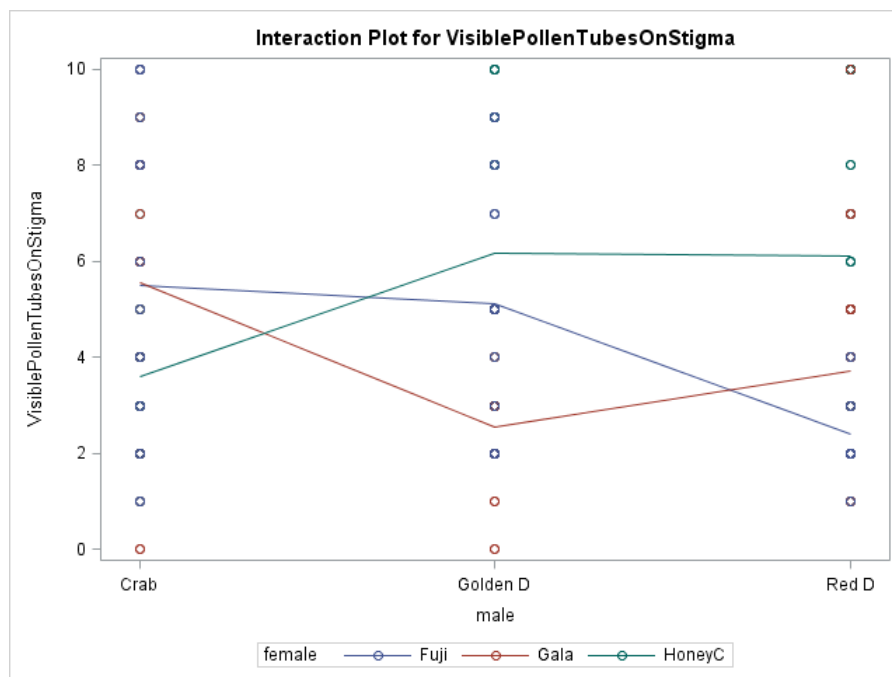


Figure 8: Male and Female Interaction, Day 1, 2013

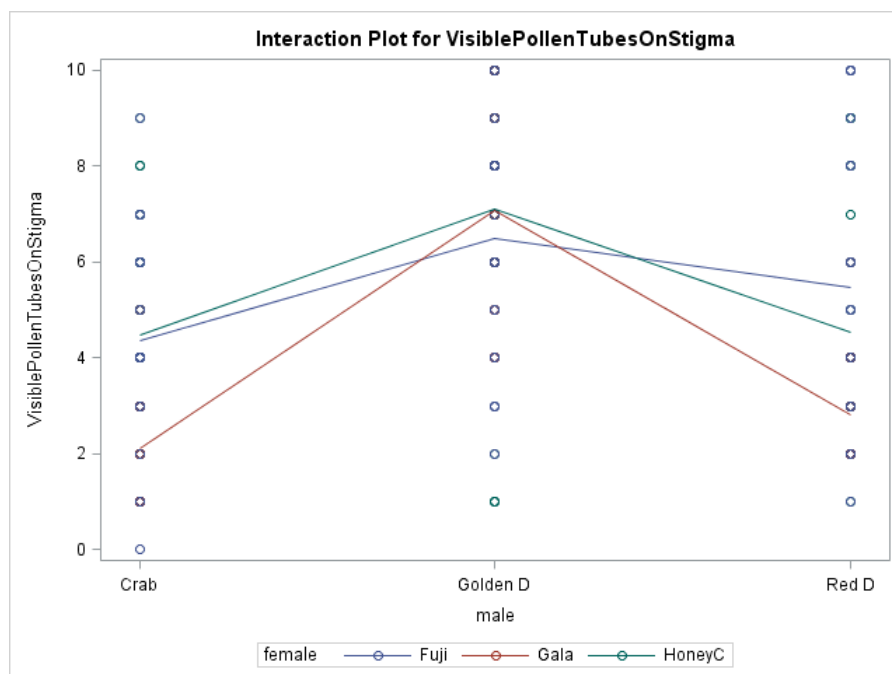


Figure 9: Male and Female interaction plot, Day 2, 2013

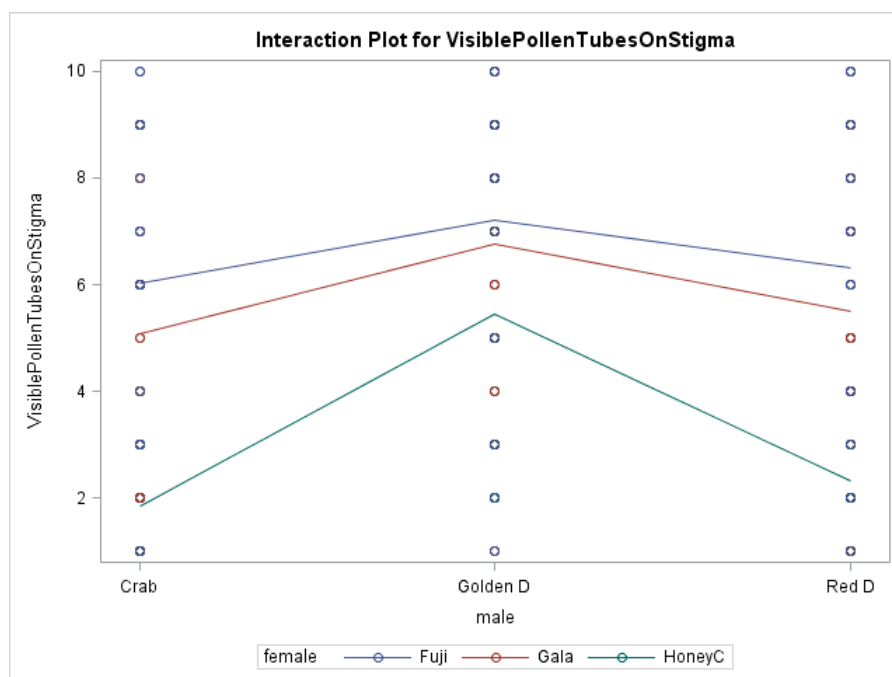


Figure 10: Male and Female interaction plot, Day 3, 2013

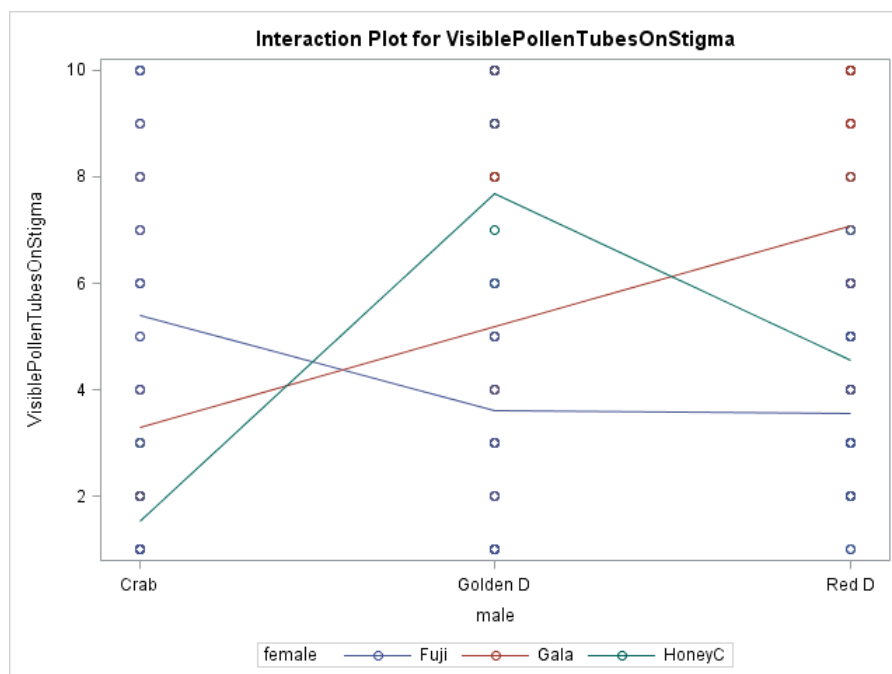


Figure 11: Male and Female interaction plot, Day 4, 2013

2. Number of visible pollen tubes penetrating the stigma: Days 1, 2, 3, 4

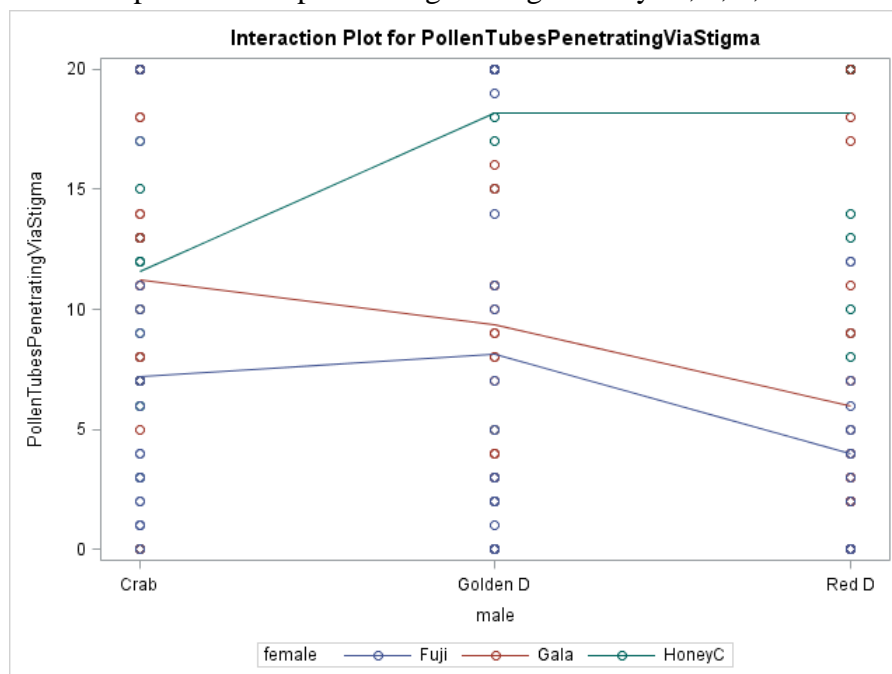


Figure 12: Male and Female interaction plot, Day 1, 2013

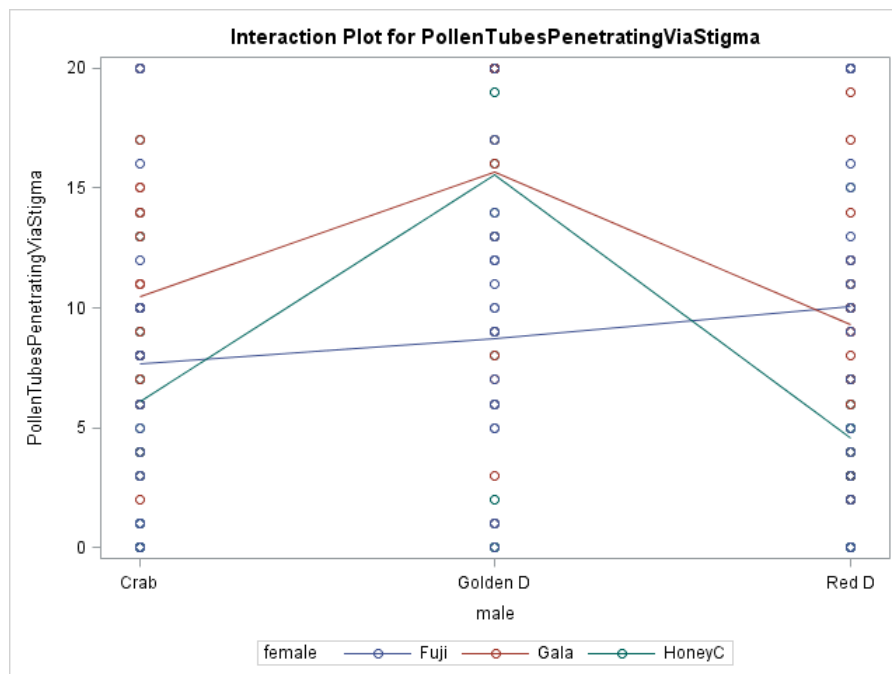


Figure 13: Male and Female interaction plot, Day 2, 2013

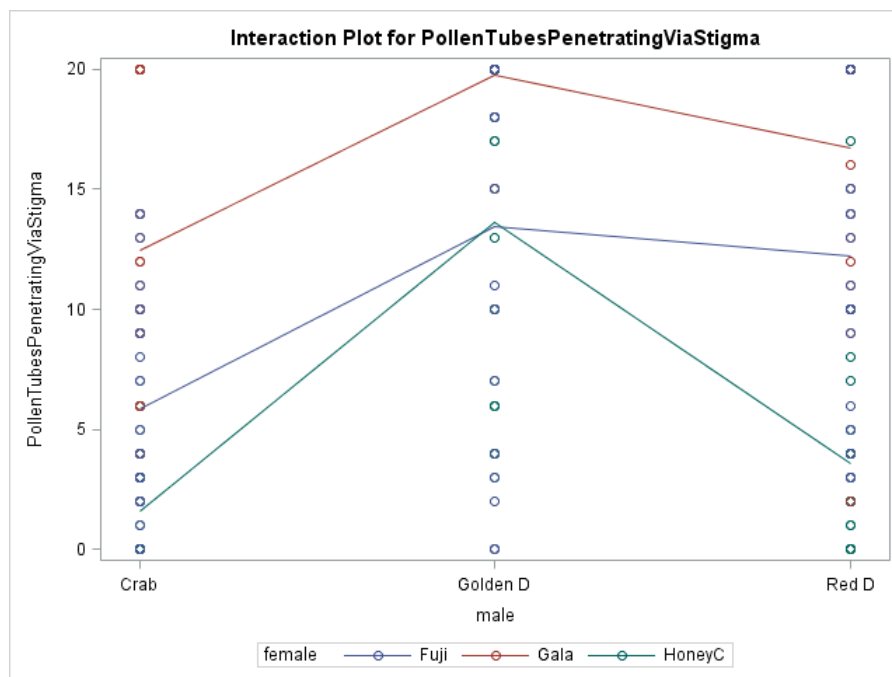


Figure 14: Male and Female interaction plot, Day 3, 2013

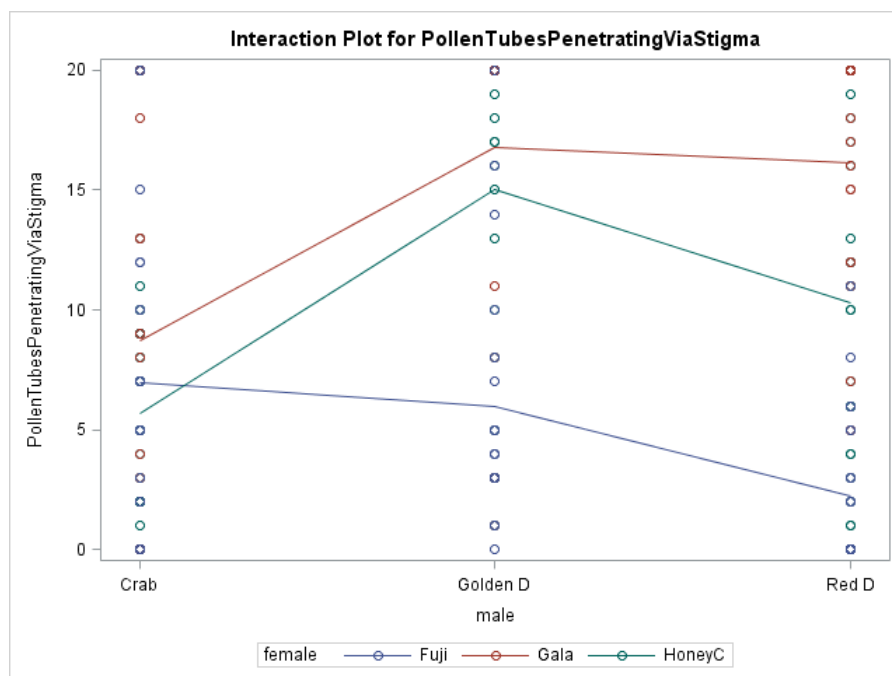


Figure 15: Male and Female interaction plot, Day 4, 2013

3. Average length of the longest pollen tubes: DAYS 1, 2, 3, 4

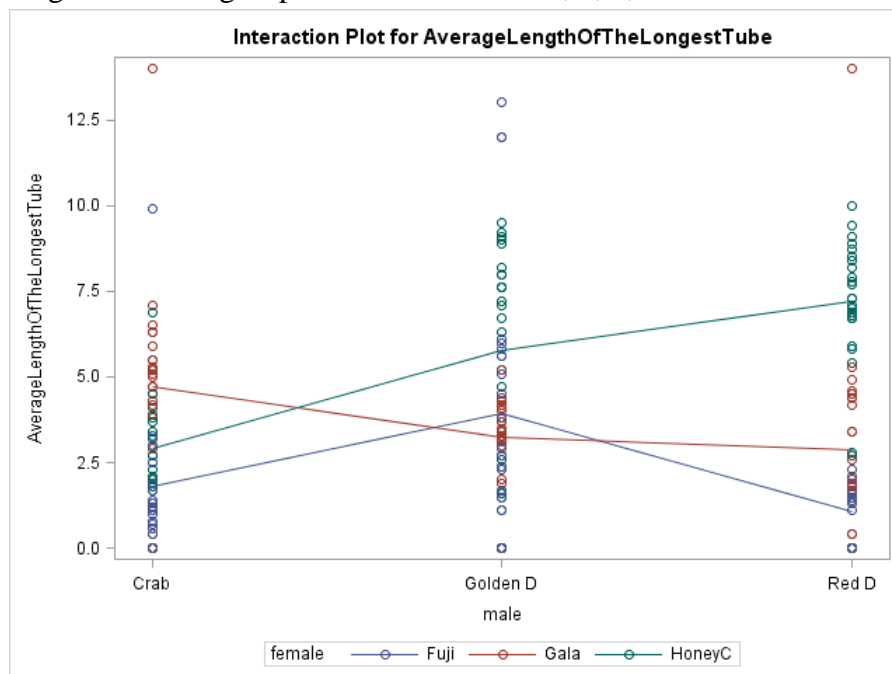


Figure 16: Male and Female interaction plot, Day 1, 2013

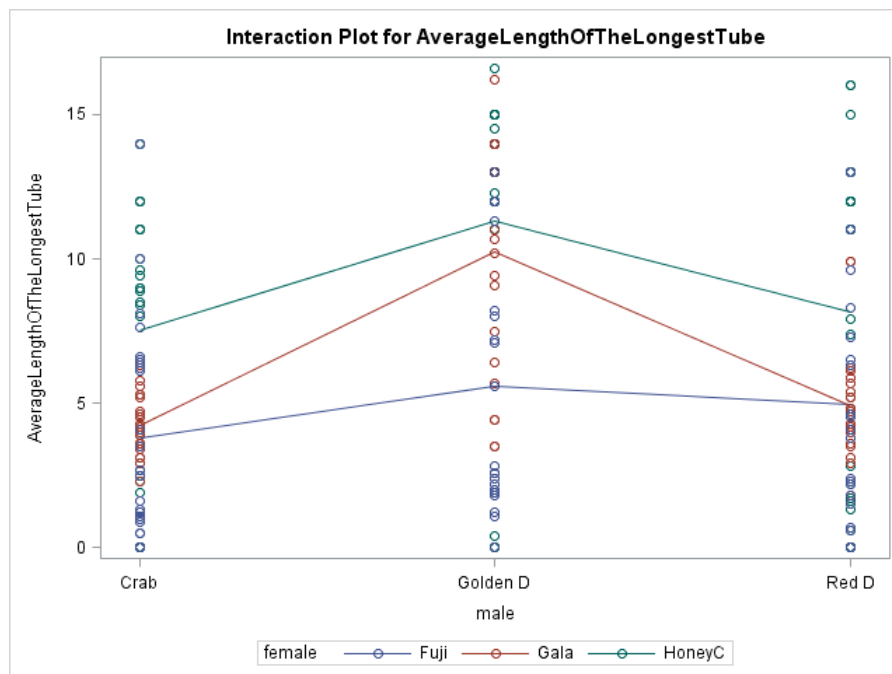


Figure 17: Male and Female interaction plot, Day 2, 2013

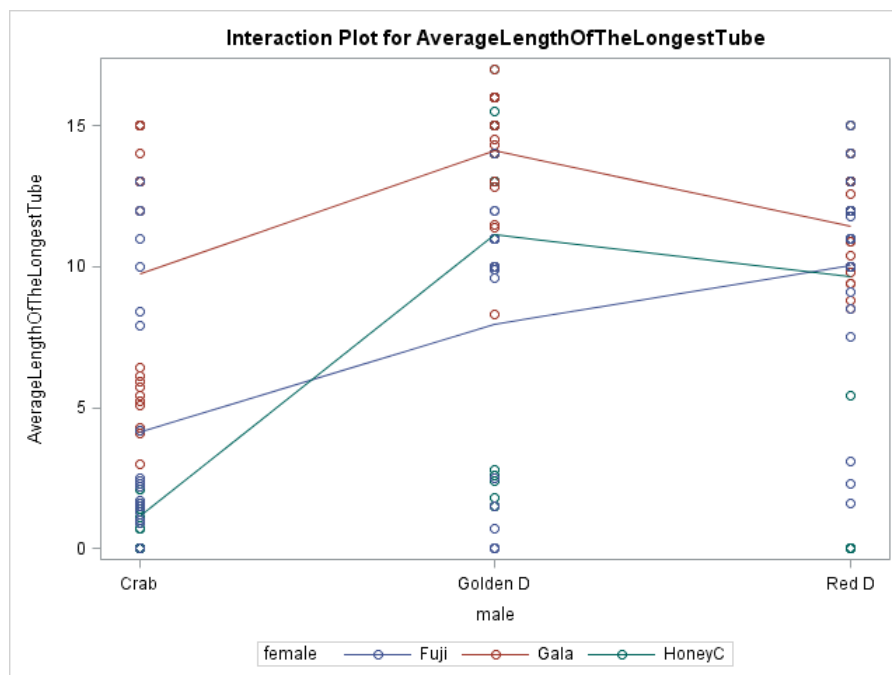


Figure 18: Male and Female interaction plot, Day 3, 2013

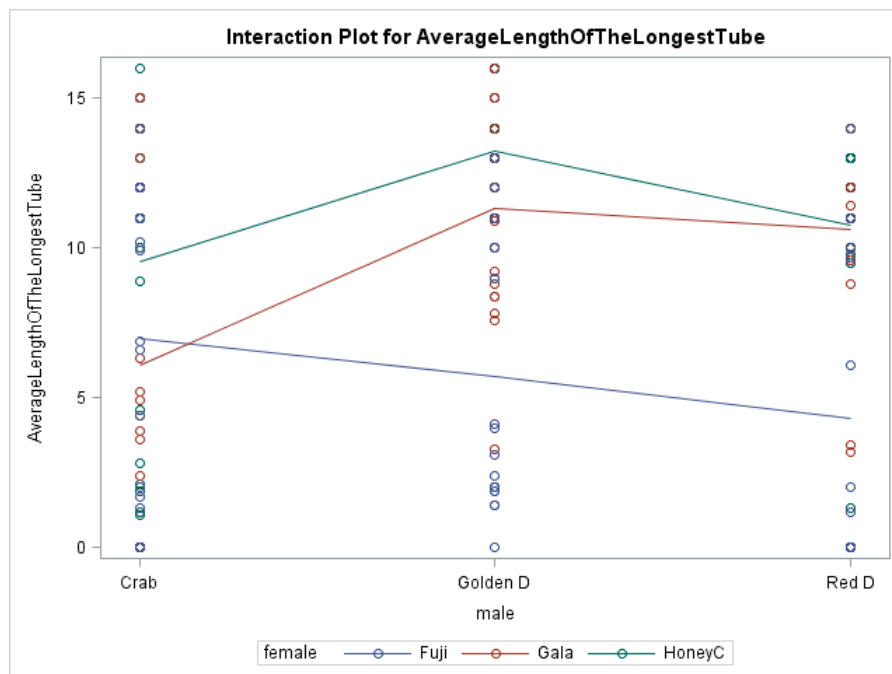


Figure 19: Male and Female interaction plot, Day 4, 2013

4. Maximum growth of pollen tubes (% style length) DAYS 1, 2, 3, 4

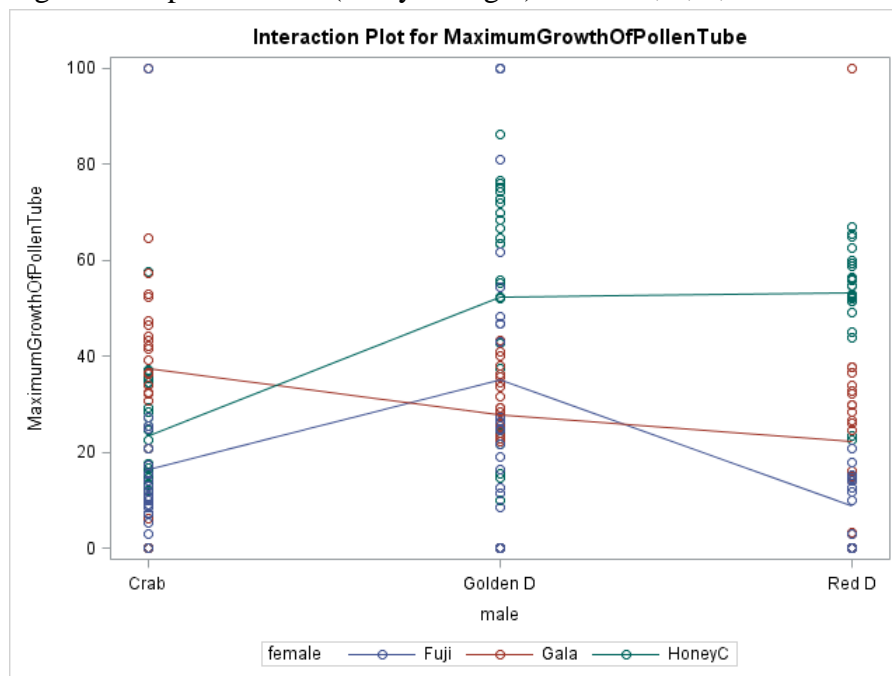


Figure 20: Male and Female interaction plot, Day 1, 2013

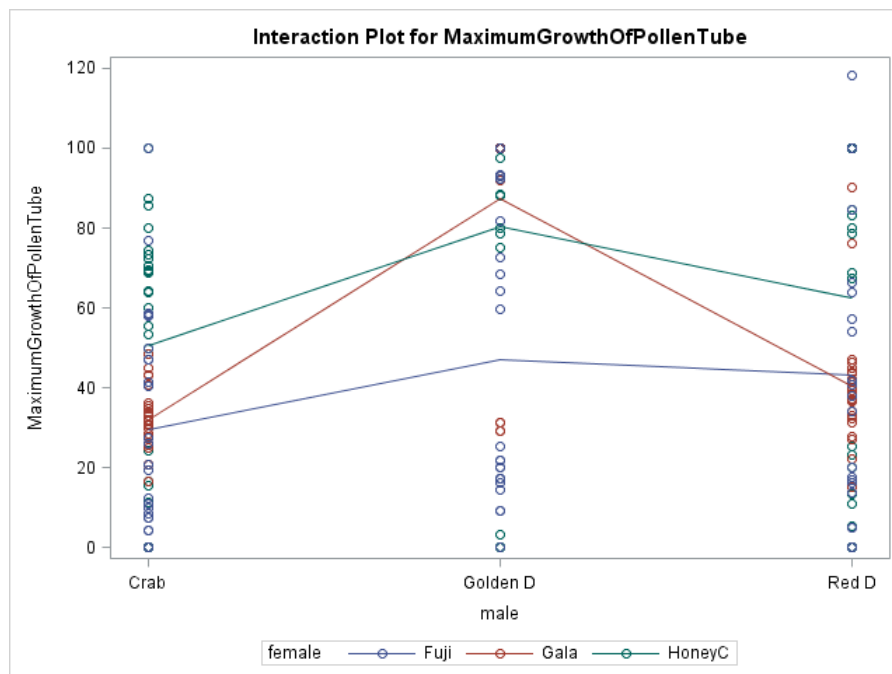


Figure 21: Male and Female interaction plot, Day 2, 2013

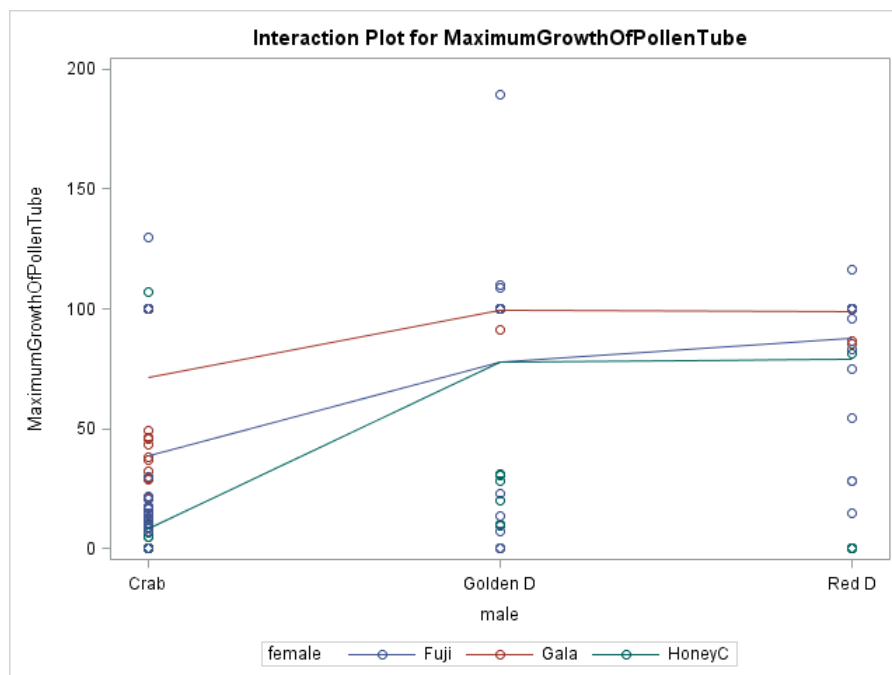


Figure 22: Male and Female interaction plot, Day 3, 2013

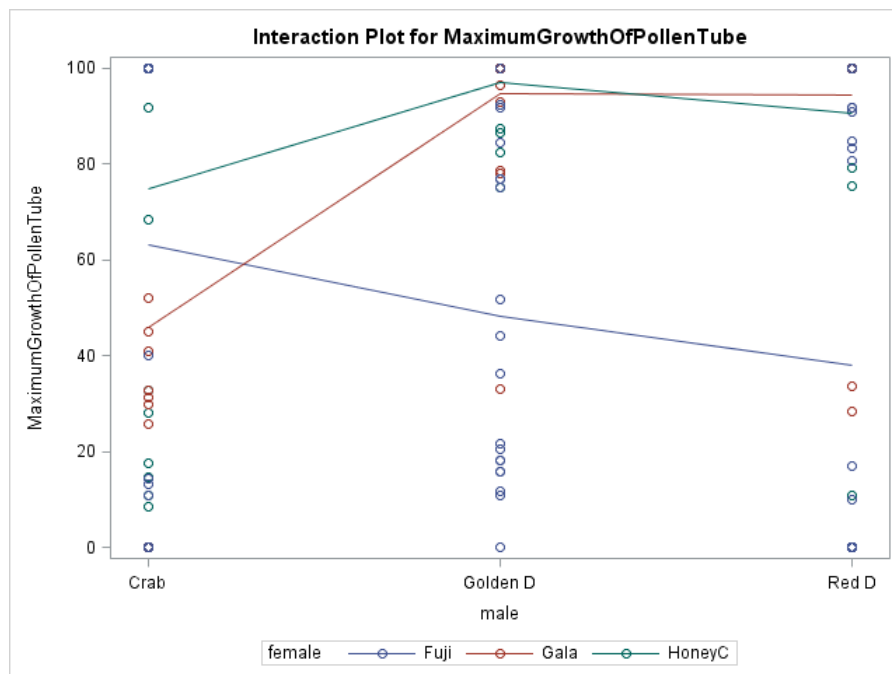


Figure 23: Male and Female interaction plot, Day 4, 2013

5. Number of pollen tubes that reached the base of style DAYS 1, 2, 3, 4

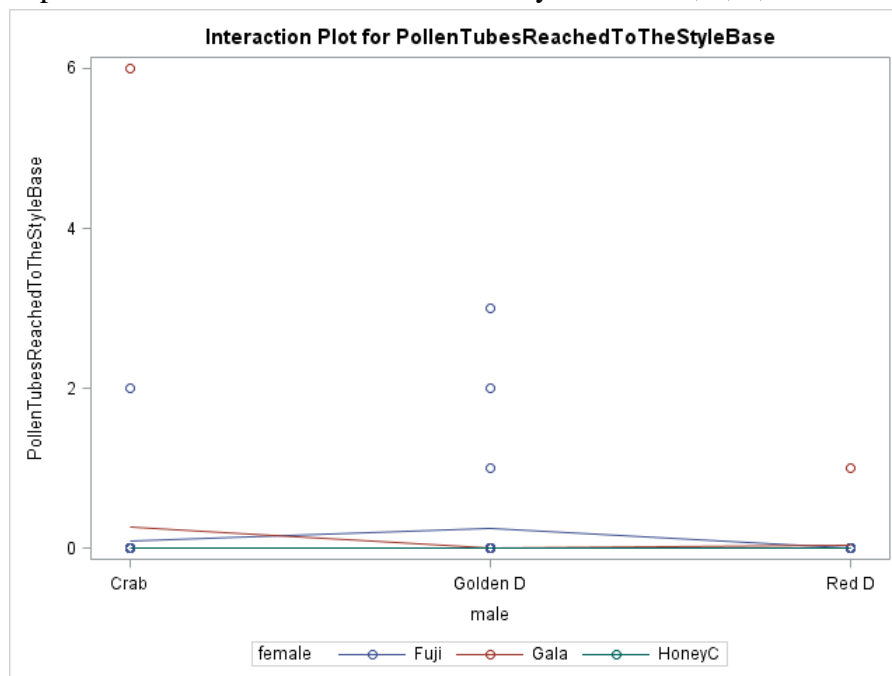


Figure 24: Male and Female interaction, Day 1, 2013

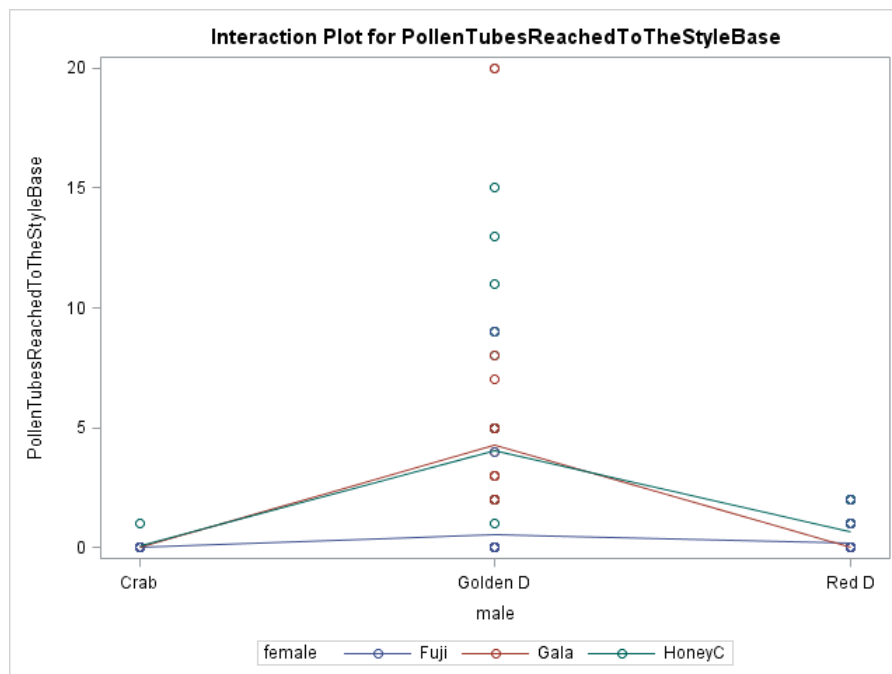


Figure 25: Male and Female interaction plot, Day 2, 2013

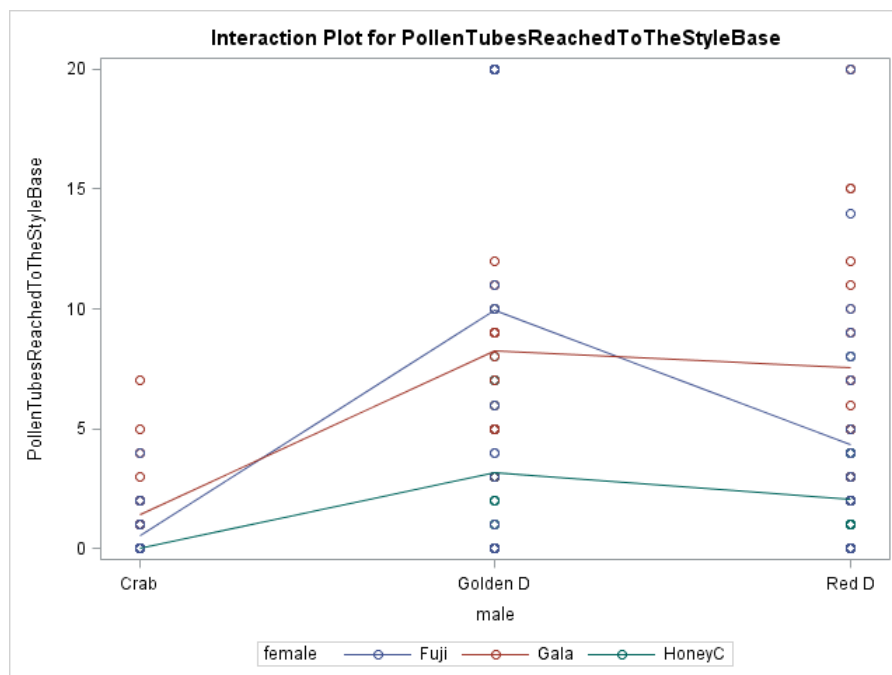


Figure 26: Male and Female interaction plot, Day 3, 2013

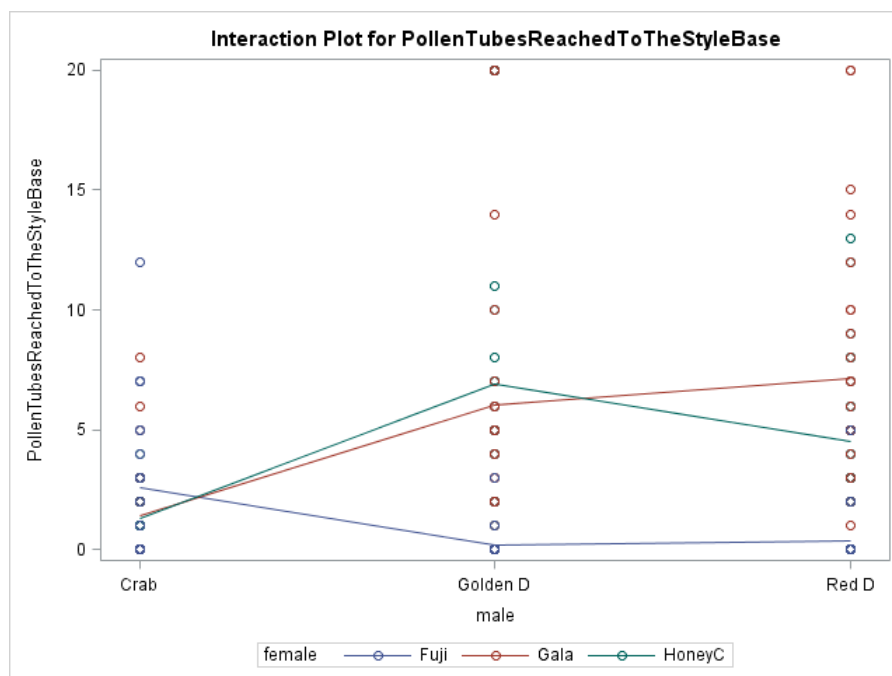


Figure 27: Male and Female interaction plot, Day 4, 2013

Experiment 1: Male and female interaction plots of pollen tubes growth, 2014

1. Rating of visible pollen germinated on stigmatic surface DAYS 1, 2, 3, 4

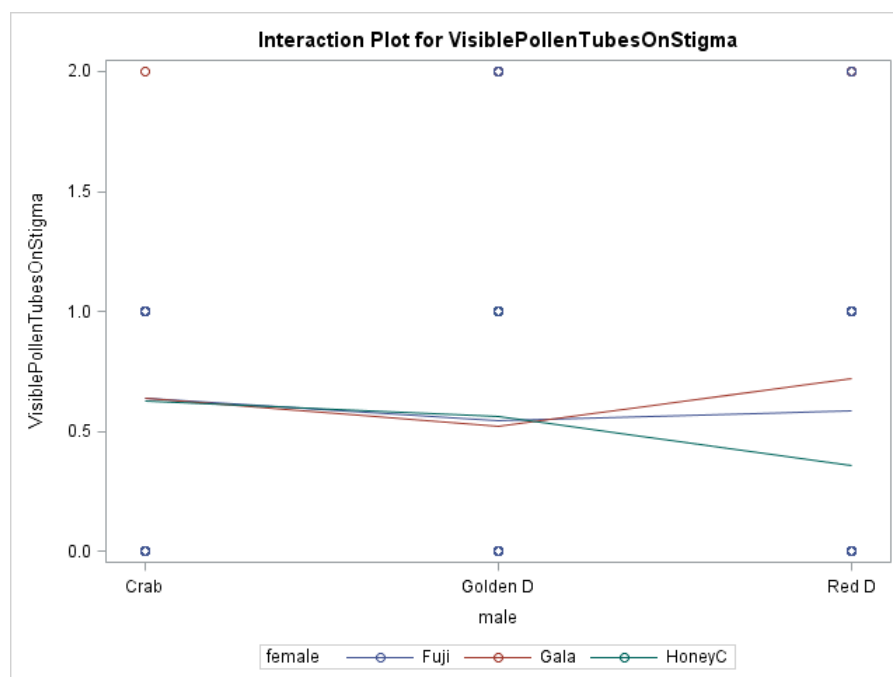


Figure 28: Male and Female interaction plot, Day 1, 2014

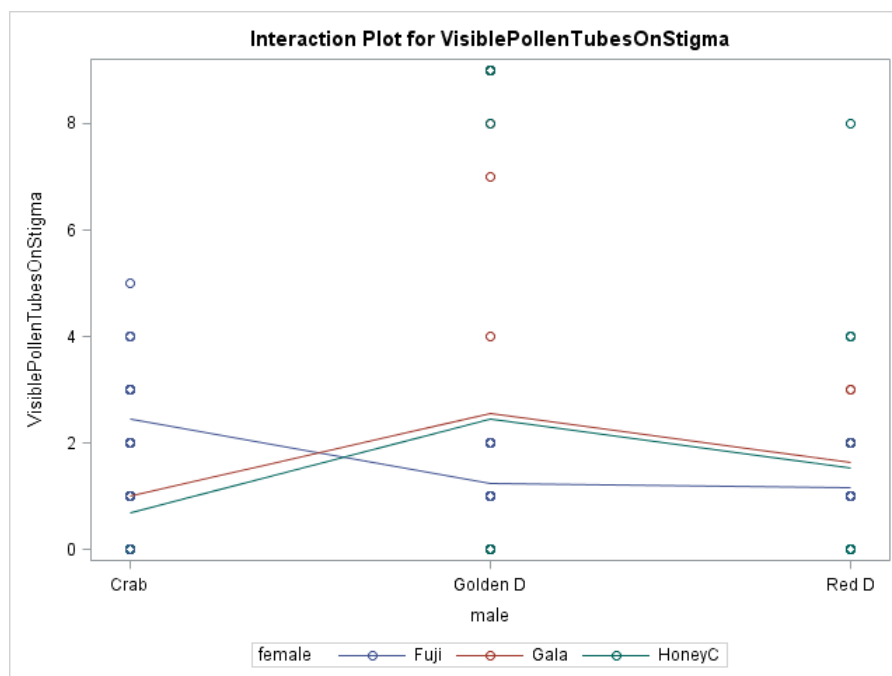


Figure 29: Male and Female interaction plot, Day 2, 2104

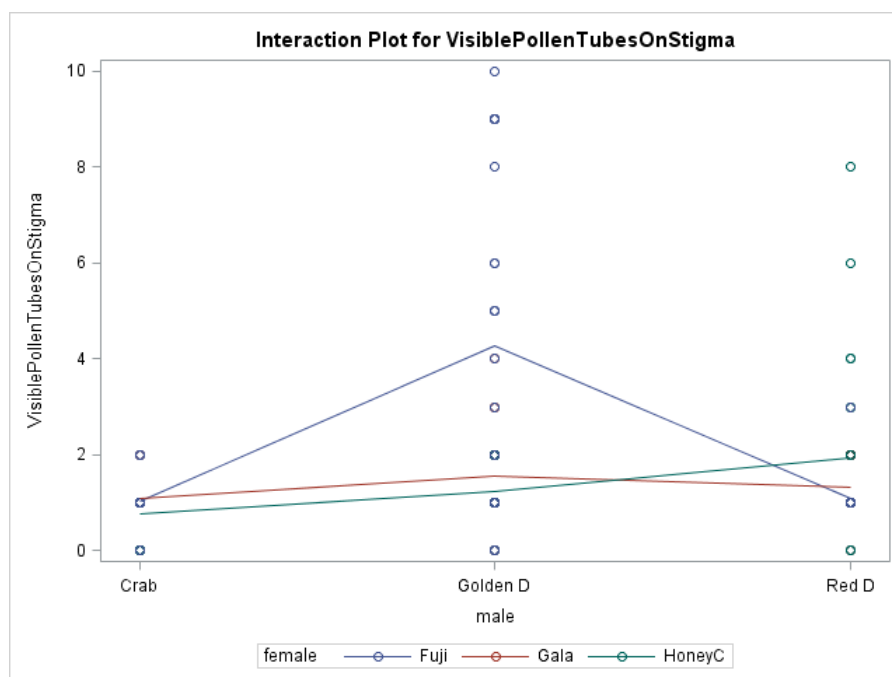


Figure 30: Male and Female interaction plot, Day 3, 2014

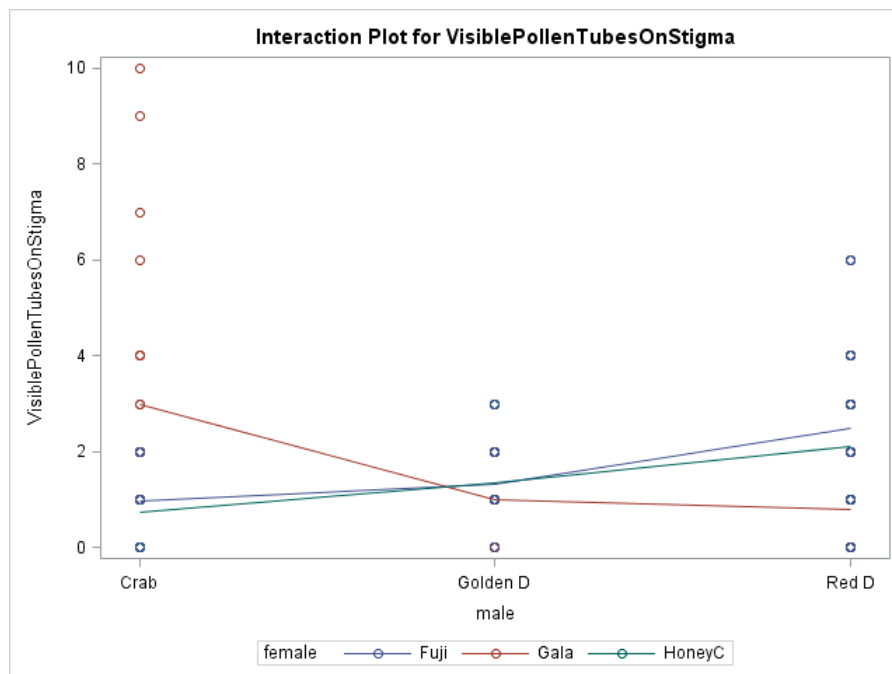


Figure 31: Male and Female interaction plot, Day 4, 2014

2. Number of visible pollen tubes penetrating the stigma DAYS 1, 2, 3, 4

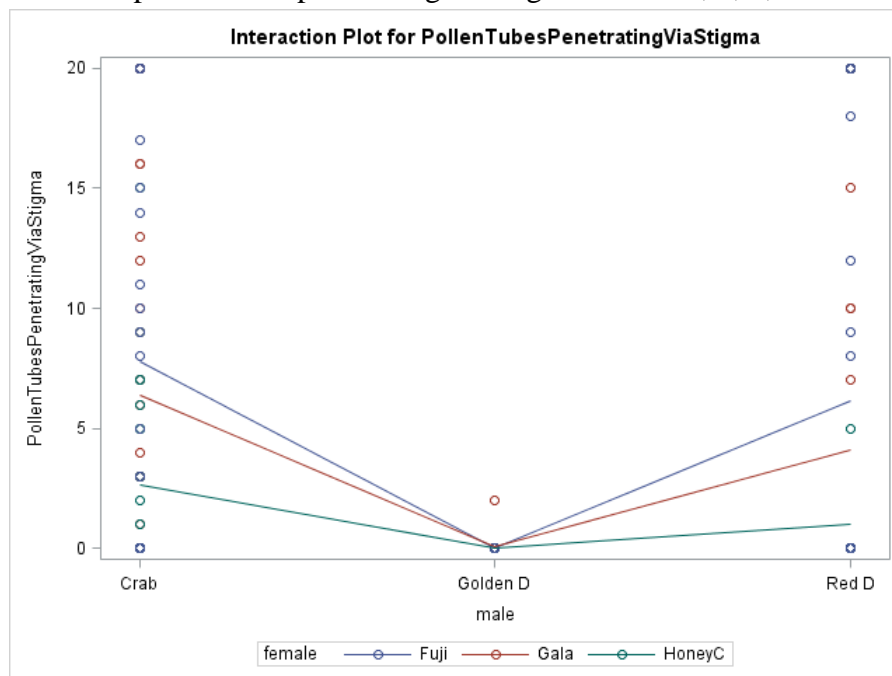


Figure 32: Male and Female interaction plot, Day 1, 2014

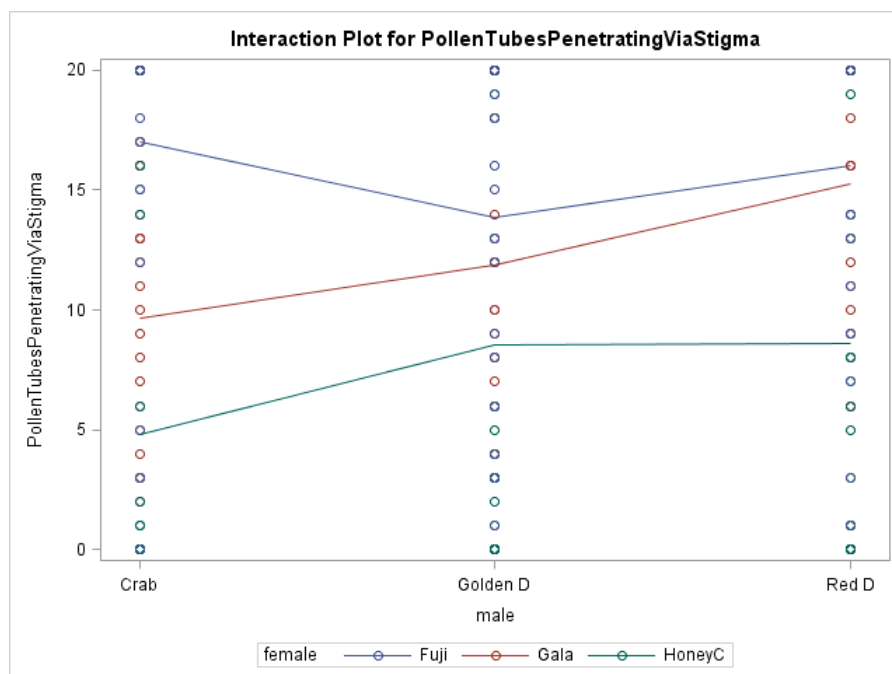


Figure 33: Male and Female interaction plot, Day 2, 2014

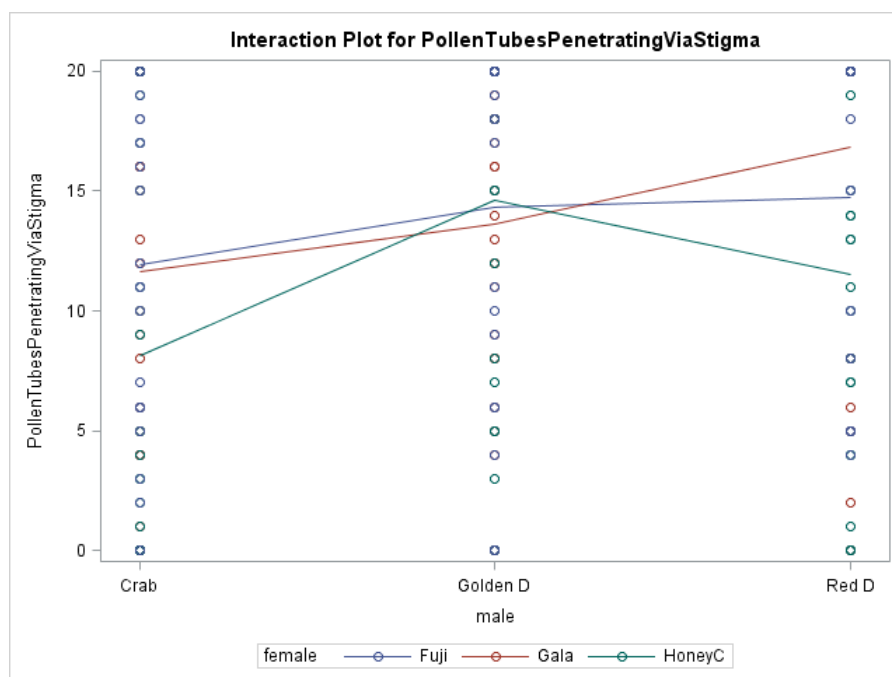


Figure 34: Male and Female interaction plot, Day 3, 2014

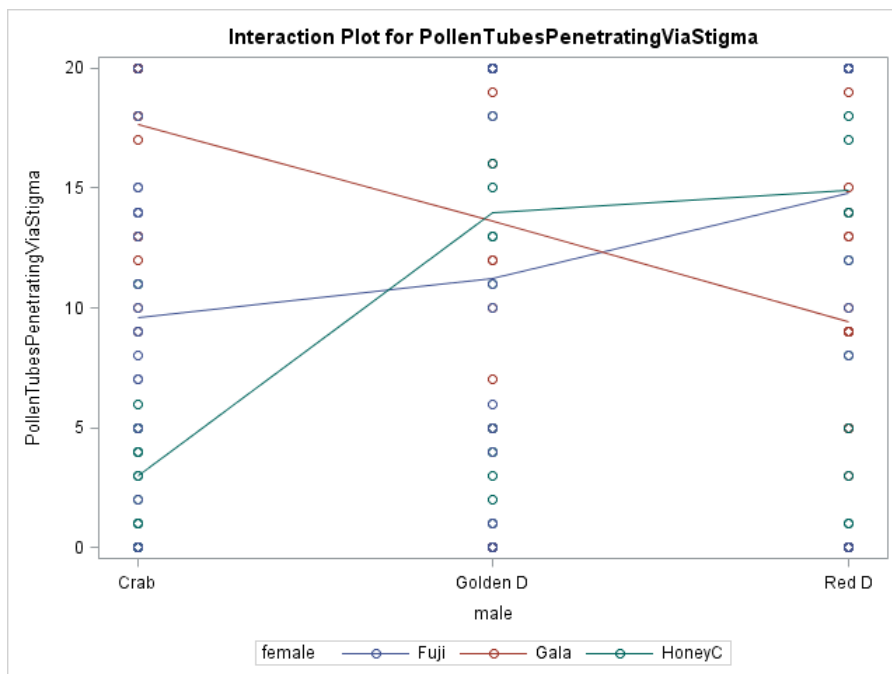


Figure 35: Male and Female interaction plot, Day 4, 2014

3. Average length of the longest pollen tubes DAYS 1, 2, 3, 4

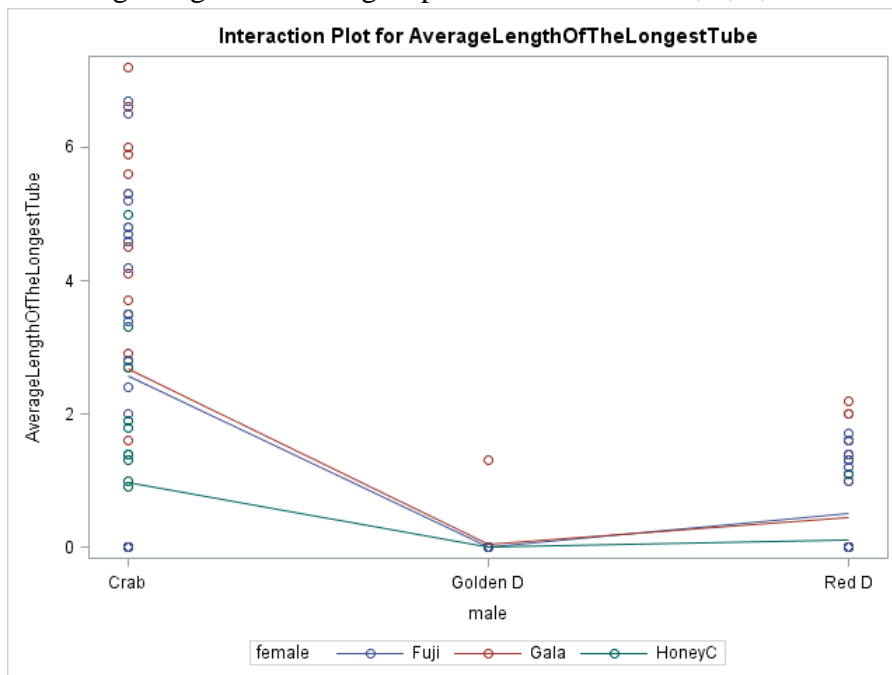


Figure 36: Male and Female interaction plot, Day 1, 2014

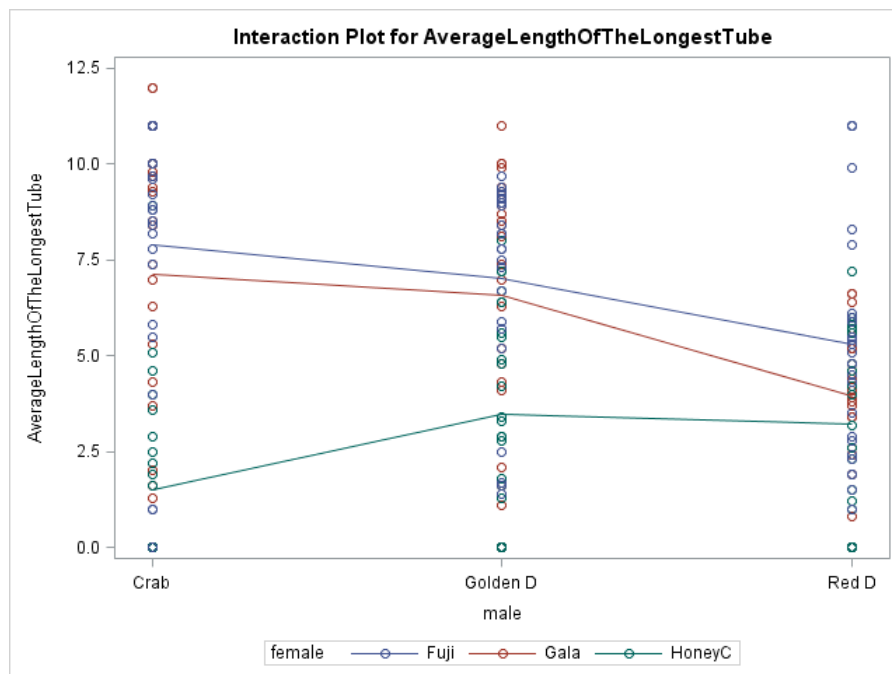


Figure 37: Male and Female interaction plot, Day 2, 2014

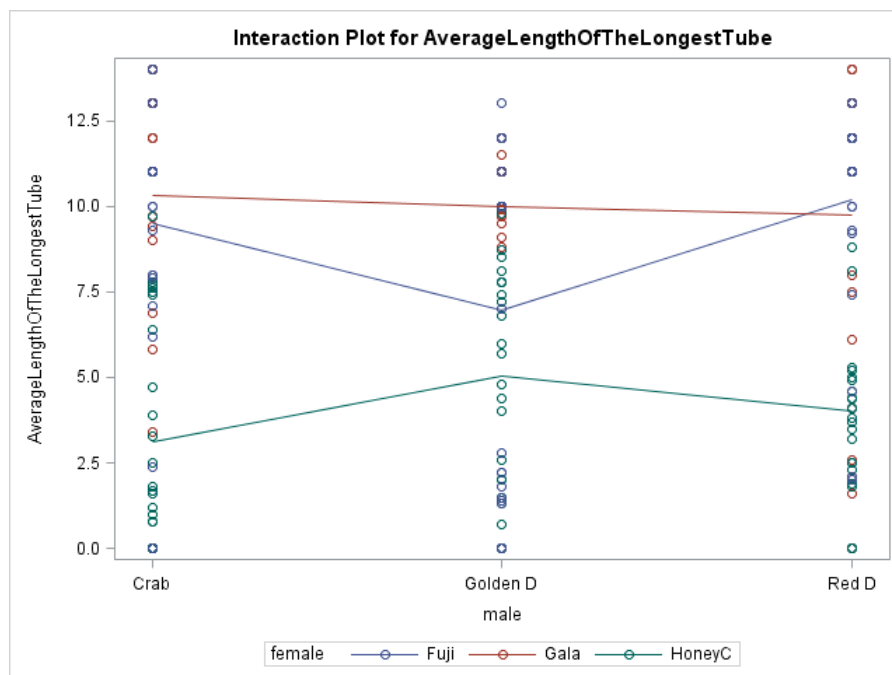


Figure 38: Male and Female interaction plot, Day 3, 2014

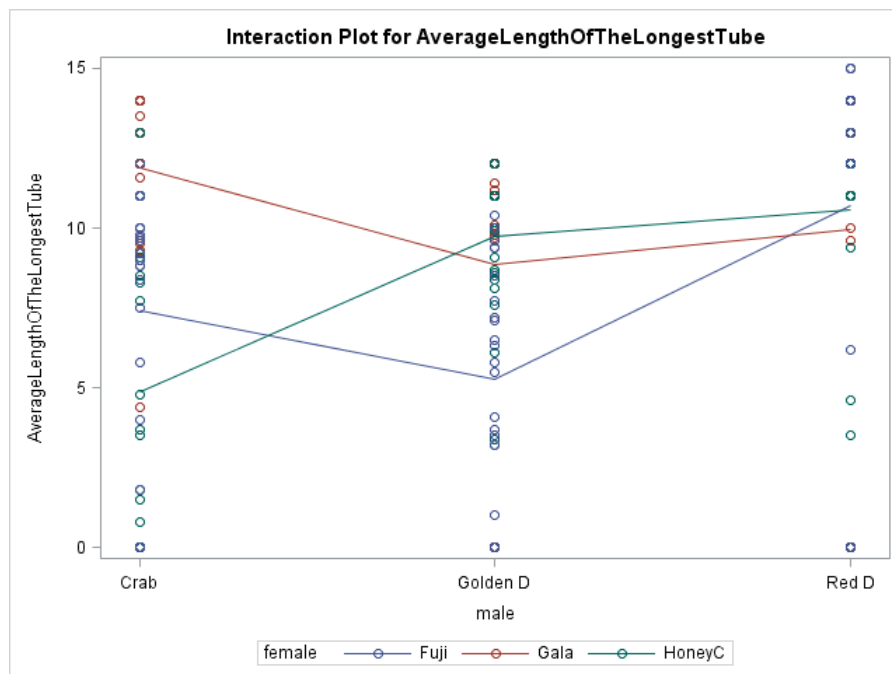


Figure 39: Male and Female interaction plot, Day 4, 2014

4. Maximum growth of pollen tubes (% style length) DAYS 1, 2, 3, 4

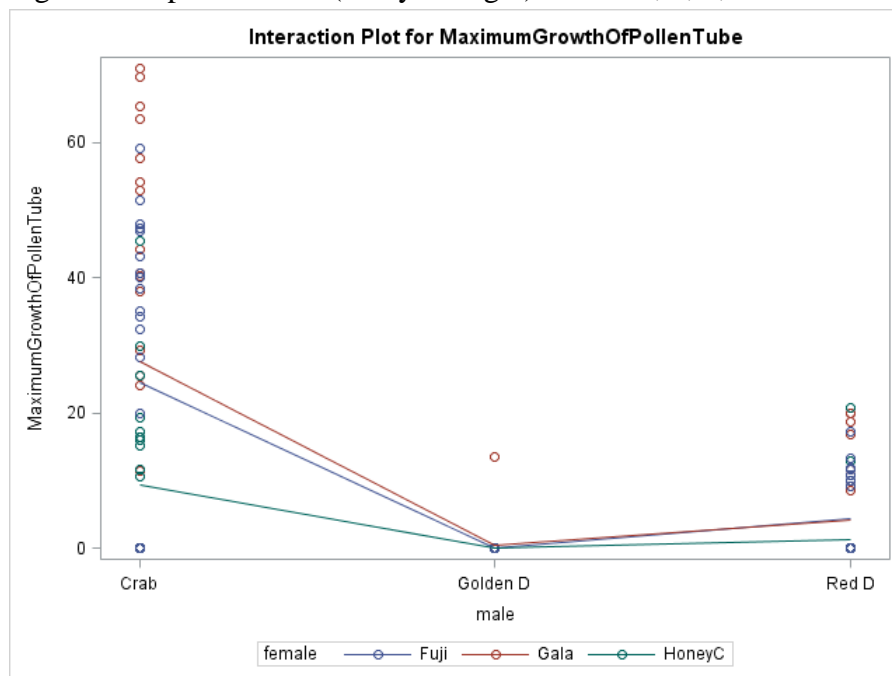


Figure 40: Male and Female interaction plot, Day 1, 2014

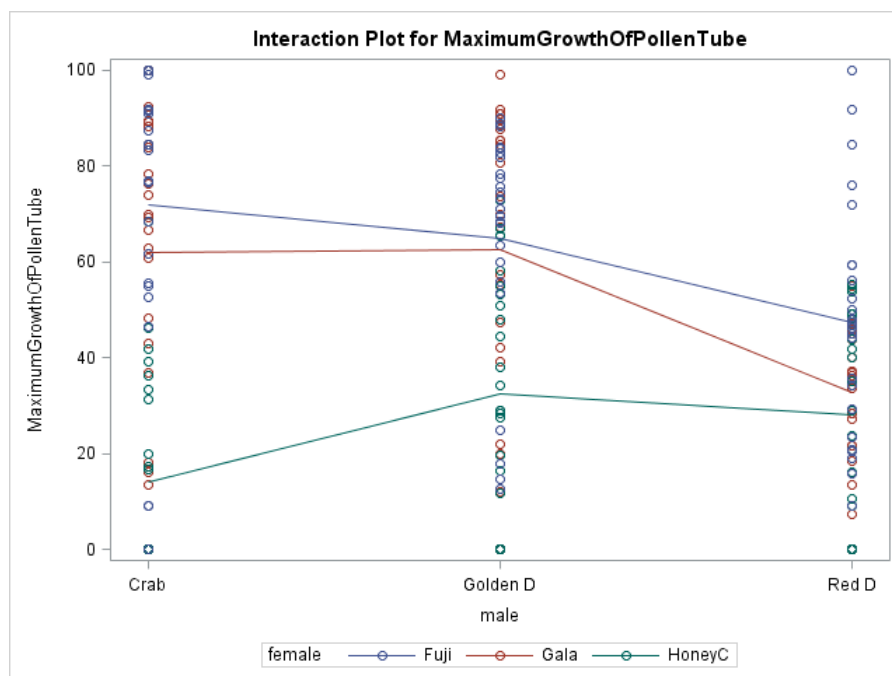


Figure 41: Male and Female interaction plot, Day 2, 2014

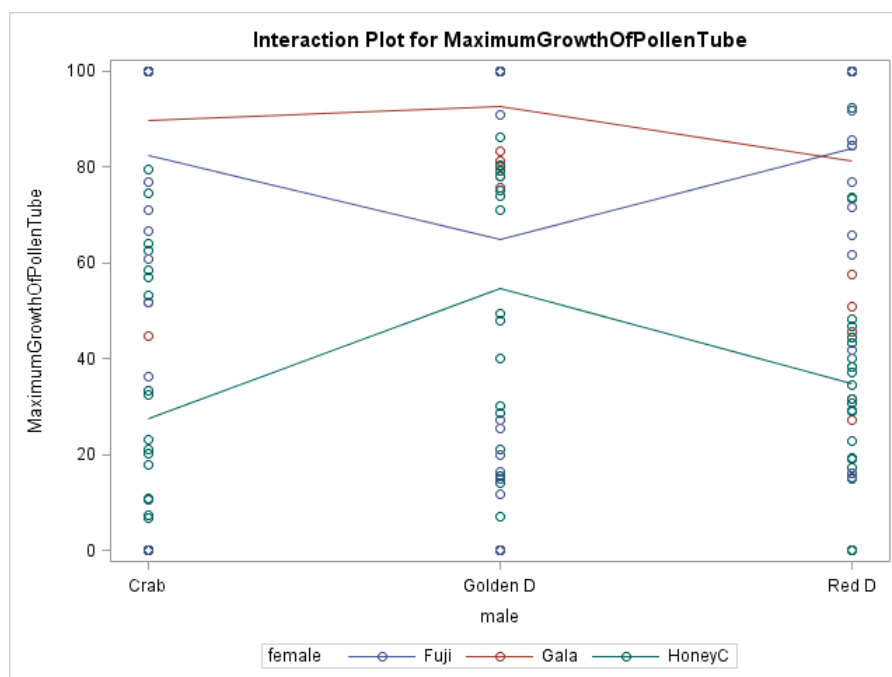


Figure 42: Male and Female interaction plot, Day 3, 2014

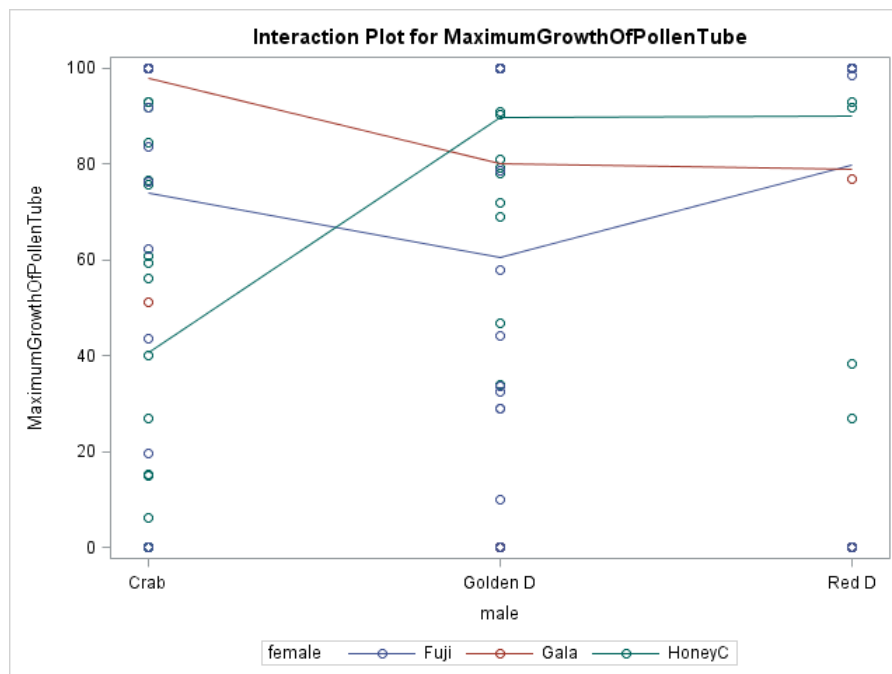


Figure 43: Male and Female interaction plot, Day 4, 2014

5. Number of pollen tubes that reached to the base of style DAYS 1, 2, 3, 4

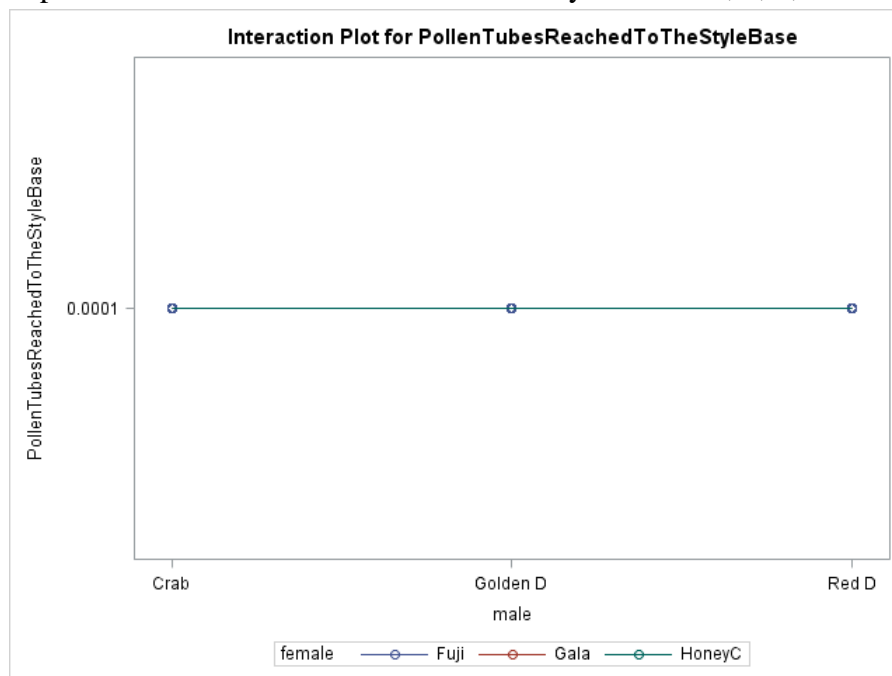


Figure 44: Male and Female interaction plot, Day 1, 2014

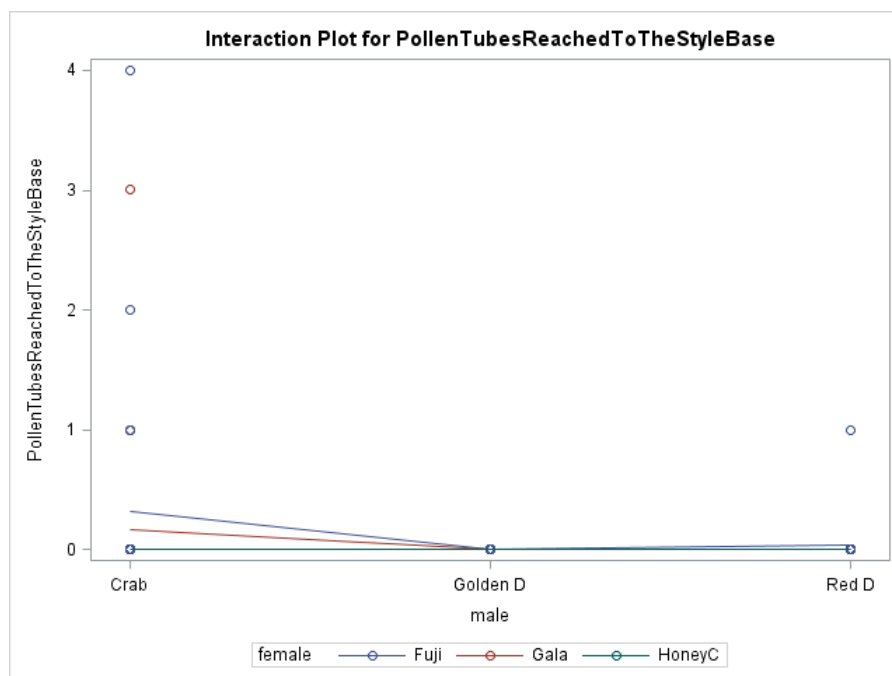


Figure 45: Male and Female interaction plot, Day 2, 2014

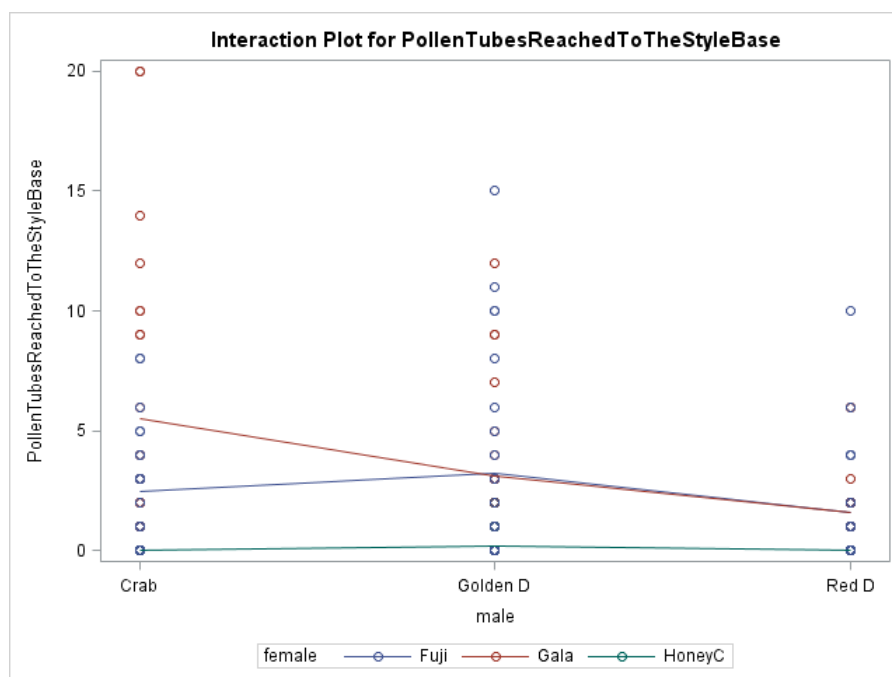


Figure 46: Male and Female interaction plot, Day 3, 2014

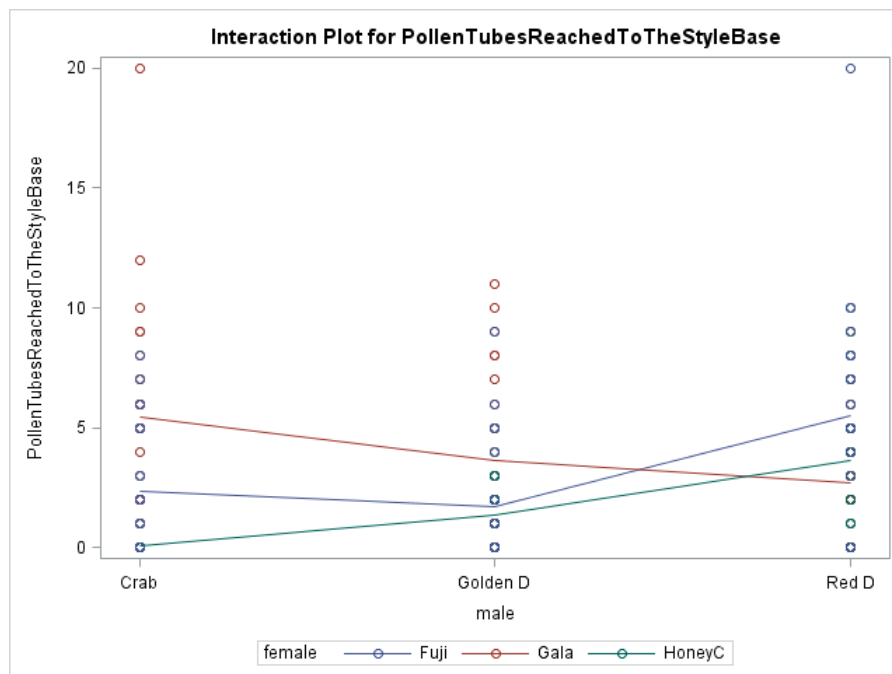


Figure 47: Male and Female interaction plot, Day 4, 2014

Appendix B Experiment two interaction plots

Experiment two: Male and female interaction plots of fruit quality attributes, 2013

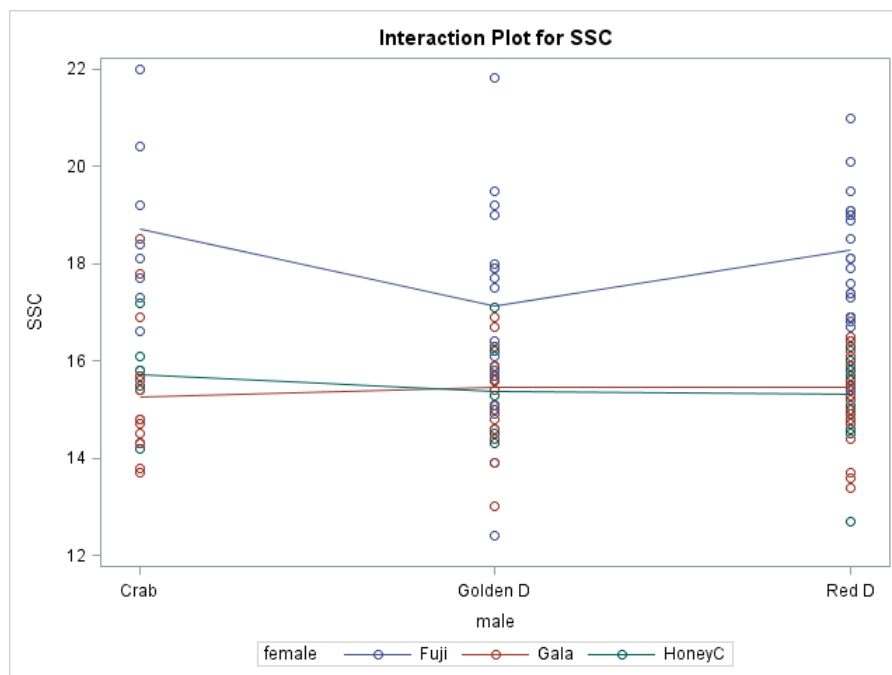


Figure 1: Male and Female interaction plot of soluble solids contents, 2013

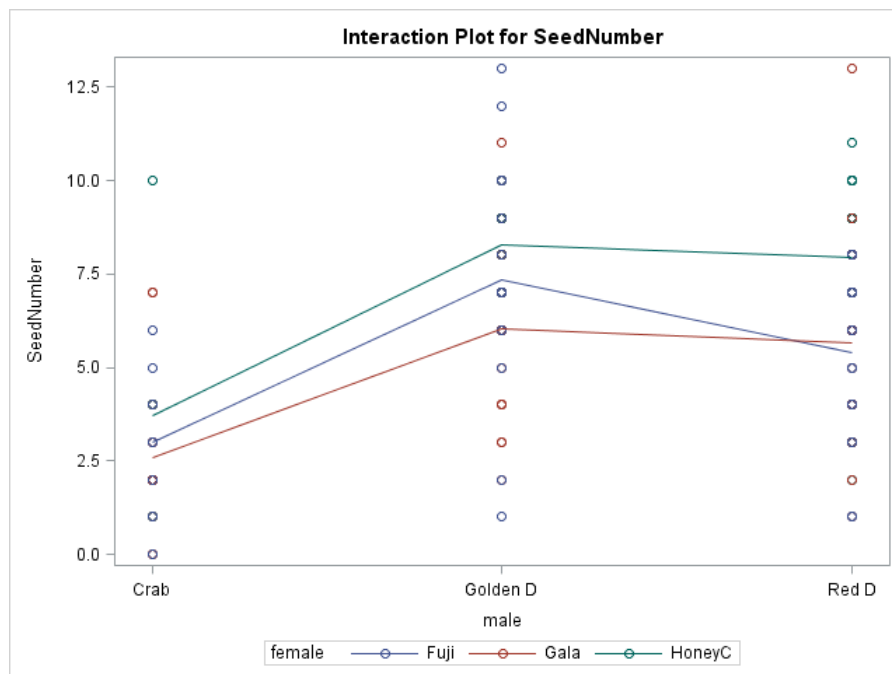


Figure 2: Male and Female interaction plot of seed number per fruit, 2013

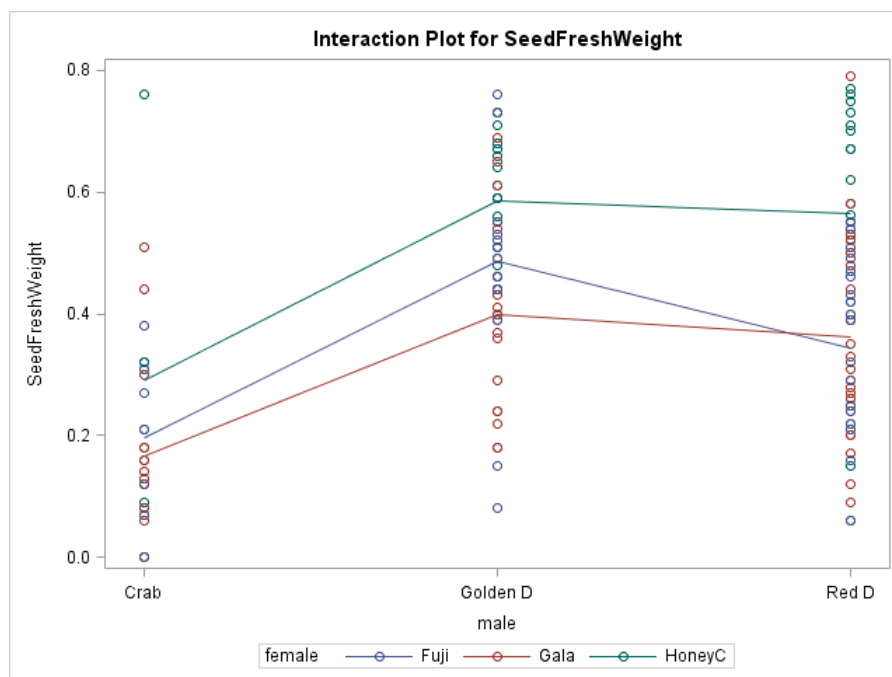


Figure 3: Male and Female interaction plot of seed fresh weight, 2013

Experiment 2: Male and female interaction plots of fruit quality attributes, 2014

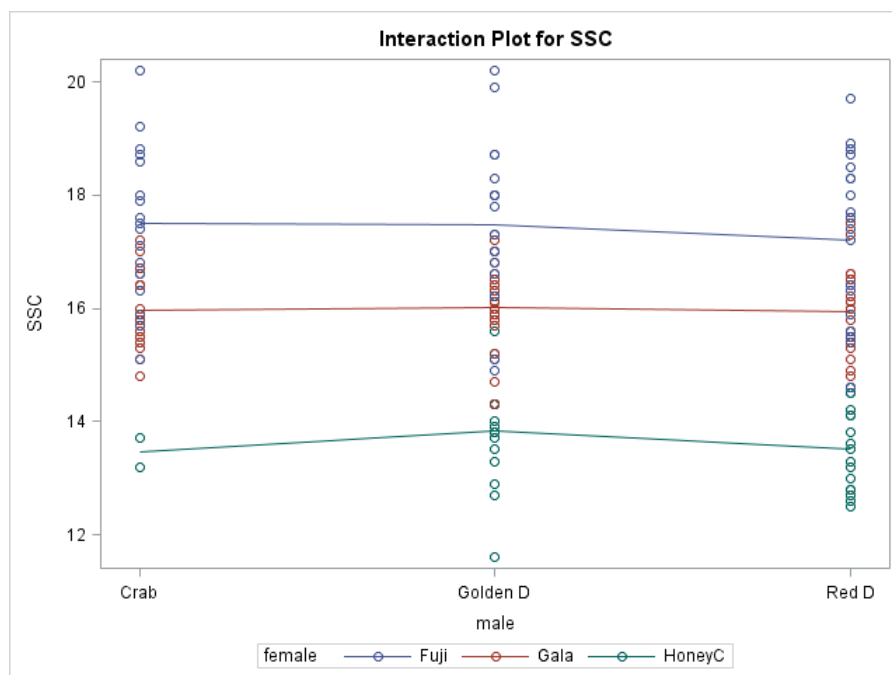


Figure 1: Male and female interaction plot of soluble solids contents, 2014

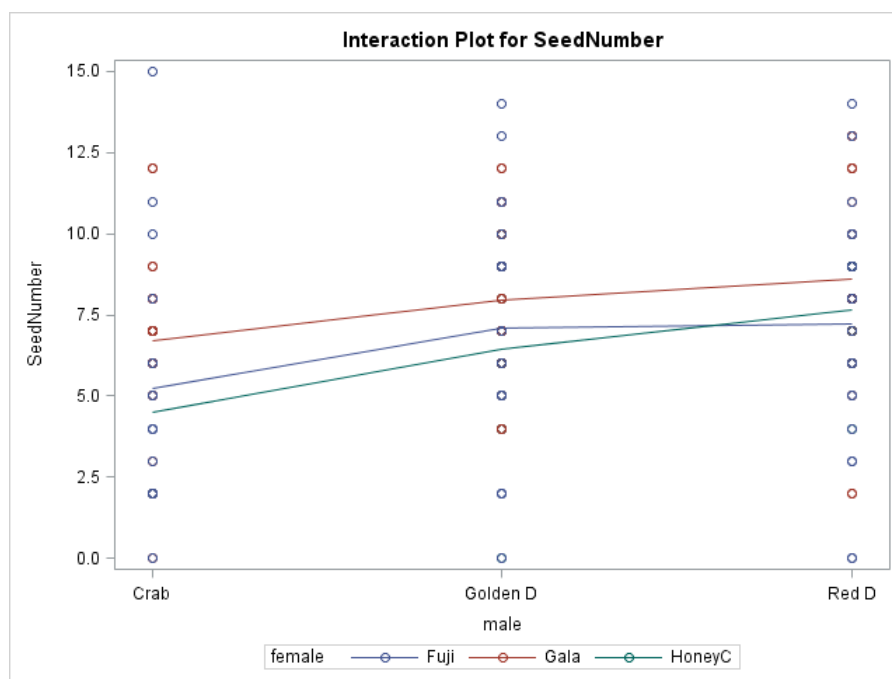


Figure 2: Male and female interaction plot of seed number per fruit, 2014

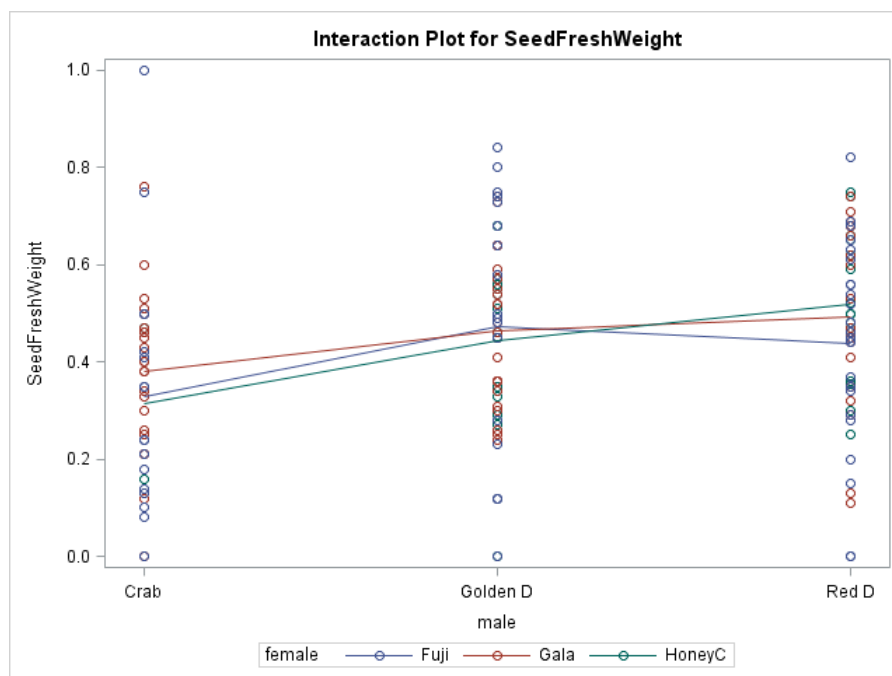


Figure 3: Male and female interaction plot of seed fresh weight, 2014