J. Amer. Soc. Hort. Sci. 136(2):83–92. 2011.

A Morphological and Histological Characterization of Bisexual and Male Flower Types in Pomegranate

Hazel Y. Wetzstein[1]
University of Georgia, Department of Horticulture, 1111 Miller Plant Science Building, Athens, GA 30602

Nadav Ravid and Erik Wilkins
Paramount Farming Company, 33141 E. Lerdo Highway, Bakersfield, CA 93308

Adriana Pinheiro Martinelli
University of São Paulo, CENA, Av. Centenário 303, 13400-970, Piracicaba, Sao Paulo, Brazil

Additional index words. *Punica granatum*, hermaphroditic, staminate, ovules, pollen, stigma

Abstract. **Pomegranate [*Punica granatum* (Punicaceae)] is characterized by having two types of flowers on the same tree: hermaphroditic bisexual flowers and functionally male flowers. This condition, defined as functional andromonoecy, can result in decreased yields resulting from the inability of male flowers to set fruit. Morphological and histological analyses of bisexual and male flowers were conducted using light and scanning electron microscopy (SEM) to characterize the different flower types observed in pomegranate plants and to better understand their developmental differences. Bisexual flowers had a discoid stigma covered with copious exudate, elongated stigmatic papillae, a single elongate style, and numerous stamens inserted on the inner wall of the calyx tube. Using fluorescence staining, high numbers of pollen tubes were observed growing through a central stylar canal. Ovules were numerous, elliptical, and anatropous. In contrast, male flowers had reduced female parts and exhibited shortened pistils of variable heights. Stigmatic papillae of male flowers had little exudate yet supported pollen germination. However, pollen tubes were rarely observed in styles. Ovules in male flowers were rudimentary and exhibited various stages of degeneration. Pollen from both types of flowers was of similar size, ≈20 μm, and exhibited similar percent germination using in vitro germination assays. Pollen germination was strongly influenced by temperature. Maximal germination (greater than 74%) was obtained at 25 and 35 °C; pollen germination was significantly lower at 15 °C (58%) and 5 °C (10%).**

Pomegranate has been grown as a fruit crop since ancient times. Native to central Asia, this tree/shrub is highly adaptive to a wide range of climates and soil conditions and is grown in many geographical regions, including the Mediterranean basin, Asia, and the United States, particularly in California (Holland et al., 2009). It has long been valued for its flavorful, juicy aril sacs and more lately for commercial juice production. Recently, numerous studies have verified the health benefits associated with pomegranates. Pomegranate juice and products are purported to show efficacy against a wide range of conditions, including cancer, coronary heart disease, atherosclerosis, hypercholesterolemia, hyperlipidemia, hypertension, HIV, infectious diseases, aging, and brain disorders (Basu and Penugonda, 2008; Holland et al., 2009; Lansky and Newman, 2007; Seeram et al., 2006). This has led to a higher awareness of the public to the benefits of pomegranate and a prominent increase in the consumption of the fruit and juice (Holland et al., 2009).

Flowering in pomegranate is characterized as having both hermaphroditic (bisexual) flowers and functionally male flowers on the same plant, a condition referred to as andromonoecy. The hermaphroditic flowers have well-formed female (stigma, style, ovary) and male (filaments and anthers) parts and have been referred to as ''fertile,'' ''vase-shaped,'' and ''bisexual'' flowers. Because the hermaphroditic flowers are the type that set fruit, they are commonly referred to as ''female'' flowers, albeit with some inaccuracy. The male flowers produce well-developed male parts, but on closer examination of the pistil contain reduced female parts. Thus, their role is more accurately depicted as functionally male flowers (i.e., flowers are not strictly male), but rather have degenerated female parts. Male flowers typically drop and fail to set fruit (Holland et al., 2009; Shulman et al., 1984). Chaudhari and Desai (1993) classified pomegranate flowers into three types: male, hermaphroditic, and intermediate. Observations of gradients of flower types in some pomegranate genotypes support this concept. Synonyms for male flowers include ''infertile'' and ''bell'' flowers. Herein, we refer to the flower types as ''bisexual'' and ''male'' for hermaphroditic and functionally male flower types, respectively.

Andromonoecy as well as other fluctuating sexual expression types is proposed to allow a species to optimize the allocation of limited resources to male and female function (Bertin, 1982; Wilson, 1983). Manipulating the relative ratio of flower types to environmental conditions can be very advantageous. As a result of the high costs associated with female expression, repression of this flower type under poor environmental conditions could be a means to conserve limited resources at a time when maturation of a high fruit/seed yield is not possible. Furthermore, having high numbers of male flowers can be a way to spread genes, because pollen spread is more efficient with more male flowers (Herlihy and Eckert, 2002; Tanurdzic and Banks, 2004).

Under agricultural production conditions, male:female flower ratios in pomegranate can impact crop productivity

Received for publication 3 Nov. 2010. Accepted for publication 6 Dec. 2010. We acknowledge Paramount Farming Company for field, research, and financial support; CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico, for a research fellowship to A.P.M. and J. G. Bracalion for assistance with micrographs.
[1]Corresponding author. E-mail: hywetz@uga.edu.

and yield. Male flowers drop and generally fail to set; thus, fruits develop exclusively from bisexual flowers. A positive relation was found between the percentage of bisexual flowers and bearing capacity (Chaudhari and Desai, 1993; El Sese, 1988). The percentage of flowers that are male in pomegranate can be significant and more than 60% to 70% depending on variety and season (Chaudhari and Desai, 1993; Mars, 2000). Furthermore, the ratio of bisexual and functionally male flowers can vary with season (N. Ravid, personal communication), cultural location, and genotype. Fluctuations of flower types within a season can proceed with a predominant appearance of bisexual flowers followed by male flowers or vice versa.

Although this crop has been grown as an agricultural crop since antiquity, scientific literature on many fundamental aspects of pomegranate development and physiology is lacking, including basic aspects of floral biology. A clear understanding of male and female flowering is lacking. As part of an ongoing project on pomegranate reproductive biology, the present work aims to describe the morphology and anatomy of bisexual and functionally male flower types in pomegranate. Morphological and histological evaluations of hermaphroditic and male flowers were conducted using light microscopy (LM) and SEM to better understand developmental differences between the flower types. Sex expression is labile in this species. A comparison of the form and function of flower parts in the two morphs, including pollen viability, stigma development, and pollen–pistil interactions, would provide information useful in the development of crop production protocols to enhance fruit production.

Material and Methods

Plant material. Pomegranate flowers were collected from 8-year-old 'Wonderful' trees grown in a commercial orchard block located near Delano, CA, that was managed under conventional methods. Bisexual and functionally male flowers were separated based on the size of the pistil, which in male flowers is expressed as a shortened style. One hundred newly opened flowers of each type were collected from several trees and taken back to the laboratory for measurements; flowering was early to midseason. Some tissues were fixed and processed for LM and SEM as described subsequently. In addition, a set of 20 female and 20 male flowers was emasculated and bagged in Delnet pollination bags (DelStar Technologies, Middletown, DE) before anther dehiscence and petal opening so that histological evaluations could be made of unpollinated flowers to facilitate stigma and stylar histological studies.

Light microscopy. Samples were prepared for LM as we have previously described (Yi et al., 2006). Tissues were fixed in a mixture of 3% paraformaldehyde (w/v) and 2% glutaraldehyde (v/v) in 0.2 M cacodylate buffer, pH 7.2 at 4 °C; dehydrated in a series of methyl cellosolve, ethanol, propanol, and n-butanol; and then infiltrated and embedded in Historesin (Leica Instruments, Heidelberg, Germany), which consists of hydroxyethylmethacrylate. Sections 5 to 6 μm thick were cut with a tungsten knife using a rotary microtome (HM350; Microm, Heidelberg, Germany). Serial sections were made to aid interpretations. Sections were stained successively in 1% acid fuchsin (w/v) and 0.05% toluidine blue O (w/v) and then mounted in Mount-quick (EMS, Fort Washington, PA). Starch was visualized by staining sections in a 50% IKI solution (Jensen, 1962). Sections were examined under bright field using a light microscope (BX51; Olympus America, Center Valley, PA) and photographed digitally. To visualize pollen tube growth in the style, pistils were dissected from flowers and then fixed in ethanol:acetic acid (3:1, v/v). To clear and soften tissues, stigma-style portions of each pistil were transferred into 10% sodium sulfite (w/v) and then autoclaved at 120 °C for 10 min. Samples were rinsed in water and then stained with aniline blue in K_3PO_4 buffer for 18 to 24 h. Whole mounts of styles were observed under both ultraviolet and transmitted light.

Scanning electron microscopy. Bisexual and male flowers were dissected and prepared using methods described by Woo and Wetzstein (2008) for SEM. Flowers were fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, dehydrated through an ethanol series, and critical point dried through carbon dioxide using a critical point drier (Samdri-790; Tousimis Research, Rockville, MD). Dried samples were mounted on aluminum stubs using carbon conductive tabs and sputter coated (SPI-Module; SPI Supplies, West Chester, PA) with gold. Samples were examined using a SEM (JSM 5800; JEOL, Tokyo, Japan) at 15 kV.

In vitro pollen germination. In vitro germination assays were conducted according to Yi et al. (2003). Freshly collected pollen was inoculated into 96-well plates (Microtest III; Falcon, Lincoln Park, NJ) containing a pollen germination medium consisting of 0.062% $CaNO_3$ (w/v), 0.024% boric acid (w/v), and 12% sucrose (w/v). Cultured pollen was maintained at one of four temperatures (5, 15, 25, or 35 °C), and percent germination assessed at 1-, 3-, and 5-h incubation times using an inverted microscope (DIAPHOT; Nikon, Garden City, NY). A pollen grain was considered germinated if tube length was longer than the diameter of the pollen grain. Eight hundred pollen grains were evaluated for each temperature (800 pollen grains × four temperatures).

Statistical analysis. Statistical analyses were conducted using the General Linear Model (SAS Version 9.1; SAS Institute, Cary, NC). Differences in flower measurements between bisexual and male flowers were compared using Student's *t* test at $\alpha = 0.05$. Pollen germination under different temperatures was compared by Duncan's multiple range test at $\alpha = 0.05$.

Results

Pomegranate trees produce large showy flowers, which in 'Wonderful' are a vivid orange–red color (Fig. 1A). Flowers are near sessile and are born either singly (Fig. 1B) or in clusters (Fig. 1C) of commonly one terminal flower subtended by two to four lateral flowers. The calyx is red, thick, leathery, fused at the base, and usually pentamerous, although infrequently tetramerous. At the distal portion of unopened buds (Figs. 1A and 1C), indentations are evident showing the region where the calyx will separate and form acuminate triangular lobes (Fig. 1B), which will persist and form the crown of the mature fruit. The normally five antesepalous petals are ovate, delicate, slightly crenulated, and may show streaks of darker pigmentation. The ovary is inferior, and the numerous stamens are composed of yellow anthers attached to long red filaments, which are inserted on the inner surface of the calyx tube (Fig. 1D–E). Anthers surround an elongated style, which at the base broadens into a conical-shaped stylopodium (Fig. 1D). When flowers are bisected longitudinally, the two flower types are readily discernable based on differences in pistil development. Bisexual flowers (Fig. 1D) possess a well-developed pistil with an elongated style that extends at or above the height of anthers.

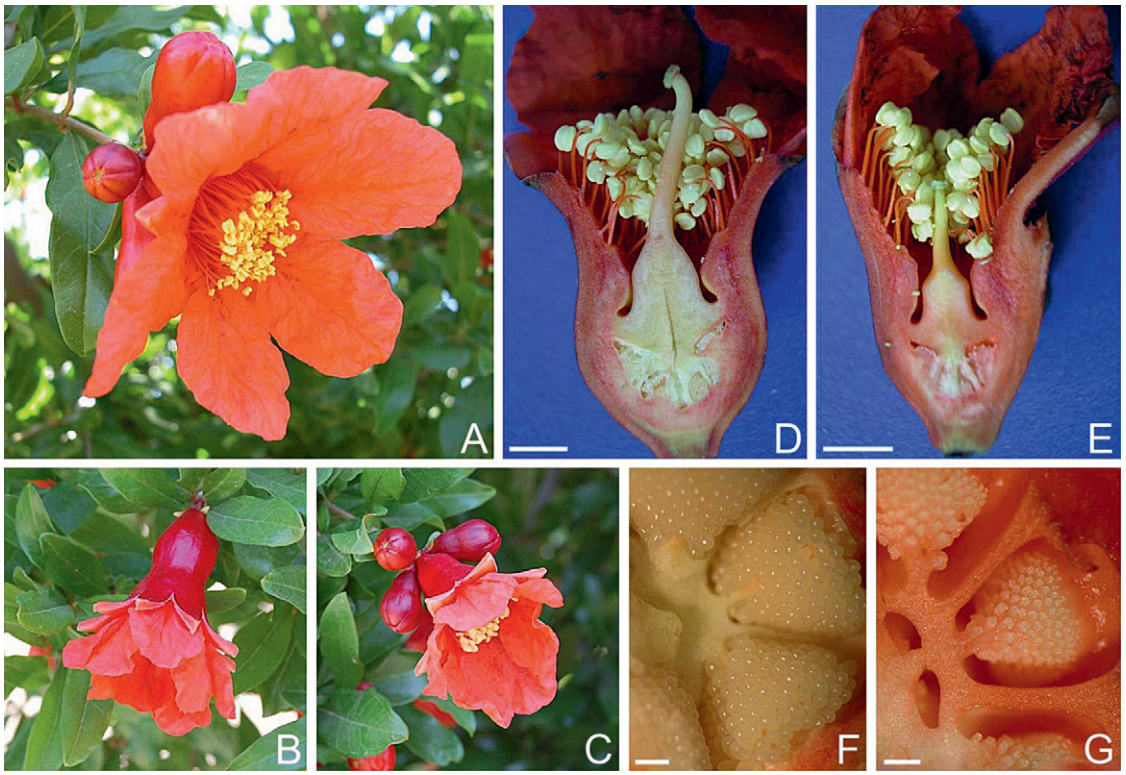

Fig. 1. Pomegranate flowers. (**A**) Fully open flower showing vivid orange–red petals and numerous anthers attached to long, red filaments. (**B**) Single near sessile flower. The leathery calyx is fused at the base. (**C**) Flower cluster with an advanced central flower subtended by closed buds. (**D**) Longitudinal section of a bisexual flower showing anthers with filaments inserted on the inner surface of the calyx tube. (**E**) Longitudinal section of a functionally male flower showing well-developed stamens but an underdeveloped pistil. (**F**) Details of well-formed ovules from a hermaphroditic flower. (**G**) Underdeveloped ovules from a functionally male flower. Scale bars: **D–E** = 5.0 mm, **F–G** = 500 µm.

Flowers have a prominent U-shaped ovary (Fig. 1D) containing numerous elliptical ovules (Fig. 1F). In contrast, male flowers (Fig. 1E) have a style that is shorter than the height of anthers and an ovary with a more V-shaped configuration. Ovules are rudimentary, much smaller in size, and have an irregular surface (Fig. 1G) compared with the rounded, cream-colored, and glistening appearance of ovules in bisexual flowers (Fig. 1F).

Male flowers are significantly smaller than bisexual flowers (Table 1). In male flowers, the length of flowers from the tip of sepals to the base of flowers was 75% of the mean length found on bisexual flowers. The mean pistil length in male flowers was also significantly shorter (70% the length of bisexual flowers). Longer pistil heights in bisexual flowers are primarily the result of more extensive growth of the ovary region (which was 300% longer in bisexual versus male flowers) and to a lesser extent the result of increases in the height of the stigma + style + stylopodium (7% taller in bisexual versus male flowers). Bisexual flowers have stigmas that are close to or greater than the height of the anthers. In contrast, the height of stigmas in male flowers is generally well below the height of the tallest anthers with the mean height ≈14 mm below anther height.

SEM observations of bisexual flowers show a prominent, slightly irregular disc-shaped stigma ending on a smooth elongated style (Fig. 2A). Elongated stigmatic papillae extend on the surface of the stigma. At anthesis, the stigmatic surface is covered with a copious exudate that partially obscures the basal portion of papillae (Fig. 2B). The style has a uniseriate epidermis and is composed of several layers of elongate cells evident in longitudinal sections (Fig. 2C). Micrographs taken of flowers that were bagged to prevent pollination (Fig. 2D) show an open, irregularly shaped, centrally located stylar canal (Fig. 2D). In cross-section, circular groups of vascular strands that transverse the length of the stigma are peripherally oriented around the stylar canal (Fig. 2E). Exudate is present lining the central stylar canal. Germinating pollen and tubes are evident

Table 1. Flower characteristics of bisexual and male pomegranate flowers showing differences in the sizes of flower organs.[z]

Flower characteristic[y]	Bisexual flowers (mean ± SD)	Male flowers (mean ± SD)
Length of pedicel to tip of sepals (mm)	36.0 ± 2.7 a[x]	26.7 ± 2.9 b
Pistil length (mm)	27.5 ± 2.1 a	19.4 ± 2.9 b
Stigma + style length (mm)[w]	17.1 ± 2.0 a	15.9 ± 2.5 b
Ovary height (mm)	10.5 ± 1.8 a	3.5 ± 2.1 b
Height of stigma exsertion (mm)	1.4 ± 1.6 a	–13.8 ± 1.8 b
Anthers (no.)	137 ± 34 b	193 ± 53 a
Sepals (no.)	5.2 ± 0.7 a	5.4 ± 0.7 a

[z]Values are means taken from 100 bisexual and 100 male flower types.
[y]Characteristics correspond to letters as noted on the accompanying figure.
[x]Means within a row (comparing bisexual versus male flowers) followed by different letters are significantly different at $P \leq 0.05$ using Student's t test.
[w]Measurement was taken from the tip of the stigma to the base of the stylopodium.

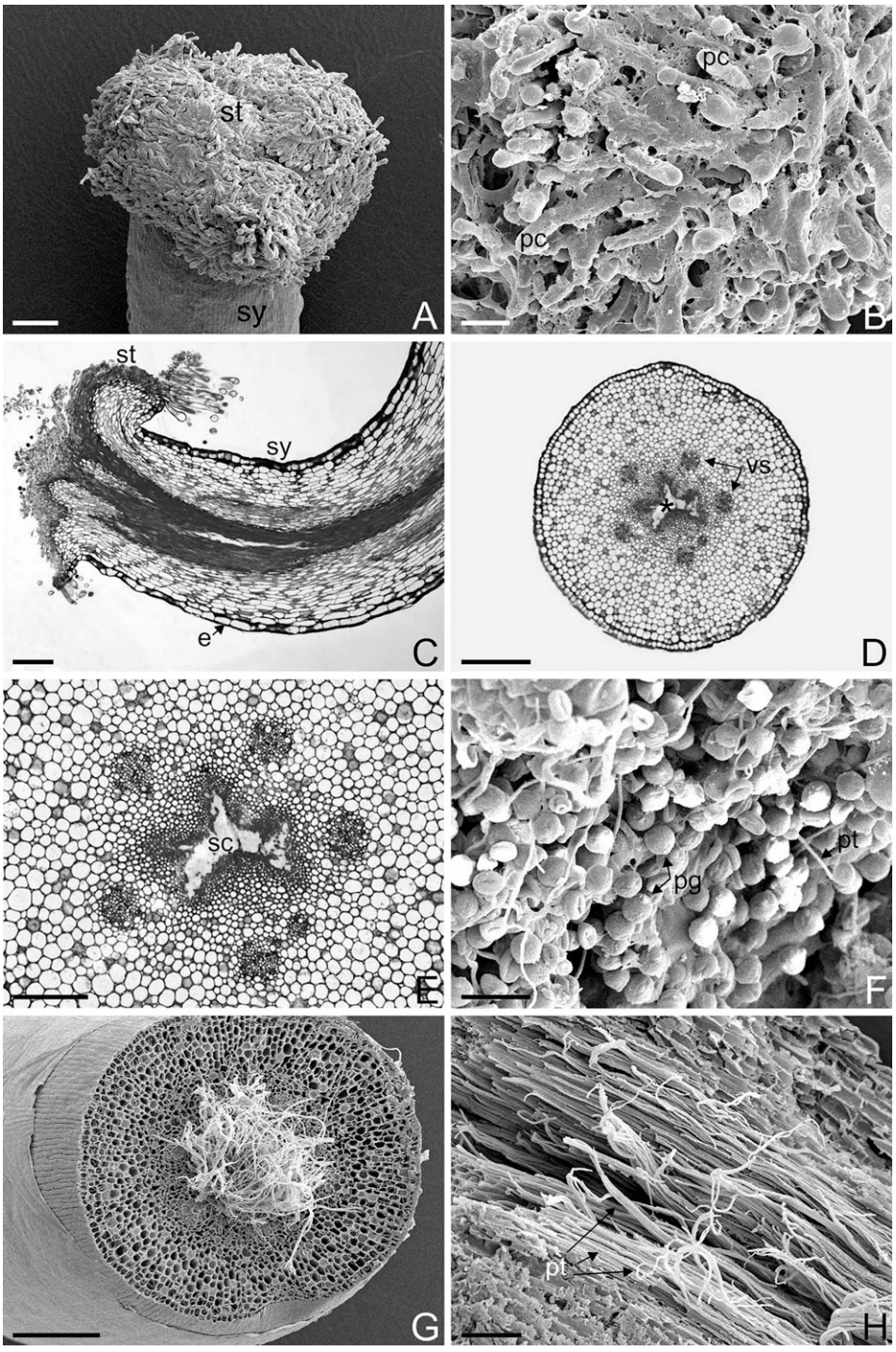

Fig. 2. Stigmas and styles of bisexual pomegranate flowers. (**A**) Scanning electron microscopy (SEM) of an unpollinated flower showing a rounded, disc-shaped stigma (st) ending on a smooth elongated style (sy). (**B**) Higher magnification of the stigmatic surface showing elongated papillary cells (pc) with abundant exudate accumulation characteristic of bisexual flowers. (**C**) Longitudinal section of the stigma (st) and style (sy) showing uniseriate epidermis (e) and elongated stylar cells. (**D**) Cross-section of the style in an unpollinated flower showing vascular strands (vs) and stylar canal. (**E**) Higher magnification of the open central stylar canal (sc). (**F**) SEM of the stigmatic surface of a pollinated flower showing numerous pollen grains (pg) and some germinated pollen and tubes (pt). (**G**) Cross-section of the style of a pollinated flower showing massive numbers of pollen tubes within the stylar canal. (**H**) Longitudinal fracture of a style showing the canal region with aggregates of pollen tubes (pt). Scale bars: **A–D, G–H** = 100 μm, **E–F** = 50 μm.

on the stigmas of pollinated flowers (Fig. 2F). Pollen grains varied from those showing various degrees of hydration with some pollen grains becoming partially submerged by stigmatic exudate. Tubes transverse the stigma and then grow within the stylar canal. An SEM cross-section shows a tuft-like collection of filamentous pollen tubes growing through the stylar canal (Fig. 2G). Styles fractured longitudinally show hundreds of pollen tubes growing within the stylar canal (Fig. 2H). Pollen tubes are numerous, forming clusters of chord-like aggregations within the canal.

The stigmas of male flowers are well developed and have a disc-shaped configuration with elongated stigmatic papillae (Fig. 3A) similar to that seen in bisexual flowers. However, at the time of petal opening, stigmas of male flowers have markedly less stigmatic exudate so that the smooth surfaces as well as the basal portions of the stigmatic papillae are clearly evident (Fig. 3B). This is in contrast to the partially submerged stigmatic cells in bisexual flowers (Fig. 2B). The shorter styles of male flowers have a dominant central canal clearly evident in longitudinal sections (Fig. 3C–D). Although stigmas on male flowers possess reduced amounts of exudates, pollen germination and tube extension were observed (Fig. 3E). However, pollen tubes often lacked directional growth and were observed growing in the air (Fig. 3E) in contrast to pollen tube growth in bisexual flowers, which was more confined to along the stigmatic surface (Fig. 2F). Pollen tubes in male flowers generally failed to penetrate the stigma and were not observed within the stylar canal even in hand-pollinated flowers (Fig. 3F).

Ovules in bisexual flowers are numerous, elliptical, and tightly packed (Fig. 4A). Ovules are anatropous and at maturity have completely inverted so that the opening to the micropyle (point) is positioned adjacent to the funiculus (Fig. 4C–D). Removed ovules reveal the attachment site of the funicle, which is shown as a scar. The outer surface of ovules in bisexual flowers has regularly shaped epidermal cells that form a smooth surface. In contrast, ovules in male flowers are considerably less developed (Fig. 4B) and can be noticeably smaller than those in bisexual flowers (ovules in Fig. 4A versus Fig. 4B are 10× larger). Ovule surfaces in male flowers have epidermal cells that appeared sunken and exhibit

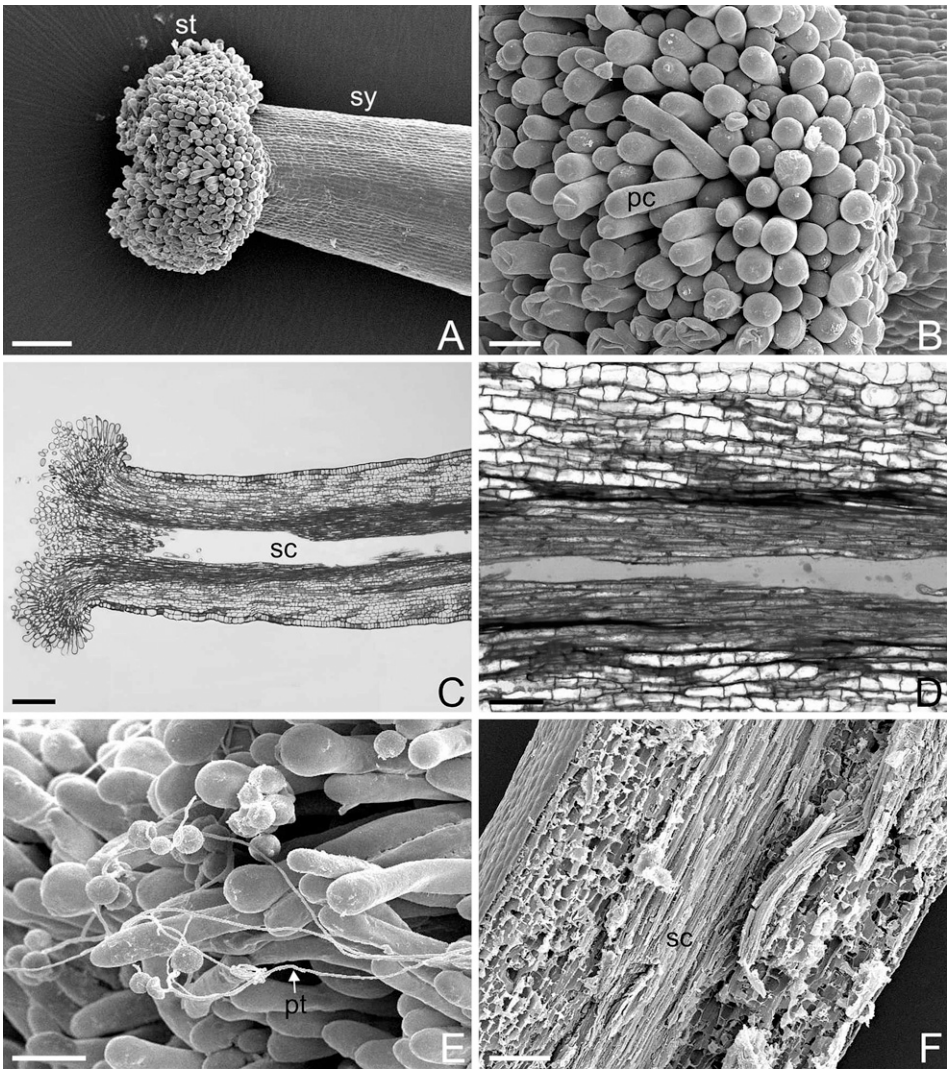

Fig. 3. Stigmas and styles of functionally male pomegranate flowers. (**A**) Scanning electron microscopy (SEM) of rounded stigma (st) from a male flower showing an elongated style (sy). (**B**) Stigmatic surface with elongated stigmatic papillary cells (pc); no accumulation of stigmatic exudates is observed. (**C**) Longitudinal light microscopy section of stigma and stylar region showing stylar canal (sc). (**D**) Higher magnification of the stylar canal. (**E**) SEM of the stigmatic surface of a pollinated flower. Some pollen has germinated; however, aerial pollen tube growth (pt) is observed. (**F**) Longitudinal fracture of stylar region showing an absence of pollen tube growth within the stylar canal (sc). Scale bars: **A, C** = 200 µm, **B, D–E** = 50 µm, **F** = 100 µm.

cellular collapse. Ovules show variable stages of arrested development ranging from those that show collapse primarily in apical regions (Fig. 4E) to those that exhibit more extensive abortive development so that ovules are rudimentary, shriveled, severely degenerated, and appear as collapsed emergences (Fig. 4F).

The orientation of ovules along the placental surface is shown in LM sections of the two flower types (Fig. 5). In bisexual flowers (Fig. 5A–B), ovules are elliptical in longitudinal section and are bitegmic with a two-layered integument. The inner integument is composed of two to three cell layers, whereas the outer integument is thicker. At floral maturity, the axis of the ovule is inverted and curved downward so that the micropylar opening faces the placenta and base of the funiculus (Fig. 5B).

Variations in ovule development were observed in male flowers (Fig. 5C–F). In some cases, some partially developed structures could be observed with ovules attached at a funicular region and enclosed within a differentiated bi-integument (Fig. 5C–D). However, integuments had incomplete development of cell layers and exhibited cell collapse and separation of the inner and outer integument layers (Fig. 5D). Ovules could be partially anatropous (Fig. 5D) or lack curvature and remain in an upright, orthotropous orientation (Fig. 4E). Some flowers exhibited a drastic failure in the developmental program (Fig. 5E–F). Undifferentiated cellular protrusions formed from limited mitotic activity (Figs. 4F and 5F). Structures emerging from placental regions lacked a differentiated embryo sac, funiculus, or integuments.

Stamens are numerous in both flower types and are composed of red-colored filaments ended by light yellow anthers (Fig. 1A–B). Bright yellow pollen is released from well-differentiated anthers, which dehisce along two abscission zones (Fig. 6A). Pollen is spherical and tricolpate with a smooth exine surface (Fig. 6B). Pollen from both bisexual and male flower types is similar in size (≈20 µm). However, male flowers have significantly more stamens per flower (i.e., 1.4 times more numerous) than bisexual flowers (Table 1). Pollen viability in both bisexual and male flowers is relatively high as assessed by in vitro germination assays with percent germination routinely at 75% to 80%. Whole mounts of a cleared pistil from a bisexual flower show an expanded stigmatic region and vascular stands (linear darker areas) that extend from the stigma (Fig. 6C) and continue down the length of the style (Fig. 6E). Aniline blue staining of tissues observed under ultraviolet light shows the path of pollen tubes throughout much of the stigma (Fig. 6D) but which become directed to grow only within the central stylar canal (Figs. 6D and 6F). Figures 6C versus 6D and Figures 6E versus 6F are companion micrographs taken of identical locations but viewed under regular transmitted versus ultraviolet light, respectively. The paths of hundreds of pollen tubes with callose plugs are shown in a higher magnification of the stylar canal in a bisexual flower (Fig. 6G). This is in contrast to male flowers, in which pollen tubes were seldom observed in stylar canals (micrographs not shown). A lack of pollen tubes in the stylar canal of male flowers is in agreement with observations made with LM-sectioned and SEM material (Figs. 3C and 3E).

Pollen germination assays conducted at different temperatures showed that pomegranate pollen germination is markedly influenced by temperature (Fig. 7). At 25 and 35 °C, percent

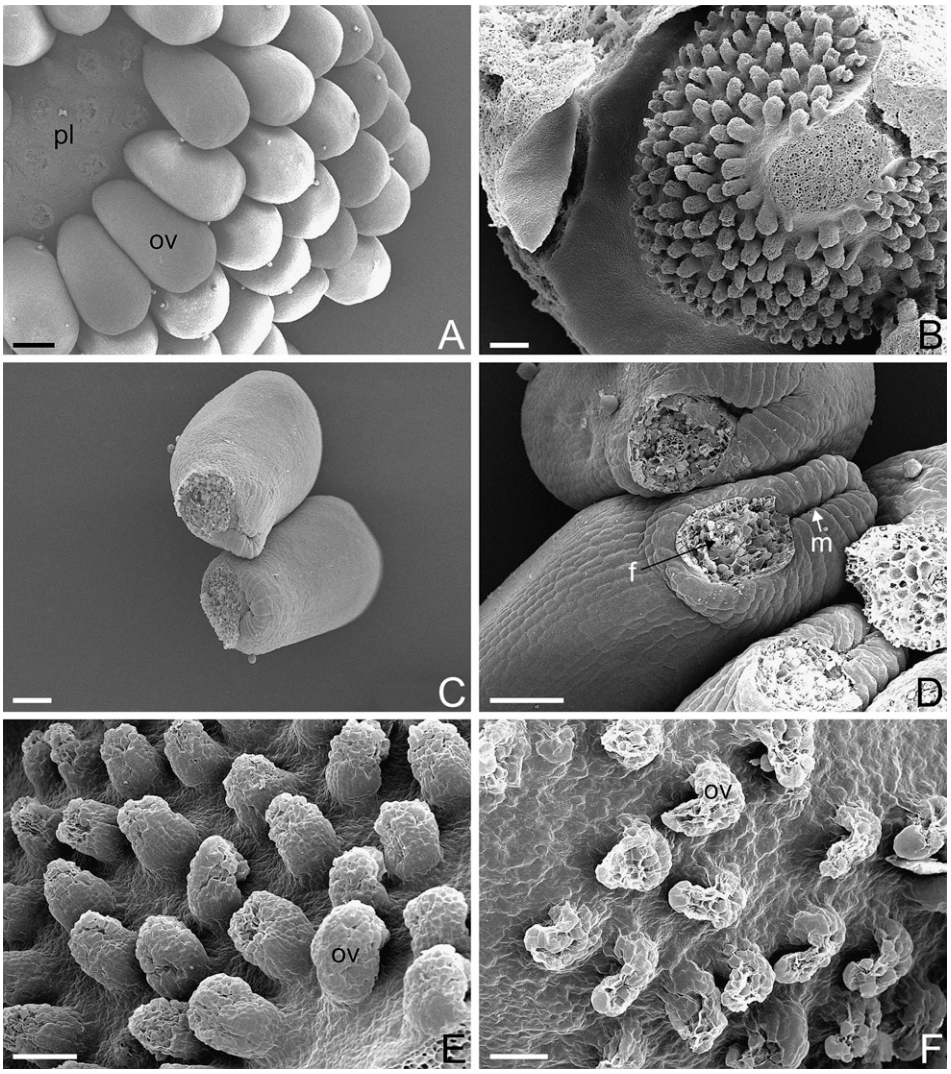

Fig. 4. Morphology of pomegranate ovules from bisexual (**A, C, D**) and functionally male (**B, E, F**) flowers. (**A**) Ovules (ov) in bisexual flowers are elliptical and tightly packed. Some ovules have been removed to show the point of attachment to the placenta (pl). (**B**) Ovules in male flowers are markedly small and underdeveloped. (**C**) Ovule from a bisexual flower showing a smooth outer surface; the attachment site of the funicule is shown as a scar. (**D**) Higher magnification of ovules from bisexual flowers. The micropylar (m) opening where pollen will enter to fertilize the zygote is adjacent to the funiculus (f). (**E**) Ovules (ov) from a male flower showing underdeveloped and collapsed regions. (**F**) Ovules (ov) from a male flower with more extensive abortive development so that ovules are rudimentary, shriveled, and severely degenerated. Scale bars: **A–B** = 200 μm, **C–E** = 100 μm, **F** = 50 μm.

pollen germination was similar and reached 74% to 79%, respectively, at 5 h incubation. Germination was rapid with a T_{50} (time to 50% germination in the population of germinating pollen grains) of ≈45 min. In contrast, germination was significantly reduced at the two lower temperatures. Percent germination at 1 h was only 6.6% and 0.5% at 15 and 5 °C temperatures, respectively, compared with ≈50% at the two higher temperatures. The T_{50} at 15 °C was ≈2 h. Maximum percent germination at 15 °C was 73% of that obtained at 25 °C. Germination was reduced by over 90% when pollen assays were conducted at 5 °C.

Discussion

Pomegranate flowers are large, showy flowers with vibrant petals and numerous anthers. Although organogenesis of both male and female flower parts occurs in all flowers, the degree of development in pistils fluctuates. Male flowers have decreased pistil development characterized with shortened stylar length and abortive ovules. Ovule degeneration can be slight or severe with structures from the placenta being rudimentary and shriveled. As a consequence of degenerated pistil development, only bisexual flowers with well-developed ovules are capable of setting fruit. Sex determination similarly results from floral organ degeneration in a number of other taxa. In dioecious date palm (*Phoenix dactylifera*), female flowers have functional carpels but vestigial sterile staminodes; male flowers have functional stamens but underdeveloped carpels (DeMason and Stolte, 1982). Likewise, pistillate and staminate flower development in dioecious pistachio (*Pistacia vera*) proceeds with androecial and gynoecial primordia initiation in both floral types, which is followed by unisexual development resulting from arrested development of male or female development (Hormaza and Polito, 1996). In mahogany (*Swietenia macrophylla*), a monoecious species, the differentiation of male and female flowers takes place during the later stages of floral development leading to arrested ovule development in male flowers and aborted microspore development in female flowers (Gouvea et al., 2008). Olive (*Olea europaea*) produces bisexual and male flower types. Functionally male, staminate flowers result from the abortion of pistils (Cuevas et al., 1999). Unisexual flower formation is not always formed from the degeneration of floral parts. For example, two monoecious tree species, *Juglans* (Lin et al., 1977; Sattler, 1973) and *Carya* (Wetzstein and Sparks, 1983, 1984), are structurally unisexual from initiation throughout development.

A difference in stigmatic exudate was another distinction observed between male and bisexual pomegranate flowers. Critical stages of pollen–stigma interactions include pollen capture, adhesion, hydration, germination, and tube growth (Hiscock and Allen, 2008) of which stigmatic secretions play an important role. In bisexual pomegranate flowers, stigmatic exudate was copious, well-developed, and covered the base of stigmatic papillae. In contrast, exudate in male flowers was much reduced. Although male flowers lacked abundant exudate, pollen germination and extensive tube growth were still noted. However, pollen tube growth appeared to lack directional growth, failed to penetrate the stigma, and was rarely observed in the style. Stigmatic exudate has been shown to be

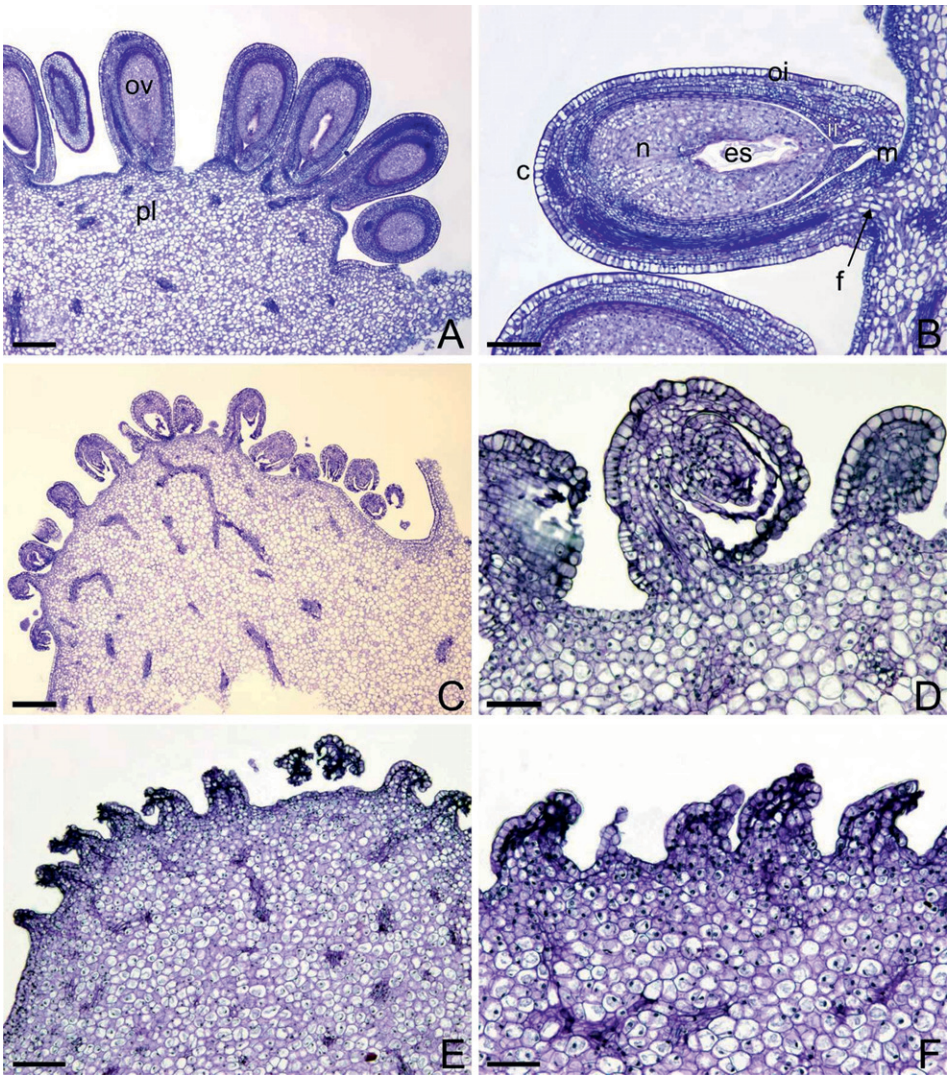

Fig. 5. Histological details of pomegranate ovules from mature hermaphroditic (**A**–**B**) and functionally male flowers (**C**–**F**). (**A**) Longitudinal section of a bisexual flower showing elliptical well-developed ovules (ov). (**B**) Closer detail of an ovule showing the funiculus (f), outer integument (oi), inner integument (ii), micropyle (m), nucellus (n), chalaza (c), and embryo sac (es). (**C**–**F**) Different degrees of ovule degeneration found in male flowers. (**C**) Ovules from a male flower, which has partially degenerated structures. Integuments and the funniculus show collapse and separation. (**D**) Higher magnification of ovules similar to those in **C**. (**E**) Severely degenerated ovules from a male flower; ovule development was arrested at an early stage and appears as limited cellular protrusions. (**F**) Higher magnification of a severely degenerated ovule. Scale bars: **A, C** = 100 µm; **B, E** = 50 µm; **D, F** = 25 µm.

important in providing directional cues for pollen tubes to grow toward and into the stigma by providing directional gradients of water potential (Lush et al., 2000) and in acting as a chemotropic attractant directing pollen tubes to grow toward stylar canals (Kim et al., 2003).

The hollow stylar canal in pomegranate can support the growth of large numbers of pollen tubes. Styles stained with aniline blue confirmed that massive numbers of pollen tubes grow through the stylar canal in pollinated bisexual flowers. Pomegranate fruits are composed of a leathery pericarp that contains hundreds of arils. Each aril is derived from an ovule and is the result of an independent fertilization event. The juice-containing translucent layer surrounds a sclarified seedcoat that encloses an embryo. The presence of a large central stylar canal would facilitate the transport of the large numbers of pollen tubes needed for fertilization of ovules required for subsequent aril development.

In contrast to the divergent pistil development observed between bisexual and male pomegranate flowers, no differences in pollen morphology or performance were observed. Pollen from bisexual and male flowers both exhibited high germination values of greater than 70%, which showed no statistical differences. A similar lack of differences in pollen viability between sexual morphs has been reported in other andromonoecious species, including olive (Cuevas and Polito, 2004), *Euphorbia nicaeenis* (Narbona et al., 2008), and *Euphorbia boetica* (Narbona et al., 2005). In pomegranate, male flowers produced significantly greater numbers of anthers suggesting that the development of staminate flowers may serve to enhance pollination by producing greater amounts of pollen. Pollen surplus has been proposed to enhance male fitness and be a selective advantage (Connolly and Anderson, 2003; Podolsky, 1993). Male flowers of *Capparis sinosa*, an andromonoecious shrub, produced larger anthers and smaller ovaries than bisexual flowers indicating that the role of male flowers is to save resources for female function and serve primarily as a pollen donor (Zhang and Tan, 2009).

Temperatures during the flowering period can have a pronounced effect on reproductive processes. The effects of temperature on pollen germination have been documented in a number of fruit tree crops. In the current study, pomegranate pollen was markedly affected by temperature. Pollen germination was significantly reduced at 5 and 15 °C, whereas the highest and similar rates of germination were obtained at 25 and 35 °C. That pomegranate pollen germination was not inhibited at 35 °C is unusual in that germination and tube growth are often reduced at such an elevated temperature. In comparisons of nine *Pistacia* species and cultivars over a range of temperatures from 4 to 40 °C, the mean temperature optimum for pollen germination was 24 °C but was severely reduced under both higher and lower temperatures; no pollen germination was observed for any of the genotypes at 40 °C (Acar and Kakani, 2010). Similarly in mango, optimum pollen germination occurred at 15 and 25 °C, whereas a decrease in germination occurred at 30 °C (Sukhvibul et al., 2000). In the Central Valley of California, which is a major production area for pomegranates in the United States, flowering occurs from May to November (Stover and Mercure, 2007); thus, a wide range of temperatures during bloom may occur. Most

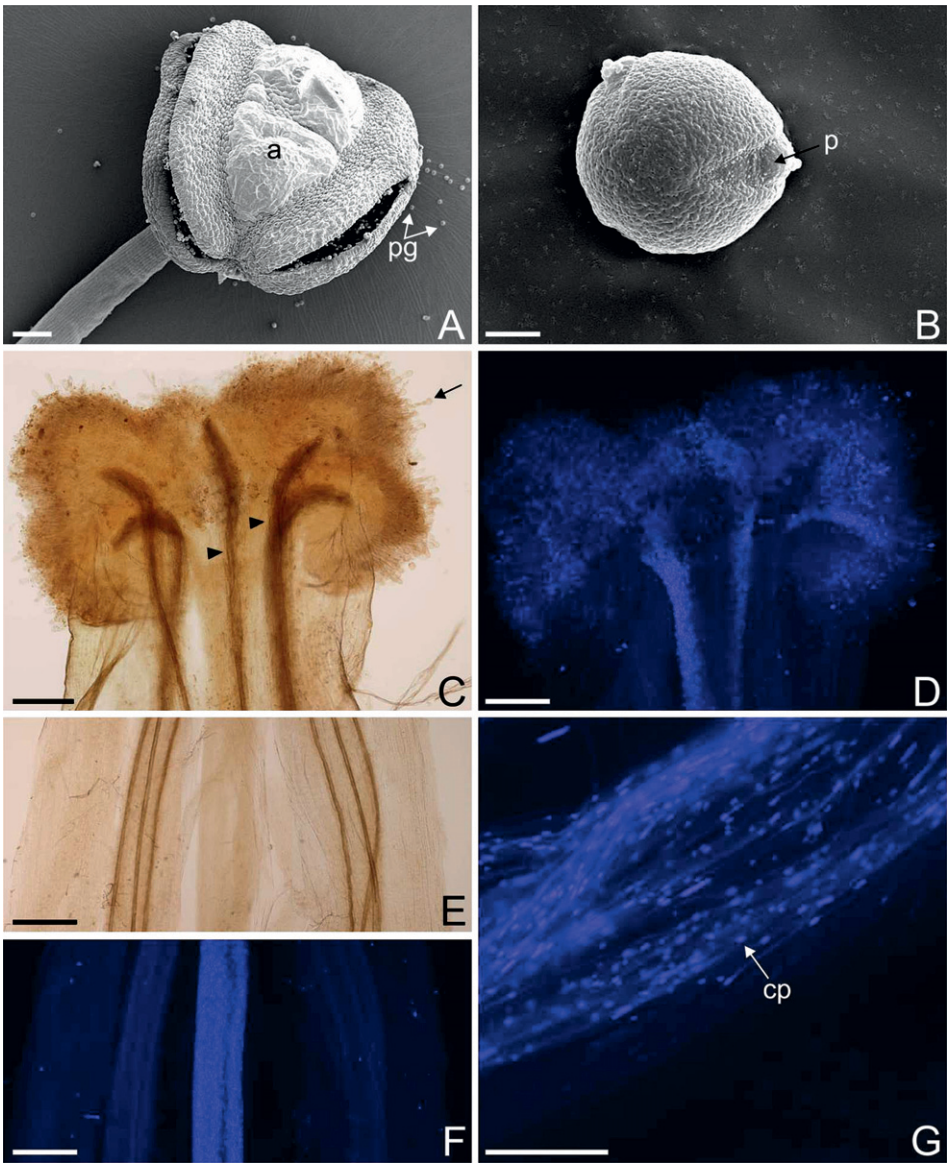

Fig. 6. Pomegranate anther, pollen, and growth of pollen tubes within the pistil. (**A**) Anther (a) after dehiscence showing release of pollen grains (pg). (**B**) Spherical and tricolpate pollen grain with a smooth exine surface (p = pore). (**C**) Cleared whole mount of a stigma stained with decolorized aniline blue and viewed under normal transmitted light. Stigmatic papillae (point) are on the surface; darkened regions are vascular strands (arrowhead). (**D**) The same region as in **C** but viewed under ultraviolet light showing fluorescing pollen tubes growing in the stigma and through the stylar canal. (**E**) Transmitted light view of a cleared portion of the style, also stained with decolorized aniline blue, showing darker vascular strands. (**F**) Identical region as in **E** viewed under ultraviolet light to show central fluorescing band composed of hundreds of pollen tubes growing within the stylar canal. (**G**) Higher magnification showing individual callose plugs (cp) of pollen tubes growing within the style. Scale bars: **A, G** = 200 µm; **B** = 5 µm; **C–F** = 100 µm.

flowering occurs in May and June, when high temperatures can be an issue. The high germination rates at elevated temperatures observed with the 'Wonderful' pollen used in this study suggest that inhibition of pollen at high temperatures during bloom may be less of a concern than in other tree crops. Temperature effects can exhibit genotypic differences. In a comparison of several olive cultivars, the optimum temperatures for pollen germination were generally 20 to 25 °C; however, 'Amigdalolia' pollen germinated similarly well at 25 and 30 °C (Koubouris et al., 2009). Considering the importance of pollination for aril and fruit development, studies comparing different pomegranate cultivars may be warranted, particularly in areas with extremes in temperature. In peach, germination assays conducted at 10, 20, or 30 °C affected primarily the rate of pollen germination with final germination only slightly affected (Hedhly et al., 2005).

Among angiosperms, labile sex determination appears to be more common among dioecious and monoecious plants than among hermaphrodites (Korpelainen, 1998). Andromonoecy or the appearance of bisexual and functionally male flowers on an individual plant can provide ecological and evolutionary advantages. Changes in sex allocation are considered a strategy to enhance fitness over the lifetime of the plant (Policansky, 1981) and can allow control of male and female function by modifying the emphasis of ovules or pollen development. With andromonoecy, the ratio of bisexual and male flowers can change with season, plant age, position within the plant, and environment. Gender diphasy or "sex choice" involves a developmental decision between two modes of prefertilization investment in femaleness (i.e., ovule production) versus maleness (Schlessman, 1988). In general, female:male flower ratios decline as environmental resources become more limiting (Korpelainen, 1998). Low nutrition, low light, limiting photosynthate, and stress from weather or water conditions can promote decreased relative numbers of female to male flowers. This is observed in a number of taxa such as *Solanum* (Miller and Diggle, 2007), Euphorbiaceae (Narbona et al., 2008), Myrtaceae (Primack and Lloyd, 1980), and Proteaceae (Walker and Whelan, 1991).

The timing of flower types can show seasonal and yearly variations. In studies conducted in Israel with 'Mule's Head' and 'Wonderful' pomegranate trees, Shulman et al. (1984) observed "normal" fertile flowering to proceed for ≈5 weeks before "abnormal" unfruitful flowering. Yet, in another season, flowering of both flower types occurred together. Also in pomegranate, Martinez et al. (2000) found that the proportion of the two flower types varied among cultivars and year to year. In *Leptospermum scoparium*, an andromonoecious montane shrub, hermaphrodite flowers tended to open in the first flush of flowering with the proportion of male flowers increasing later in the season (Primack and Lloyd, 1980). Chaudhari and Desai (1993) showed that applications of plant growth regulators can influence the sex expression distribution of flower types in pomegranate.

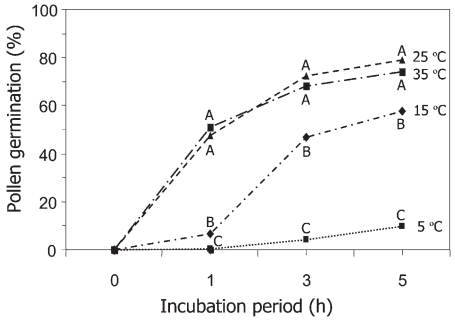

Fig. 7. Percent germination of pomegranate pollen over time using in vitro germination assays conducted at different temperatures. Mean separations compare different temperatures within an assay time using Duncan's multiple range test; means with a different letter are significantly different at $P \leq 0.05$. Values are means of eight replications with 100 pollen grains each.

Gibberellic acid induced more male flowers and reduced hermaphrodite flowers, whereas ethrel and maleic hydrazide induced more hermaphrodite and fewer male flowers.

Although pomegranate flowers are commonly described as falling into either male or bisexual flower types, sex expression appears to follow a spectrum where flowers range from those that have strong pistil development with elongated styles and well-developed ovules to those with severely degenerate ovules having only vestigial development. Intermediate forms exist within this range in which male flowers can express different degrees of ovule degeneration.

Furthermore, our more recent work evaluating flower quality indicates that female flowers vary in size and number of ovules (H.Y. Wetzstein, unpublished data). Verification of this gradient condition of flower quality can have important implications in crop production applications. Sex expression in pomegranate can vary with season and time of bloom. Studies directed toward determining when male and bisexual flower determination occurs are warranted and would provide insight into what environmental and physiological factors may be contributing to sex determination in pomegranate. Optimizing cultural conditions may be a means to promote the development of greater numbers of bisexual flowers with high vigor to obtain maximum fruit set and yield.

Literature Cited

Acar, I. and V.G. Kakani. 2010. The effects of temperature on in vitro pollen germination and pollen tube growth of *Pistacia* spp. Sci. Hort. 125:569–572.

Basu, A. and K. Penugonda. 2008. Pomegranate juice: A heart healthy fruit juice. Nutr. Rev. 67:49–56.

Bertin, R.I. 1982. The evolution and maintenance of andromonoecy. Evol. Theory 6:25–32.

Chaudhari, S.M. and U.T. Desai. 1993. Effects of plant growth regulators on flower sex in pomegranate (*Punica granatum* L.). Indian J. Agr. Sci. 63:34–35.

Connolly, B.A. and G.J. Anderson. 2003. Functional significance of the androecium in staminate and hermaphroditic flowers of *Solanum carolinense* (Solanaceae). Plant Syst. Evol. 240:235–243.

Cuevas, J., K. Pinney, and V.S. Polito. 1999. Flower differentiation, pistil development and pistil abortion in olive (*Oleo europaea* L.). Acta Hort. 474:293–296.

Cuevas, J. and V.S. Polito. 2004. The role of staminate flowers in the breeding system of *Oleo europaea* (Oleraceae): An andromonoecious, wind-pollinated taxon. Ann. Bot. (Lond.) 93:547–553.

DeMason, D.A. and K.W. Stolte. 1982. Floral development in *Phoenix dactylifera*. Can. J. Bot. 60:1437–1446.

El Sese, A.M. 1988. Physiological studies on flowering and fruiting habits of some pomegranate cultivars under Assiut conditions. Assuit J. Agr. Sci. 19:320–336.

Gouvea, C.F., M.C. Dornelas, and A.P. Martinelli. 2008. Characterization of unisexual flower development in the endangered mahogany tree *Swietenia macrophylla* King. (Meliaceae). Bot. J. Linn. Soc. 156:529–535.

Hedhly, A., J.I. Hormaza, and M. Herrero. 2005. The effect of temperature on pollen germination, pollen tube growth, and stigmatic receptivity in peach. Plant Biol. 7:476–483.

Herlihy, C.R. and C.G. Eckert. 2002. Genetic cost of reproductive assurance in a self-fertilizing plant. Nature 416:320–323.

Hiscock, S.J. and A.M. Allen. 2008. Diverse cell signaling pathways regulate pollen-stigma interactions: The search for consensus. New Phytol. 179:286–317.

Holland, D., K. Hatib, and I. Bar-Ya'akov. 2009. Pomegranate: Botany, horticulture, breeding. Hort. Rev. (Amer. Soc. Hort. Sci.) 35: 127–191.

Hormaza, J.I. and V.S. Polito. 1996. Pistillate and staminate flower development in dioecious *Pistacia vera* (Anacardiaceae). Amer. J. Bot. 83:759–766.

Jensen, W.A. 1962. Botanical histochemistry. Freeman, San Francisco, CA.

Kim, S., J.-C. Mollet, J. Dong, K. Zhang, S.-Y. Park, and E.M. Lord. 2003. Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. Proc. Natl. Acad. Sci. USA 100:6125–6130.

Korpelainen, H. 1998. Labile sex expression in plants. Biol. Rev. Camb. Philos. Soc. 73:157–180.

Koubouris, G.C., I.T. Metzidakis, and M.D. Vasilakakis. 2009. Impact of temperature on olive (*Olea europaea* L.) pollen performance in relation to relative humidity and genotype. Environ. Exp. Bot. 67:209–214.

Lansky, E.P. and R.A. Newman. 2007. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J. Ethnopharmacol. 109:177–206.

Lin, J., B. Shabany, and D. Ramos. 1977. Pistillate flower development and fruit growth in some english walnut cultivars. J. Amer. Soc. Hort. Sci. 102:702–703.

Lush, W.M., T. Spurck, and R. Joosten. 2000. Pollen tube guidance by the pistil of a solanaceous plant. Ann. Bot. 85:39–47.

Mars, M. 2000. Pomegranate plant material: Genetic resources and breeding, a review. Options Mediterraneennes Serie A 42:55–62.

Martinez, J.J., P. Melfagejo, and F. Martinez. 2000. Study of the floral morphology of the pomegranate clones: PTO8, CRO1 and ME14. Options Mediterraneenees Serie A 42:105–113.

Miller, J.S. and P.K. Diggle. 2007. Correlated evolution of fruit size and sexual expression in andromonoecious *Solanum* sections *Acanthophora* and *Lasiocarpa* (Solanaceae). Amer. J. Bot. 94:1706–1715.

Narbona, E., P.L. Ortiz, and M. Arista. 2005. Dichogamy and sexual dimorphism in floral traits in the andromonoecious *Euphorbia boetica*. Ann. Bot. (Lond.) 95:779–787.

Narbona, E., P.L. Ortiz, and M. Arista. 2008. Sexual dimorphism in the andromonoecious *Euphorbia nicaeensis*: Effects of gender and inflorescence development. Ann. Bot. (Lond.) 101:717–726.

Podolsky, R.D. 1993. Evolution of a flower dimorphism—How effective is pollen dispersal by male flowers. Ecology 74:2255–2260.

Policansky, D. 1981. Sex choice and the size advantage model in jack-in-the-pulpit (*Arisaema triphyllum*). Proc. Natl. Acad. Sci. USA 78: 1306–1308.

Primack, R.B. and D.G. Lloyd. 1980. Andromonoecy in the New Zealand montane shrub manuka, *Leptospermum scoparium* (Myrtaceae). Amer. J. Bot. 67:361–368.

Sattler, R. 1973. Organogenesis of flowers. Univ. Toronto Press, Toronto, Canada.

Schlessman, M.A. 1988. Gender diphasy ('sex choice'), p. 139–153. In: J.L. Doust and L.L. Doust (eds.). Plant reproductive ecology patterns and strategies. Oxford Univ. Press, New York, NY.

Seeram, N.P., R.N. Schulman, and D. Heber (eds.). 2006. Pomegranates: Ancient roots to modern medicine. CRC Press, Boca Raton, FL.
Shulman, Y., L. Fainberestein, and S. Lavee. 1984. Pomegranate fruit development and maturation. J. Hort. Sci. 59:265–274.
Stover, E. and E.W. Mercure. 2007. The pomegranate: A new look at the fruit of paradise. HortScience 42:1088–1092.
Sukhvibul, N., A.W. Whiley, V. Vithanage, M.K. Smith, V.J. Doogan, and S.E. Hetherington. 2000. Effect of temperature on pollen germination and pollen tube growth of four cultivars of mango (*Mangifera indica* L.). J. Hort. Sci. Biotechnol. 75:214–222.
Tanurdzic, M. and J.A. Banks. 2004. Sex-determining mechanisms in land plants. Plant Cell 16:S61–S71.
Walker, B.A. and R.J. Whelan. 1991. Can andromonoecy explain low fruit:flower ratios in the Proteaceae? Biol. J. Linnaean Soc. 44:41–46.
Wetzstein, H.Y. and D. Sparks. 1983. The morphology of pistillate flower differentiation in pecan. J. Amer. Soc. Hort. Sci. 108:997–1003.
Wetzstein, H.Y. and D. Sparks. 1984. The morphology of staminate flower differentiation in pecan. J. Amer. Soc. Hort. Sci. 109:245–252.
Wilson, M.F. 1983. Plant reproductive ecology. Wiley, New York, NY.
Woo, S.M. and H.Y. Wetzstein. 2008. Morphological and histological evaluations of in vitro regeneration in *Elliottia racemosa* leaf explants induced on media with thidiazuron. J. Amer. Soc. Hort. Sci. 133:167–172.
Yi, W., S.E. Law, D. McCoy, and H.Y. Wetzstein. 2006. Stigma development and receptivity in almond (*Prunus dulcis*). Ann. Bot. (Lond.) 97:57–63.
Yi, W., S.E. Law, and H.Y. Wetzstein. 2003. An in vitro study of fungicide effects on pollen germination and tube growth in almond. HortScience 38:1086–1088.
Zhang, T. and D.Y. Tan. 2009. An examination of the function of male flowers in an andromonoecious shrub *Capparis spinosa*. J. Integr. Plant Biol. 51:316–324.