

FLORAL REPRODUCTION IN NORTHERN PRICKLY ASH
(*ZANTHOXYLUM AMERICANUM* MILL., RUTACEAE)

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Abstract

Zanthoxylum americanum floral reproductive biology was investigated in two Minnesota populations, concentrating on possible areas of interest for a future *Zanthoxylum* / citrus breeding effort. The study examined whether (1) *Z. americanum* pollen was viable, (2) autonomous apomixis was the predominant floral reproductive mechanism, (3) seedless fruits were produced, (4) instances of hermaphroditism occurred, and (5) citrus pollen showed any compatibility with *Z. americanum* pistils. *Zanthoxylum americanum* pollen viability was 95.8+/-0.3% (fresh) and 78.6+/-1.1% (stored 18 months at -12° C). Autonomous apomixis did not appear to be the primary floral reproductive mechanism. Seedless (stenospermocarpic) fruits occurred in 13% of the female fruit clusters examined. Hermaphroditism occurred sporadically in males but was not observed in females. Pollen from some citrus cultivars was sufficiently compatible with *Z. americanum* for pollen germination and pollen tube growth, but offspring from attempted *Z. americanum* x citrus crosses showed only *Z. americanum* morphology.

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The following manuscript is prepared for journal submission.

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David A. Munter

Abstract

Key message Reproductive biology

Zanthoxylum americanum floral reproductive biology was investigated in two Minnesota populations, concentrating on possible areas of interest for a future *Zanthoxylum* / citrus breeding effort. The study examined whether *Z. americanum* pollen was viable, autonomous apomixis was the predominant floral reproductive mechanism, seedless fruits were produced, instances of hermaphroditism occurred, and citrus pollen showed any compatibility with *Z. americanum* pistils. *Zanthoxylum americanum* pollen viability was 95.8±0.3% (fresh) and 78.6±1.1% (stored 18 months at -12°C). Autonomous apomixis did not appear to be the primary floral reproductive mechanism. Seedless (stenospermocarpic) fruits occurred in 13% of the female fruit clusters examined. Hermaphroditism occurred sporadically in males but was not observed in females. Pollen from some citrus cultivars was sufficiently compatible with *Z. americanum* for pollen germination and pollen tube growth, but offspring from attempted *Z. americanum* x citrus crosses showed only *Z. americanum* morphology.

Keywords Autonomous apomixis, Citrus, Seedlessness, Hermaphroditism, Intergeneric hybridization

Introduction

Cold hardy citrus cultivars have long been a goal of citrus breeders, though the desired level of cold hardiness varies depending on the breeding program. In the context of this paper, cold hardiness is defined as citrus plants that can withstand winter temperatures found in USDA Plant Hardiness Zone 3 (Z3), where temperatures can reach -40° C (USDA 2014).

Citrus lack some key features that would enable them to survive under Z3 climatic conditions (Yelenosky 1996; Sherman and Beckman 2002; Zhang et al. 2005):

- Citrus are not deciduous – they lack mechanisms such as autumnal leaf drop and cold acclimation employed by many species in cold climates prior to the onset of winter.
- Citrus lack physiological attributes that would enable plant tissues to withstand temperatures down to -40° C.

- Citrus fruits typically take longer to reach maturity than the length of the growing season in cold climates.

Poncirus trifoliata (a member of the citrus family, *Rutaceae*) is a deciduous citrus relative native to colder regions of China that has been used to increase cold hardiness in citrus (Swingle and Robinson 1923; Spiegel-Roy and Goldschmidt 1996). However, *P. trifoliata* is hardy only to USDA Z5b and, thus, is not hardy enough for areas such as Minnesota, which lies in Z3 and Z4 (Spiegel-Roy and Goldschmidt 1996; Missouri Botanical Gardens 2014; USDA 2014).

Citrus crosses with *Z. americanum* Mill., a.k.a. northern prickly ash, may be an alternative way of developing cold hardy citrus able to withstand the climatic conditions in Z3 (Stace et al. 1993; Chase et al. 1999). *Zanthoxylum americanum* is native to the northern portions of the United States and southern Canada, having a northernmost range in USDA Z3 (Porter 1976; Ownbey and Morley 1991; USDA 2014). *Zanthoxylum americanum* fruits have some citrus characteristics such as a strong citrus/lemon scent and an outer covering like that of a citrus peel. However, *Z. americanum* fruits lack horticulturally important citrus traits such as juice vesicles and large size.

One potential avenue for combining citrus and *Z. americanum* traits is via sexual hybridization. This study examines some aspects of *Z. americanum* floral biology that might be useful in a breeding program between *Z. americanum* and citrus, especially one involving sexual crosses: pollen viability; autonomous apomixis; seedless fruit production (parthenocarpy/stenospermocarpy); hermaphroditism; citrus pollen compatibility with *Z. americanum*.

Pollen Viability

Flowering in *Z. americanum* and citrus occur in different geographic regions and at different times of the year. Citrus grow in warm climates such as California and Florida, and flower predominantly in late winter (Citrus 1981). *Zanthoxylum americanum* grows in cold climates such as Minnesota and blooms in April or May (Porter 1976). In any *Z. americanum* / citrus breeding program, the ability to store citrus pollen or *Z. americanum* pollen until one or the other are in bloom will, therefore, likely become necessary. This

study documents whether: (1) pollen in *Z. americanum* populations is viable and (2) *Z. americanum* pollen retains viability in long-term cold storage.

Autonomous Apomixis

Apomixis is common in citrus (Garcia et al. 1999; Aleza et al. 2010). Some citrus such as sweet oranges (*Citrus sinensis*; Khan 2007) are almost complete obligate apomicts. Obligate apomixis complicates citrus breeding efforts since normal sexual crossing techniques with an obligate apomictic maternal parent almost always result in embryos that are maternal asexual clones rather than a cross with the intended pollen parent (Asker 1979; Koltunow 1993).

Apomixis has also been reported in the genus *Zanthoxylum* (Desai 1962; Yinghong et al. 1987; Naumova 1992). One study tested for autonomous apomixis in four *Zanthoxylum* species: *Z. americanum*, *Z. bungei*, *Z. simulans*, and *Z. planispinum* (Desai 1962). The study used specimens from the Royal Botanical Gardens (Kew) and the author stated that “*Xanthoxylum* (sic) is a dioecious plant and only pistillate flowers are found in Great Britain”. Desai (1962) found that the four *Zanthoxylum* species are autonomous apomicts, with no indication of fertilization. According to Naumova (1992) “Nucellar embryony is a single mode of seed formation in all studied *Zanthoxylum*: *Z. bungei*, *Z. alatum*, *Z. planispinum*, *Z. americanum*, and *Z. simulans*”. Naumova also stated that “In a few species of the genera *Sarcococca*, *Zanthoxylum*, *Zeuxina*, and *Euphorbia*, seed production is achieved by adventive embryony exclusively”.

Apomixis and Dioecy Paradox

Since apomixis produces maternal clones, in the long term any apomixis in dioecious species would have to be facultative; species-wide obligate apomixis would eventually remove all males from the species (Asker 1979; Koltunow 1993; Bicknell and Koltunow 2004). This assumes though that sexual expression in a species is completely under genetic control. It may be that sexual expression in a dioecious species such as *Z. americanum* is determined by a combination of genetics and environment (Mosseler and Zsuffa 1989).

If *Z. americanum* is an obligate apomict or strongly apomictic, this would complicate sexual breeding efforts between *Z. americanum* and citrus. *Zanthoxylum americanum* could not then be readily used as the maternal parent since offspring from sexual hybridization attempts would be maternal clones of the *Z. americanum* parent and not sexual crosses.

Seedless Fruits (Parthenocarpy/Stenospermocarpy)

Parthenocarpy could be a useful trait in a *Z. americanum* breeding program since consumers prefer seedless fruits (Elmstrom and Gray 1991; Campbell et al. 2004). Seedlessness may also indicate that apomixis is neither present nor predominant (Bicknell and Koltunow 2004). Seedlessness in the form of stenospermocarpy may give the breeder clues about incongruity issues or deleterious alleles since incongruous genomes or homozygous-lethal deleterious alleles could cause embryo abortion (Chaudhury et al. 2001; Vilorio et al. 2005; Jacquemart 2007; Chai et al. 2011).

Hermaphroditism

Dioecious plant species are theorized to develop via several pathways (Darwin 1877; Webb 1979; Ross 1982; Ainsworth 2000; Pannell 2002). The particular pathway taken by a species can be partially inferred by examining its flower sexual types – hermaphrodite, pistillate, staminate, or distylous – and determining which flower types co-exist on individual plants in the species populations being studied (see Table 1 for a list of sexual variants delineated in evolutionary pathways to dioecy).

According to Barrett (2002), the most common pathways are some variant of hermaphrodite-monoecious-dioecious (Fig. 1d) or hermaphrodite-gynodioecious-dioecious (Fig. 1e). Additional pathways have also been described (Fig. 1; Webb 1979; Bawa 1980; Ross 1980 and 1982; Barrett 2002; Pannell 2002).

Dioecious species often are not completely dioecious (termed subdioecious), exhibiting signs that they either have not yet completed their transition to dioecy, have reached some equilibrium state, or are evolving beyond a pure dioecious state (Darwin 1877; Webb 1979; Ross 1982; Pannell 2002). These subdioecious species often have

individuals that show some aberrant flower types such as monoecious flowers (indicating a pathway with monoecy as an intermediate step; Figs. 1b and 1d) or hermaphroditic flowers (indicating gynodioecy, andromonoecy, or androdioecy as an intermediate step; Figs. 1e, 1f, and 1g; Webb 1979; Ross 1982; Pannell 2002). From a breeding perspective, these aberrant individuals in a dioecious species are potentially useful because they may allow breeding techniques such as self-pollination in an otherwise self-incompatible species.

For male (staminate) plants, hermaphroditic flowers on otherwise male plants allow the breeder to assess fruiting characteristics of males when choosing which males to include in the next breeding cycle. Hermaphroditic flowers might also be chemically inducible, allowing the breeder to use males as the maternal parents in cases where it would be advantageous to do so (Heslop-Harrison 1956; Chailakhyan 1979; Marchetti et al. 1992; Ainsworth 2000). Such cases might appear in a *Z. americanum* / citrus breeding program, where it may be advantageous to retain maternally inherited cytoplasmic DNA in crosses involving *Z. americanum* x citrus offspring (Froelicher et al. 2011). In particular, cytoplasmic DNA may be a crucial contributor to *Z. americanum* cold hardiness and by extension cold hardiness in *Z. americanum* x citrus crosses.

Citrus Pollen Compatibility

Successful sexual crosses between *Z. americanum* and citrus require compatibility between the two genera. For crosses where *Z. americanum* is the maternal parent, this means that citrus pollen must be able to germinate on *Z. americanum* stigmas and that the resulting pollen tubes must reach the ovaries so that fertilization can take place.

Many species or cultivars have self incompatibility (SI) mechanisms that prevent selfing or crossing between closely related individuals (Brewbaker 1957; Haring et al. 1990; Takayama and Isogai 2005; Eberle 2012). This is the case with some citrus cultivars such as the Clementine mandarin orange (*Citrus clementina*; Yamamoto et al. 2006; Distefano et al. 2009a and b). SI mechanisms can also cause incompatibility with other cultivars or species (Lewis 1958; Murfett et al. 1996; Eberle 2012). In unilateral

incompatibility, SI species reject pollen from self-compatible species, while pollen is not rejected in the reciprocal cross (Beecher et al. 2001; Eberle 2012).

Taking into account unilateral incompatibility and the nature of dioecy, a *Z. americanum* / citrus crossing approach that may avoid SI barriers is to use citrus as the pollen parent and *Z. americanum* as the maternal parent. This avoids known citrus SI barriers because it is the maternal parent that imposes SI barriers (Lewis 1958; Murfett et al. 1996; Eberle 2012). Since *Z. americanum* is dioecious and therefore has separate male and female plants that prevent selfing, it may not possess additional stigmatic or stylar SI reproductive barriers.

There are two additional benefits to using *Z. americanum* as the maternal parent in *Z. americanum* x citrus sexual crossing attempts: (1) Citrus occasionally produce 2N gametes ($2n = 2x = 18$, i.e. twice the number of chromosomes as normal 1N gametes; Krug 1943; Chen et al. 2008; Gmitter et al. 2012). Citrus 2N gametes would bring their ploidy level in better alignment with that of the $x \sim 18$ *Z. americanum* egg cells (Walker 1942; Desai 1960; Stace et al. 1993), and the large number of gametes in pollen may increase the chances of 2N gametes being available for fertilizing the *Z. americanum* ovules; (2) Using *Z. americanum* as the maternal parent avoids the complication of citrus apomixis. Using citrus as the maternal parent would rule out sexual crosses with obligate citrus apomicts, e.g. sweet oranges (*Citrus sinensis*), as the maternal parent.

Research Objectives

Pollen Viability

Sexual crosses between citrus and *Z. americanum* with *Z. americanum* as the pollen parent will require viable *Z. americanum* pollen. This study documents whether *Z. americanum* pollen in the study populations is viable and if so, whether the pollen remains viable in long-term cold storage.

Autonomous Apomixis

This study tests whether there is any significant difference between the fruit:flower ratio of unpollinated branches as compared to the fruit:flower ratio of branches pollinated by fresh *Z. americanum* pollen. A fruit:flower ratio on unpollinated branches greater than or

equal to that of *Z. americanum* pollinated branches (H_0 ; the null hypothesis) would indicate that autonomous apomixis is the predominant form of floral reproduction in *Z. americanum* since the presence of pollen would not have increased the fruit production rate. A fruit:flower ratio on *Z. americanum* pollinated branches that is significantly higher than that of unpollinated branches (H_1) would indicate that other factors such as sexual reproduction or pseudogamous apomixis play a substantial role in *Z. americanum* floral reproduction, since the presence of pollen would have increased the fruit production rate.

Seedless Fruits (Parthenocarpy/Stenospermocarpy)

Seedless fruits might be a goal of a *Z. americanum* breeding program, and might also present clues to other aspects of *Z. americanum* floral reproduction such as whether selfing occurs. This study documents whether the *Z. americanum* study populations produce seedless fruits and, if so, whether the fruits are parthenocarpic or stenospermocarpic.

Hermaphroditism

This study documents instances of hermaphroditism found in the study populations. Pollen viability of hermaphroditic flowers from a group of hermaphroditic plants is compared to that of primarily staminate plants (H_0 : the pollen viability rates are equal; H_1 the pollen viability rates are not equal). It also investigates whether fruits from hermaphroditic flowers might be the result of selfing or apomixis. Fruits that develop on branches of predominantly staminate flowered plants when pollen has been excluded from those branches would indicate that those fruits resulted from selfing or apomixis.

Citrus Pollen Compatibility

This study tests whether there is any compatibility between citrus pollen and *Z. americanum* pistils. If citrus pollen germinates on *Z. americanum* stigmas, then there is some degree of compatibility. It also tests whether using mentor pollen affects the fruit:flower ratio when mixed with citrus pollen to pollinate *Z. americanum* flowers (H_0 : the fruit:flower ratios are equal; H_1 : the fruit:flower ratios differ).

Materials and Methods

Two Minnesota *Z. americanum* populations were used in this study. One population consisted of 120 purported clonal stands found in St. Croix Bluffs Regional Park (Washington County, Minnesota, USA; the western-most clonal stand positioned at 44° 47' 41.7" N, 92° 48' 04.0" W; Google Maps 2014). The second population consisted of 30 individual purported clonal stands in Cottage Grove Ravine Regional Park (Washington County, Minnesota, USA; the eastern-most clonal stand located at 44° 48' 11.0" N, 92° 54' 10.2" W). The westernmost St. Croix Bluffs Regional Park clonal stand studied was 8.08 kilometers E-NE of the easternmost Cottage Grove Ravine Regional Park clonal stand studied (Google Maps 2014).

Individual clonal stands were identified by distinct separations (gaps) between sympatric plant groupings. Whether members within individual clonal stands were truly the same clone (clonal ramets) was not determined. One plant (*i.e.* stem rising out of the ground) from each clonal stand was tagged and numbered. Each tagged plant was then the primary member of the clone used in subsequent interactions. At times it was necessary to also use other plants from a clone, such as during the pollination studies when there were insufficient branches on the primary plant of a clone. In such cases, the nearest neighboring stem within the same clonal stand to the tagged plant was used.

Forcing

The sex of each tagged plant as male or female was determined in 2013 by forcing cuttings into bloom indoors from each tagged plant (hermaphroditism was not part of the study until 2014). Cuttings were taken in mid-winter 2013 (January and February). Each cutting was approximately 15 cm long (the lengths varied depending on the location of flower buds on each cutting). The cuttings were placed vertically (terminal bud at the top) in plastic cups (Kordite® 532 ml cups, ASIN B00E7FUFMR, Reynolds Consumer Products Inc.) in 100 ml of floral preservative solution (10 g Aquaplus® powder per liter of distilled water; Aquaplus®, Syndicate Sales, Inc.) and kept at room temperature (22/15°C, day/night) under T5 type grow lights (~30 cm distance from the terminal bud

to the lights; AgroBrite® T5 lights, 54W, 6400K, Hydrofarm Ltd.) with a long day photoperiod (0600 – 2200 HR). The floral preservative solution in each cup was replaced weekly. Flowers were evaluated after bud break when either the styles (females) or anthers (males) became apparent (Fig. 2). The sex of the flowers on each forced cutting was determined by examining all of the inflorescences on a cutting and determining whether the cutting was male (staminate flowers) or female (pistillate flowers; Fig. 2). Since hermaphroditism was not yet part of the study at this stage, no attempt was made to look for hermaphroditic flowers. The sex of each plant was later reconfirmed when the plants reached anthesis in May 2013.

Pollen Sources

Citrus pollen used in controlled pollinations with *Z. americanum* came from two sources: (1) Citrus plants growing indoors and (2) flowers shipped from the USDA Germplasm Facility in Riverside, California (Table 2). Fresh citrus pollen was used for pollination studies when possible. Otherwise, citrus pollen was stored in a freezer using established pollen storage protocols (Towil 2010; see Pollen Storage section) for subsequent pollinations. *Zanthoxylum americanum* pollen was collected from two sources: (1) forced cuttings (see Forcing section) and used in indoor pollination studies March-April 2013; (2) study population plants growing outdoors in the wild during the blooming periods in May 2013 and 2014.

Mentor pollen in previous studies was prepared using irradiation (Stettler 1968; Pandey 1977 and 1978; Den Nijs and Oost 1980). No irradiation source was available for this study, so alternative pollen-killing techniques were used. Mentor pollen – killed *Z. americanum* pollen – was prepared in two ways: (1) heating the pollen in aluminum foil (Reynolds Wrap®, Reynolds Consumer Products Inc.) for 48 hours at ~ 55°C (DeLonghi® model 2507L 1500 watt heater); mentor pollen derived from this technique was used in the 2013 study; (2) keeping pollen at room temperature (22/15°C, day/night) in a sealed plastic Ziplock® medium size (28 ounce) container (S. C. Johnson & Son, Inc.) with Drierite® desiccant (<http://www.drierite.com>) for one year (May 2013 - 2014);

this pollen was used in the 2014 study. Viability tests indicated that the pollen was no longer viable (see pollen viability methodology).

In 2013, compatibility tests were done between citrus pollen and *Z. americanum* pistils using indoor pollinations and outdoor pollinations to check whether *Z. americanum* x citrus sexual crosses would produce viable seed. Non-pollinated branches were used as (1) a negative control and (2) to check for the presence of autonomous apomixis.

Indoor Pollinations (March 2013 – April 2013)

Cuttings were forced into bloom using the same technique that had been used to determine sex type (see Forcing section). Pollen was applied a random number of days after style elongation (zero to five days because the time from style elongation to stigma receptivity was not known) using a small paintbrush. Style elongation was visually identified when styles began to separate within inflorescences. (Styles within a *Z. americanum* inflorescence emerge from the bud in a fused state, elongate, and then spread apart. The stigmas come back together and fuse late in the bloom cycle. See Fig. 2a for an example of an inflorescence shortly before the styles separate; see Fig. 11a and 11c for examples of fused stigmas). The Microsoft Excel® (Microsoft Corporation, Redmond, WA 98052) Rand function was used to generate random numbers for pollination days. Separate paintbrushes were used for each pollen type (citrus cultivar or *Z. americanum*) and were dipped in 70% ethanol between pollinations. Pollinated flowers were removed from the stems with tweezers and placed in FAA (formalin/acetic acid/alcohol; 1 part formalin: 1 part acetic acid: 8 parts ethanol; Martin 1959; Gardner et al. 2009) preservative solution. See Table 2 for the pollen types used for indoor pollinations.

Outdoor Pollinations (May 2013)

Outdoor pollinations were conducted *in situ* on wild *Z. americanum* plants at the Cottage Grove Ravine and St. Croix Bluffs locations. Eight branches per tagged female were bagged with pollination bags (DelStar Technologies Inc. 18 inch x 16 inch pollination bags, item number 310023-1) and randomly assigned one of four treatment

types, with two of each treatment type replicated/female. In cases where the tagged females did not have eight branches, adjacent plants that appeared to be part of the same clone (same plant grouping) were included in the bagging. Each bag was sealed with a color-coded clothespin that indicated its treatment type.

Outdoor *in situ* pollinations were done in a five-day period (May 14 - 19), as the female plants did not all bloom on the same day. Each bag was briefly removed from its branch, flowers were counted, pollinated, and then the bag was placed back on the branch. This was done one bag at a time to minimize the length of time for potential pollen exposure. Pollinations were done in the following order and all pollinations of the same treatment type were done before moving onto the next treatment type to prevent accidental contamination with the wrong pollen type: (1) No pollen – the flowers were counted; half of the bagged portion of the branch was stimulated with an empty artist's paint brush to simulate pollination contact without pollen being present; no stimulation and no pollination was done to the remaining flowers on the bagged portion of the branch. (2) A mixture of citrus pollen – the flowers were counted and then a mixture of citrus pollen (Table 2) was applied with a paintbrush. (3) *Z. americanum* pollen – the flowers were counted and then a mixture of *Z. americanum* pollen (bulked in approximately equal amounts from the tagged males in bloom at that time) was applied with an artist's paintbrush. (4) The citrus mixture again, this time as a control for the mentor study; preparing the mentor pollen delayed pollination with the mentor pollen and so the citrus mixture was used again as a control in case pistil receptivity had changed in the intervening period since the other pollinations. (5) The citrus mixture with mentor pollen added in an approximate citrus pollen / mentor pollen ratio of 2:1 by volume.

Separate brushes were used for the different pollen types with separate brushes for each plant. Mentor pollen ran out after 11 pollinations. For the remaining plants, the bagged branches intended for the mentor study were instead used to check for autonomous apomixis, in this case with the bags never removed until flowering finished as a control in case of pollen contamination when then other bags were briefly removed during pollination (Table 3).

Pollination bags were kept on the branches until three weeks after pollinations to ensure that unintended pollinations would not occur. Fruits on each of these branches were counted in late May – June (May 25, 2013 to June 22, 2013) and again just prior to harvest. The harvest date (August 7, 2013 to August 20, 2013) for each plant was determined by selecting a date when fruit color changed from green to red. All fruits on a given plant were harvested within a two-day period rather than waiting for all fruits to ripen since the seeds are ejected from well ripened capsules. Harvested fruits were placed in paper bags labeled with the plant ID and bagged branch type and kept at room temperature (22/15°C, day/night) until dry, after which seeds were cleaned and counted.

Seeds were placed in moist horticultural grade vermiculite (Hoffman Good Earth®) and placed in cold storage (3-4°C) from December 2013 (December 1, 2013 to December 11, 2013; various preparation dates) until spring/summer 2014 (March – June; various planting dates). Initial trial germinations were poor (less than 3%; data not shown). Soaking the seeds in 1000 ppm GA3 for twenty-four hours at room temperature (22/15°C, day/night) aided germination, resulting in rates of about 20% to 30%.

Fruiting males were discovered serendipitously in the study populations. Since the discovery happened late in the blooming period, 2013 investigations concerning the fruiting males were limited to documenting the presence or absence of fruits on each tagged male in the study populations. Every branch of each tagged male was visually inspected for developing fruits in late May and early June 2013. The same visual inspections were done again at harvest time and any fruits on those males were then harvested.

2014 Pollination Study

Research objectives in 2014 were to: (1) Confirm autonomous apomixis in *Z. americanum*, (2) Identify individual citrus cultivars compatible with *Z. americanum*, (3) Evaluate mentor pollen effects on *Z. americanum* x citrus compatibility, and (4) Check for selfing or apomixis in fruiting males.

Female parents in 2014 were limited to plants that showed possible compatibility with citrus in 2013 as indicated by the presence of fruits on bagged branches in 2013, or potential apomixis in 2013. In 2014, pollinations on *Z. americanum* were done using pollen from individual citrus cultivars as male parents as well as a citrus pollen mixture as a control (Table 2). Some of the citrus cultivars from 2013 were not available in 2014. Pollination bags were placed on branches prior to bud break with bags on most of the branches of the entire clonal stand, not just the tagged plant. Pollination bags were randomly assigned treatment types: (1) No pollination (as both a control and to check for autonomous apomixis), (2) Bulked *Z. americanum* pollen, (3) Pollen from a citrus cultivar or the citrus mixture control group, or (4) Citrus pollen mixed with mentor pollen (i.e. killed *Z. americanum* pollen). The citrus / mentor pollen mixture consisted of a citrus pollen / mentor pollen ratio of approximately 2:1 by volume.

Pollination was performed when most styles on a given clonal stand reached the elongation stage (see Indoor Pollinations section). The 2014 flowering spanned a two-week period, with some clonal stands flowering earlier while others flowered later in the period. Pollinations used the same methodology as in 2013.

Since possible unintended pollinations may have occurred during 2013 while the bags were briefly removed to count the flowers, the methodology for the non-pollinated controls was modified in 2014. Double bagging of non-pollinated branches was used in 2014 to ensure isolation of the bagged branches from extraneous pollen. For double bagging, a branch was sealed within one bag, which was then sealed within a second bag. In 2014, flowers were counted for the non-pollinated controls after each clonal stand finished flowering when the styles were beginning to drop off the pistils (i.e. flowers were counted after flowering finished but before flower remnants fell off the plants). Since some flowers could have dropped off into the pollination bags during that time, dried flowers in the bags were included in the no-pollen flower counts. Flower counts were, therefore, likely lower for these non-pollinated branches than the actual number of flowers on those branches, meaning that fruit to flower ratios were likely less than reported here.

To test whether the technique of Desai (1962) in a prior *Z. americanum* apomixis study would give different results, six of the non-pollinated control bagged branches from one large clonal stand used this treatment. The stigmas and styles were manually removed from seven non-pollinated bagged branches so that no stigmatic pollinations could occur, and the bags were then immediately placed back over those branches. Bags were removed three weeks after all male flowering had finished. For fruiting males in 2014, only the two males with the largest 2013 fruit crop (plant IDs CGR023 and SCB041) were chosen for bagging studies. Six branches were bagged on each of those two males prior to bud break. The same methodology was followed for these bags as was used for the 2014 non-pollination controls (see above).

Pollen Viability Test

Fresh Pollen

Fresh pollen was collected in 2013 from 30 males of *Z. americanum* at anthesis (11 of these were later found to be fruiting males) during the March-April forcing study from forced cuttings. Fresh pollen from the hermaphroditic group was collected *in situ* in May 2014. Within one hour of collection, the pollen was stained with 0.1% aniline blue in 80% propionic acid and observed under a microscope (Anderson and Ascher 2000). Viable pollen grains (stained) and non-viable pollen grains (not stained) were counted in 12 microscope fields containing at least 20+ pollen grains.

Stored Pollen

Pollen was prepared for long term cold storage using established pollen storage protocols (Towil 2010). Fresh pollen was placed in gel caps immediately after collection. The gel caps were then placed in a sealed plastic bag (Ziplock® Gallon Storage polyethylene bags, 26.8 cm x 27.3 cm; S. C. Johnson & Son, Inc.) containing Drierite® desiccant (<http://www.drierite.com>) and kept in the plastic bag for 24 hours at room temperature (22/15°C, day/night). The gel caps with pollen were then transferred to a freezer for long-term cold storage at approximately -12°C. Once each month, a sample of the stored

pollen was removed from cold storage. Pollen from the sample was evaluated for viability using the same methodology as for fresh pollen.

Fluorescence Microscopy

Pistils were placed in FAA under refrigeration until ready to use. When ready to study under fluorescence, the pistils were removed from the FAA and placed in 8N NaOH for 45 minutes to soften the styles (Kho and Baer 1968; Gardner et al. 2009). The pistils were removed from the 8N NaOH, rinsed with dH₂O, and then soaked in dH₂O for 1 hour. The pistils were removed from the dH₂O and placed in decolorized aniline blue (0.05% aniline blue, 65mM Na₂HPO₄) for at least 1 hour. After treatment with decolorized aniline blue, the pistils were removed from the aniline blue solution and placed on microscope slides, using drops of the aniline blue solution as needed to fill gaps under the microscope slide coverslip. The slides were microscopically observed with an ultraviolet light source and filter (Gardner et al. 2009).

Statistical Analysis

Pollen viability (stainability) data were analyzed using Microsoft Excel® and the associated statistical package StartPlus:mac® (AnalystSoft, Alexandria, VA 22314) except for ANOVA and Tukey HSD tests, which were conducted via SPSS® (IBM Corporation, Armonk, NY 10504). Stainability ratios were arcsin square root transformed before being used in ANOVA and Tukey HSD tests.

Fruit:flower ratio comparisons of non-pollinated flowers versus *Z. americanum* pollinated flowers, and mentor-aided citrus pollinated flowers versus non-mentored citrus pollinated flowers, were conducted using Microsoft Excel® and the associated statistical package StartPlus:mac®. Fruit:flower ratios were arcsin square root transformed before being used in paired T test calculations.

Results

Sex Ratios

The study populations consisted of 150 separate clonal stands, 120 of which were at the St. Croix Bluffs location and 30 at the Cottage Grove location. There were 85 females and 60 males (58.6% females, 41.4% males) in the combined populations (the remaining 5 plants did not produce flowers). The Cottage Grove Ravine population consisted of 12 females and 18 males (40% females, 60% males). The St. Croix Bluffs location consisted of 73 females and 42 males (63.5% females, 36.5% males).

Pollen Viability

Fresh pollen had a grand mean viability of 95.8 \pm 0.3%, with genotype differences accounting for most of the variance (Fig. 3; Tables 4 and 5). Frozen pollen viability was 78.6 \pm 1.1% after 18 months at -12 $^{\circ}$ C (Fig. 4; Table 6).

Autonomous Apomixis

In 2013, 38.9% of non-pollinated branches had one or more fruits, with a mean fruit count of 11.9 \pm 1.2 and a median fruit count of 4 for those branches bearing one or more fruits. The fruit:flower ratio for non-pollinated branches was 6.2 \pm 1.7% (Table 7). By contrast, 85.6% of the branches pollinated with *Z. americanum* pollen (the control group) had one or more fruits, with a mean fruit count of 33 \pm 3.2 and a median of 23 fruits for the branches that bore fruits. The fruit:flower ratio for this control group was 46.5 \pm 5.3% (Table 7). Fruit:flower ratios for *Z. americanum* pollinated branches were significantly different (p -value < 0.05) from the fruit:flower ratios of non-pollinated branches.

In 2014, 15.3% of non-pollinated branches (non-cropped) had one or more fruits, with a mean fruit count of 1.6 \pm 0.1 and a median fruit count of 1 for those branches bearing one or more fruits. The fruit:flower ratio was 0.3 \pm 0.0% (Table 7). None of the cropped (stigmas/styles removed), non-pollinated branches bore fruit. By contrast, 91.3% of the branches pollinated with *Z. americanum* pollen had one or more fruits, with

a mean fruit count of 39.5 \pm 10.9 and a median of 29 fruits for the fruiting branches. The fruit:flower ratio for this control group was 56.2 \pm 11.5% (Table 7).

Parthenocarpy/Stenospermocarpy

No instances of true parthenocarpy were observed in the study populations. Stenospermocarpy was common in the study populations, with one or more stenospermocarpic fruits occurring in 13% of the female fruit clusters examined. Stenospermocarpic fruits were much smaller than normal, seed-containing *Z. americanum* fruits, but varied in size (Figs. 5 and 6). Stenospermocarpic fruit size was relative to the size of the remnant aborted seed inside.

Hermaphroditism

Three flower types were identified in the populations: (1) Pistillate flowers with no stamens, (2) Functionally staminate flowers with fully developed anthers and rudimentary pistils, and (3) Hermaphroditic flowers containing fully developed stamens as well as fully developed pistils.

The flowers occurred on four sexual variants (Fig. 2): (a) Females, i.e. plants with only pistillate flowers, (b) Non-fruiting males, i.e. plants with only functionally-staminate flowers observed and producing no fruits, (c) Fruiting males, i.e. plants that appeared to have only functionally staminate flowers but yet produced fruits, and (d) One hermaphroditic group of plants (possibly one clonal stand) with both hermaphroditic and functionally staminate flowers on the same plant. The distinction between the two types of male variants may be an artifact of observational limitations as non-fruiting males may have produced some hermaphroditic flowers that did not produce fruits.

In 2013, 30% of the males (18/60 males in the combined study populations) bore at least one fruit. The mean fruit count was 14.9 \pm 6.5 per fruiting male, with a median fruit count of 4.5. Two outliers, one of which bore more fruits than all of the other males combined, skewed the mean (Fig. 7). The most fruitful male genotype bore >100 though probably less than <200 fruits. By contrast, females often bore more than one thousand

fruits per plant (data not shown). Fruit clusters on fruiting males never exceeded five fruits/cluster, with a high incidence of stenospermocarpic fruits (Fig. 8). Females typically bore fruits in clusters exceeding 20 fruits (Fig. 9).

In 2014, the male fruit production rate dropped to 10% of males (6/60 males in the combined study populations) bearing at least one fruit. The mean fruit count was 4.2 \pm 1.6 fruits per fruiting male with a median fruit count of 2.5. Fruits sometimes set on the same branches as in 2013, but in no case did fruits appear at the same location (same axillary cluster location) in 2013 and 2014. Fruits appeared in seemingly random locations on the plants except that in 2013 fruits were often observed just below the site where cuttings were taken in the winter of 2013 (Figure 10).

Based on remnant flower parts when the male fruits were first observed (Fig. 11), flowers in fruit-bearing flower clusters on male plants had both hermaphroditic and staminate flowers. Flower remnants on male fruit-bearing branches were staminate other than the hermaphroditic fruit-bearing flowers. Staminate flowers appeared both acropetally and basipetally to the fruit-bearing flowers.

In 2014, male fruits appeared only on males that bore fruits in 2013. The highest male fruit producer from 2013 (plant ID CGR023) did not bear any fruits in 2014. Genotype CGR023 is in the Cottage Grove Ravine population, where 2014 fruit production was very low. Four Cottage Grove Ravine males bore fruits in 2013 (including the top male fruit producer, CGR023), but no Cottage Grove Ravine males bore fruits in 2014.

Pollination bags were placed on the top two 2013 male fruit producers in 2014 to see whether fruits would appear without outcrossing pollen (*i.e.* whether selfing or apomixis might be involved in male fruit production). The bagged branches were double-bagged, with one bag inside a second bag, to ensure that outside pollen was excluded from the bagged branches. No other males were bagged due to concerns that this might affect the observed rate of 2014 male fruit production.

As previously mentioned, the top 2013 male fruit producer (plant ID CGR023) bore no fruits in 2014. This same male was one of the two males involved in the 2014

bagging study. One of the bagged branches on the other 2014-bagged male (plant ID SCB041) did produce fruits (Fig. 12).

Pollen viability tests conducted on pollen from hermaphroditic flowers indicated a high degree of viability, ranging from 95.1% to 100% in the samples tested. Fresh pollen viability rates were 95.8 \pm 0.3% for all males, 96.1 \pm 0.4% for fruiting males, 95.6 \pm 0.4% for non-fruiting males, and 98.7 \pm 0.4% for the hermaphroditic group (Table 5). Pollen viability of males (fruiting and non-fruiting; mean group a) was significantly different than the pollen viability of the hermaphroditic group (mean group b) ($\alpha = 0.05$).

Stigmas and styles on hermaphroditic flowers from the hermaphroditic group (Fig. 2d) appeared somewhat misshapen compared to stigmas and styles on female plants (Fig. 2a). The stigmas on these hermaphroditic flowers were protogynous, extending above the anthers before the anthers reached anthesis. By contrast, the staminate flowers were protandrous, with the anthers developing before the pistils and with pistil development often minimal; pistil development was delayed in the staminate flowers with pistils still incomplete at anthesis. Despite their misshapen appearance, stigmas on the hermaphroditic flowers were able to support pollen germination (Figure 13b). These observations are based on a small sample of hermaphroditic flowers and may not be representative of *Z. americanum* hermaphroditic flowers in general.

Despite a relatively large number of hermaphroditic flowers in the hermaphroditic group, only thirteen fruits developed on the seven plants. This may be explained, in part, by hermaphroditic samples removed from plants, but even areas of the plants not sampled (i.e. the top of the canopy) had low fruiting rates.

Citrus Compatibility and Congruity

Zanthoxylum americanum pollen germinated and grew normally on *Z. americanum* stigmas (Figs. 13a, 14a), in comparison with citrus cultivar Ponderosa lemon (Figs. 14b, 15). Clementine mandarin (Fig. 14c) and Meyer lemon (Fig. 14d) showed relatively more limited compatibility with *Z. americanum* stigmas and styles.

In 2013, outdoor *in situ* pollinations, a pollen mixture of citrus cultivars rather than separate cultivars was used due to a limited supply of citrus pollen. In 2014, the research

objective shifted to whether citrus pollen for specific citrus cultivars could successfully fertilize *Z. americanum* and produce viable seed. Crossing success was based on whether any fruits developed on citrus-pollinated branches and, if so, what percentage of the flowers produced fruits (Table 7).

To check whether the fruits on pollinated, bagged branches were actually a *Z. americanum* x citrus cross, the resulting seeds were germinated and morphologically compared to seedlings from intraspecific *Z. americanum* crosses. There was no noticeable difference in morphology between the *Z. americanum* x citrus seedlings and the *Z. americanum* x *Z. americanum* seedlings (Fig. 16).

Mentor-aided pollinations in 2013 showed no significant difference (p-value 0.2) in fruit:flower ratios (3.8+/-1.5%) compared to pollinations without mentor pollen (4.5+/-5.8%). However, the sample size was small (n=11). In 2014, mentor-aided pollinations were done using a mixture of pollen from several citrus cultivars (containing pollen from different citrus cultivars than in 2013; see Table 7) and also separately using Clementine pollen. Clementine pollen was chosen because there was more Clementine pollen available than for other citrus cultivars. Mentor-aided pollinations in 2014 using the citrus pollen mixture had a fruit:flower ratio of 5.2+/-5.9% while pollinations using just the citrus mixture had a fruit:flower ratio of 7.4+/-2.2%. Mentor-aided pollinations in 2014 using Clementine pollen had a fruit:flower ratio of 4.3+/-1.7% while pollinations using just Clementine pollen had a fruit:flower ratio of 0.8+/-0.0% (Table 7).

Discussion

Fresh Pollen Viability

The high pollen viability rate and the vigorous pollen germination visible under fluorescence (Fig. 13a, 14a) indicate that pollen can play a role in *Z. americanum* floral reproduction in these populations via either sexual crosses or pseudogamous apomixis because both require viable pollen and pollen germination. Pollen viability could be expected to decline during *Z. americanum* evolution if autonomous apomixis were the driving reproductive force since pollen would no longer be needed for reproduction. The high rate of males in the study populations (41.4% males for the combined populations)

implies that sexual reproduction continues in this species, unless there is a facet of sex determination in *Z. americanum* that could otherwise account for the presence of males in the species.

Stored Pollen Viability

The stored pollen viability rate might have been higher if storage conditions were colder and drier (Towil, 2010) than the -12° C temperature used in this study. A previous collection of *Z. americanum* pollen that was stored at -18° C still had a viability rate of $87.5\pm 1.6\%$ after 30 months (unpublished data) compared with $78.6\pm 1.1\%$ for pollen stored 18 months at -12° C in the present study. However, the earlier pollen came from different plants, years, and environmental conditions than the pollen used in this study, so the results may not be directly comparable. Regardless, *Z. americanum* pollen storage could be useful in a citrus x *Z. americanum* breeding program since citrus could be pollinated with *Z. americanum* pollen at normal citrus blooming periods and not just when *Z. americanum* are in bloom.

Autonomous Apomixis

The substantially different fruit:flower ratios between 2013 ($6.2\pm 1.7\%$) and 2014 ($0.3\pm 0\%$) for the non-pollinated branches could be an indication that the single bagging method employed in 2013 did not exclude all extraneous pollen or that the branches were contaminated with extraneous pollen during bag removal. If either scenario is true, then the actual 2013 autonomous apomixis rate may have been substantially less than the 2013 observed rate of $6.2\pm 1.7\%$.

If autonomous apomixis was present in the *Z. americanum* study populations, the upper limit (at least on the non-pollinated branches) for non-aborted fruits was the 2013 fruit:flower ratio of $6.2\pm 1.7\%$. This is contrary to a previous study where only autonomous apomixis was observed in a population consisting of only pistillate flower types (Desai 1962).

Non-autonomous apomixis (pseudogamy) might also be present in *Z. americanum*. Other *Zanthoxylum* (Desai 1962; Yinghong et al. 1987; Naumova 1992) and some citrus can reproduce via nucellar embryony, which is a form of apomixis that is often polyembryonic and may require fertilization of a zygotic embryo in order for the apomictic embryo to develop (Koltunow 1993; Koltunow et al. 1995). In citrus, nucellar embryony has one or more embryos arising from nucellar tissue and often one or more zygotic embryos arising from sexual union of the maternal and paternal gametes. The zygotic embryos in these cases usually die, leaving only the nucellar embryos (Koltunow 1993; Koltunow et al. 1995). Non-autonomous apomixis may explain the fruits that developed on citrus-pollinated *Z. americanum* branches – the absence of any citrus morphology in seedlings derived from *Z. americanum* x citrus crosses may indicate that seeds from these crosses actually resulted from some form of apomixis and rather than the intended sexual crosses. If this was the case, the upper bound for all forms of apomixis on citrus-pollinated branches is the rate of fruit production on the citrus-pollinated branches (Table 7). This indicates an apomixis upper bound of 5.2+/-1.2% (2013) and 7.8+/-4.9% (2014) for citrus-pollinated branches using the fruit:flower ratio as this upper bound. Likewise, the rate for apomixis in intraspecific *Z. americanum* crosses has an upper bound of 46.5+/-5.3% (2013) and 56.2+/-11.5% (2014) using the fruit:flower ratio for such crosses as the upper bound.

Parthenocarpy/Stenospermocarpy

Stenospermocarpic fruit size may indicate when embryo abortion took place. Early abortion would result in small fruits and later abortion producing larger fruits. Why the stenospermocarpic fruit appeared at high rates in the male fruit clusters is unclear. Some possibilities are that the pistils were not fully functional or that the aborted embryos resulted from selfing and so exhibited the effects of inbreeding depression.

Hermaphroditism

Zanthoxylum americanum is considered a dioecious species, but reports of hermaphroditism in *Z. americanum* go back to at least 1898 (Felter and Lloyd 1898). This

dichotomy may stem from a low rate of hermaphroditism in the species, with the predominant form being dioecious but having occasional aberrant hermaphroditic flowers. This same dichotomy exists in the Minnesota populations studied in this report.

One marked aspect of hermaphroditism in the study populations is the apparent randomness. Hermaphroditic flowers appeared sporadically and their location varied between 2013 and 2014. Even on plants that had a high incidence of hermaphroditism, hermaphroditic flowers appeared to be randomly distributed on the branches. This suggests that hermaphroditism in the study populations is controlled both genetically (only males had hermaphroditic flowers) and environmentally (random appearance on branches and inconsistent hermaphroditism in axillary clusters that previously produced fruits).

The rare and sporadic appearance of fruits (and by extension, hermaphroditic flowers) on males and the apparent lack of stamens/anthers on any female plants provide clues to the evolutionary pathway to dioecy taken by *Z. americanum*. This observation suggests a pathway of hermaphrodite-gynodioecious-dioecious (Ross 1982; Fig. 1e). The sporadic appearance of hermaphroditic flowers on male plants but never on female plants suggests that this pathway could be further refined to hermaphrodite-gynodioecious-gynodioecious/andromonoecious, with the current gynodioecious/andromonoecious (subdioecious) state possibly on an evolutionary path towards complete dioecy (Fig. 17a). An alternative path (Fig. 17b) that involves andromonoecy is hermaphrodite-andromonoecious-gynodioecious-dioecious (Webb 1979). This alternate path could also account for the floral types exhibited in the *Z. americanum* study populations.

A direct path from hermaphroditism to dioecy is unlikely because it would require two simultaneous mutations: (1) male sterility to give the pistillate plant type and (2) loss of pistil development/functionality to give the staminate or andromonoecious plant types (Ross 1982; Ainsworth 2000). Alternatively, perhaps these mutations could have arisen simultaneously but separately in disparate parts of the population, then converged over time.

Assuming that the pistillate plant type and the andromonoecious plant type developed sequentially, one scenario (Fig. 17a) is that: (1) a mutation arose in a hermaphroditic ancestor, conveying male sterility and resulting in a mixed population of pistillate plants

and hermaphroditic plants (*i.e.* gynodioecy) and (2) mutation(s) that damaged or delayed pistil development in non-pistillate plants resulted in a population of pistillate and andromonoecious plants (Ross 1982). The final step would be the gradual elimination of hermaphroditic flowers, which may still be progressing (Ross 1982). In an alternative scenario (Fig. 17b), the intermediate steps are essentially flipped, with the andromonoecious plant type appearing first, followed by the pistillate plant type (Webb 1979). Occasional hermaphroditism may have been retained by *Z. americanum* because it conveys some benefit to the species.

Since the hermaphroditic flowers in the hermaphroditic group found in the study populations were protogynous and the staminate flowers were protandrous, it may be that protogyny and protandry play a role in the evolution towards dioecy in *Z. americanum*. One scenario is that balanced pistil/stamen development in ancestral *Z. americanum* hermaphroditic flowers gradually gave way to increasingly protandrous flowers, resulting in andromonoecious plants that were increasingly functionally staminate (Webb 1979).

As observed in the study populations, any given non-pistillate *Z. americanum* plant might fall somewhere on a range from being completely hermaphroditic to being completely staminate; only those plants lying somewhere between the two extremes might therefore be considered andromonoecious. If there is an environmental component to the presence or absence of hermaphroditic flowers on non-pistillate *Z. americanum* plants, then any given non-pistillate plant might also hover between hermaphroditic/andromonoecious/staminate states from year to year, depending on the environmental influences on that plant.

From a breeding perspective, the apparent environmental role in hermaphroditic flower development may allow breeders to purposely induce hermaphroditism in *Z. americanum*. One key may be the variable rate of pistil development, ranging from full development in pistillate and hermaphroditic flowers, to varying degrees of pistil development in staminate flowers (ranging from barely discernable pistil primordia to nearly fully developed pistils). The varying degree of pistil development in male *Z. americanum* flowers suggests a developmental race. Anther dehiscence in *Z. americanum* may signal floral abscission (van Doorn and Stead 1997). Pistils that

develop embryos before anthers reach anthesis may be able to halt abscission and so be able to develop their fruit to maturity. If this scenario is true, then treating flowers on male *Z. americanum* plants with gibberellins or other chemicals known to feminize staminate flowers in other species or inhibit fruit drop may raise the hermaphroditism rate in male *Z. americanum* (Heslop-Harrison 1956; Chailakhyan 1979; Marchetti et al. 1992; Ainsworth 2000; Pozo 2001).

Fruits on male *Z. americanum* plants indicate that those plants are at least partially hermaphroditic. The reproductive mechanisms (outcrossing, selfing, or apomixis) responsible for those fruits are unclear. One clue comes from the high incidence of stenospermocarpy in those fruits, indicating that selfing resulting in inbreeding depression may be responsible for the failed embryos in those stenospermocarpic fruits. An additional clue is the presence of fruits on double-bagged male branches, which should have resulted in no fruits being produced on those branches unless apomixis or selfing were involved. While not conclusive, these observations support the possibility that male fruits may be due to selfing or apomixis. Further evidence comes from two other observations. In 2013, one of the fruiting males had delayed flowering. Flowering began about two weeks after all other *Z. americanum* flowering in the area had finished (the late flowering male was deeply shaded, which may explain the late flowering). Heavy thunderstorms during that two-week period should have washed residual pollen from the area, yet the late flowering male bore fruits. If there was no non-self pollen available, then the remaining explanations for seed production or fruit set are selfing or apomixis. Likewise, in 2014, nine branches in the hermaphroditic group were mistakenly thought to be part of one of the female clones and so pollination bags were placed on those hermaphroditic branches prior to bud break. Three of those hermaphroditic bagged branches bore fruits (total of five fruits on those bagged branches). The double bagging used should have excluded all non-self pollen. Branches that were double-bagged on the adjacent female clone bore no fruits, indicating that the double bagging method effectively excluded pollen. If fruits on male *Z. americanum* arise via apomixis, then *Z. americanum* has the unusual ability to produce seed asexually on male (primarily staminate) plants – in other words, male apomixis.

Citrus Compatibility and Congruity

Some limited compatibility between citrus and *Z. americanum* (Figs. 14 and 15) was indicated based on fluorescence observed in stylar preparations, but whether *Z. americanum* x citrus fertilization and viable offspring are possible from sexual crosses is unclear. Three of the citrus cultivars tested (Ponderosa lemon, Meyer lemon, and Clementine mandarin) showed pollen germination and pollen tube growth on *Z. americanum* stigmas/styles when examined under fluorescence (Fig. 14). Ponderosa lemon also had the highest fruit:flower ratio (7.8 +/-4.9%) of the citrus cultivars used in 2014 *in situ* pollinations.

While fluorescence images of other cultivars did not show compatibility, this does not necessarily mean that those cultivars are incompatible. Factors such as citrus pollen viability, pollen freshness, pollen quantity, application stage, and pollen application time of day may have affected the results. Compatibility tests were done only on indoor pollinations, and not all pollen types were available.

Seedlings germinated from *Z. americanum* x citrus crossings showed only *Z. americanum* morphology with no obvious indications of any citrus parentage. The seedlings will be examined as they mature for any indications of citrus parentage as it is possible that the *Z. americanum* x citrus seedlings have not yet exhibited their citrus characteristics. Nevertheless, the *Z. americanum* x citrus crosses were likely unsuccessful in producing intergeneric hybrids (Grosser and Gmitter 1990). If *Z. americanum* x citrus sexual crosses do prove to be futile, somatic hybridization or microprotoplast fusion are future avenues to explore for intergeneric gene exchange to develop truly cold hardy citrus that combine *Z. americanum* cold hardiness with citrus fruiting traits (Deng et al. 1992; Guo and Deng 1998; Louzada et al. 2002; Zhang and Deng 2006; Bona 2007).

Conclusion

Developing cold hardy citrus by taking advantage of cold tolerance traits found in the citrus relative *Z. americanum* requires information about many facets of its floral

reproduction. Some aspects of *Z. americanum* floral reproduction such as pollen viability and stenospermocarpy may be of interest in a breeding program.

Although commonly described as being dioecious, some *Z. americanum* plants in the study populations also occasionally produced hermaphroditic flowers. The sporadic, seemingly random appearance of hermaphroditic flowers on otherwise male (staminate) plants raises a number of research possibilities, such as determining the cause of hermaphroditic flowers in *Z. americanum* and determining whether hermaphroditism is an inducible trait in *Z. americanum*.

Zanthoxylum americanum appears to have some compatibility with pollen from certain citrus cultivars. Further research will determine the breadth of that compatibility with citrus genomes. Compatibility is not sufficient to ensure reproductive success though. Future investigations need to determine whether the *Z. americanum* and citrus genomes are sufficiently congruous to form viable embryos.

Fig. 1 Proposed paths to Dioecy or Subdioecy (Webb 1979; Ross 1980 and 1982; Bawa 1980; Barrett 2002; Pannell 2002). Gynodioecious plants may have only hermaphroditic flowers on the non-pistillate (male) plants (diagram e), or may include both staminate and hermaphroditic flowers on the non-pistillate (male) plants (diagram g), depending on the definition used

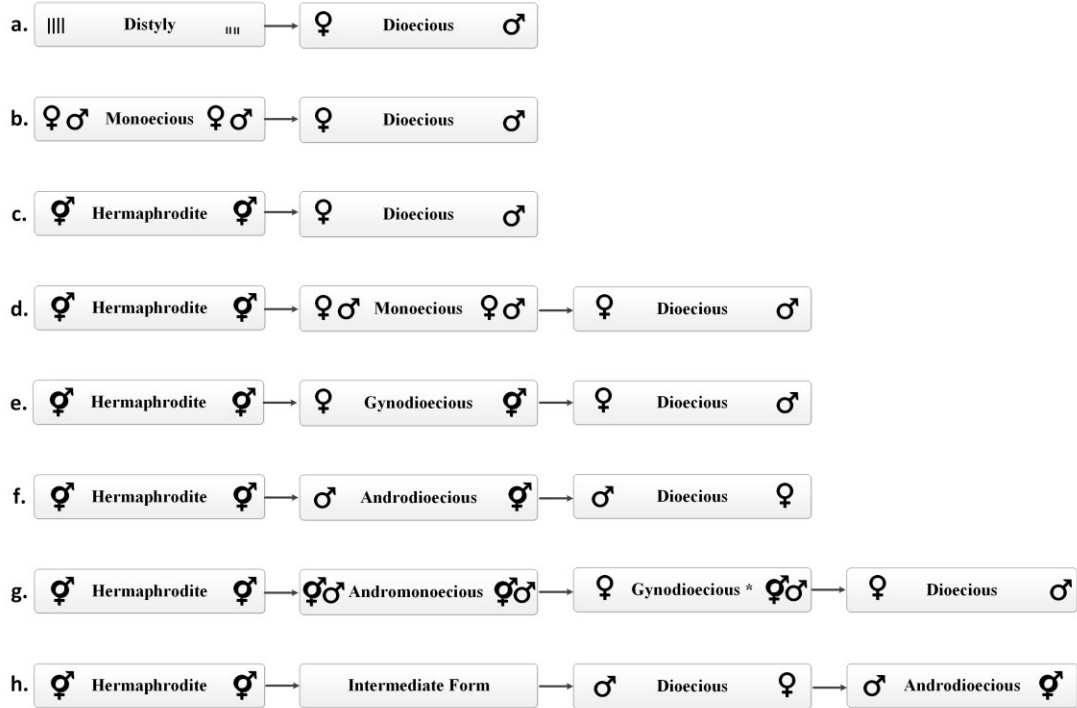


Fig. 2 Flowers from *Zanthoxylum americanum* sexual variants: (a) Flower from a pistillate plant. Styles (solid arrow) are relatively thick with somewhat bulbous stigmas; pollen (yellow) is adhering to a stigma. (b) Flower from a male that did not produce fruits. Pistil development is minimal (solid arrow). Anthers are approaching anthesis (dashed arrow). (c) Flower from a fruit-producing male (i.e. plant with predominately staminate flowers). Anthers (dashed arrow) are approaching anthesis but pistil development (solid arrow) is minimal. (d) Hermaphroditic flower from a plant (hermaphroditic group) that had both hermaphroditic and staminate flowers. The stigmas/styles (solid arrow) emerged before the anthers (dashed arrow) reached anthesis. Styles and stigmas appear malformed, with thin styles and non-bulbous stigmas. Some pollen (yellow) is present on the styles and stigmas.

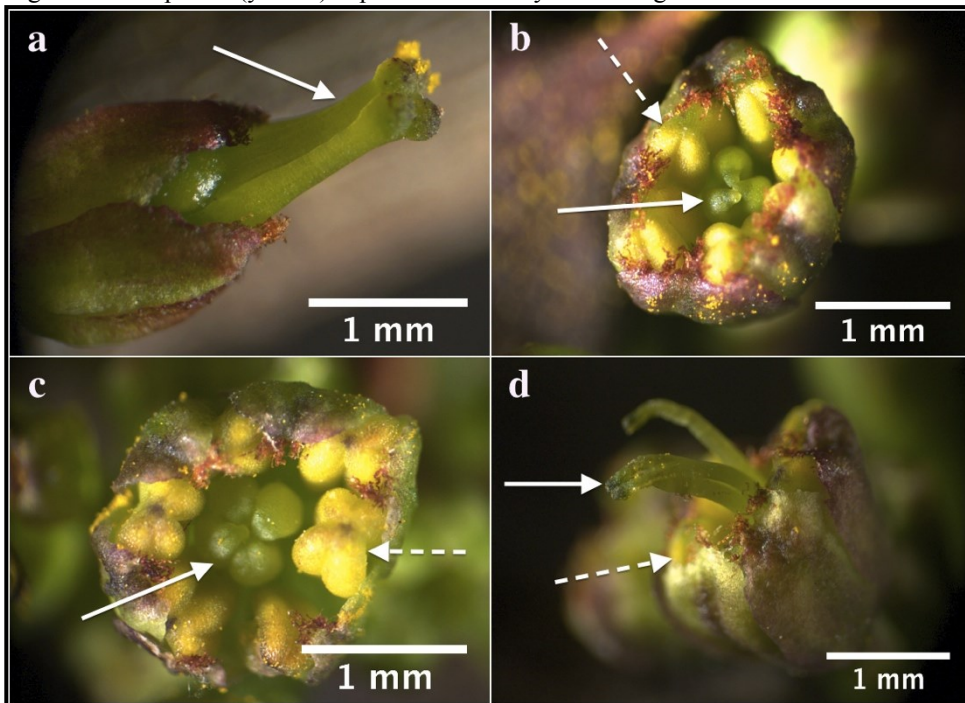


Fig. 3 *Zanthoxylum americanum* populations and males sampled for % pollen stainability (mean +/-S.E.) using freshly collected pollen; stained with 0.1% aniline blue in 80% propionic acid. Y axis starts at 50.

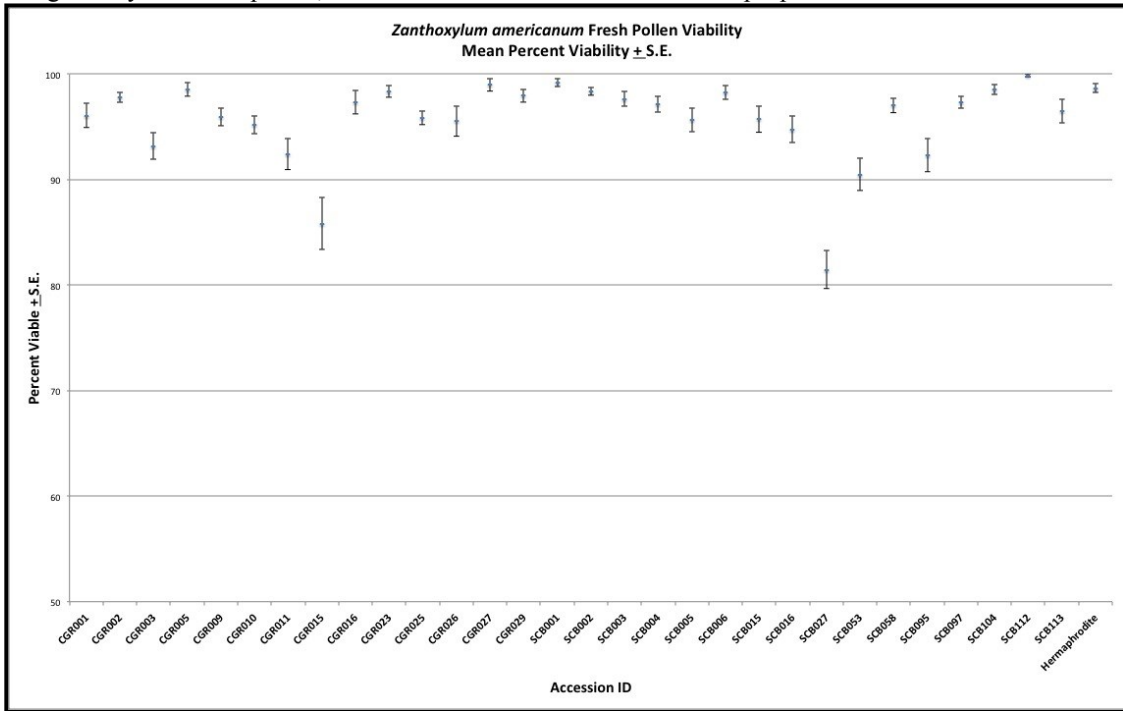


Fig. 4 Stored *Zanthoxylum americanum* % stainability pollen (mean +/-S.E.) of bulked pollen stored at -12° C; stained with 0.1% aniline blue in 80% propionic acid. Y axis starts at 50.

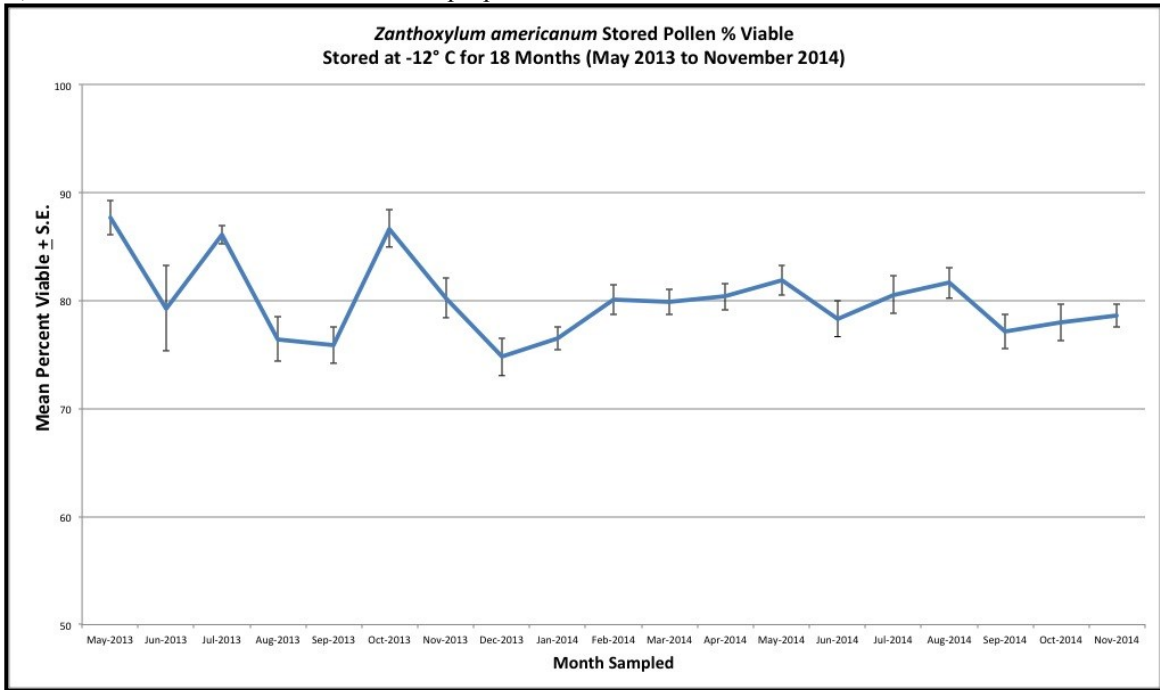


Fig. 5 (a) A *Zanthoxylum americanum* fruit cluster containing both fully developed fruits and the smaller stenospermocarpic fruits. (b) A cross section of a fully developed fruit. The seed fills the entire fruit cavity. (c) Cross section of a stenospermocarpic fruit. The aborted seed remnant is shriveled and fills only a portion of the seed cavity.

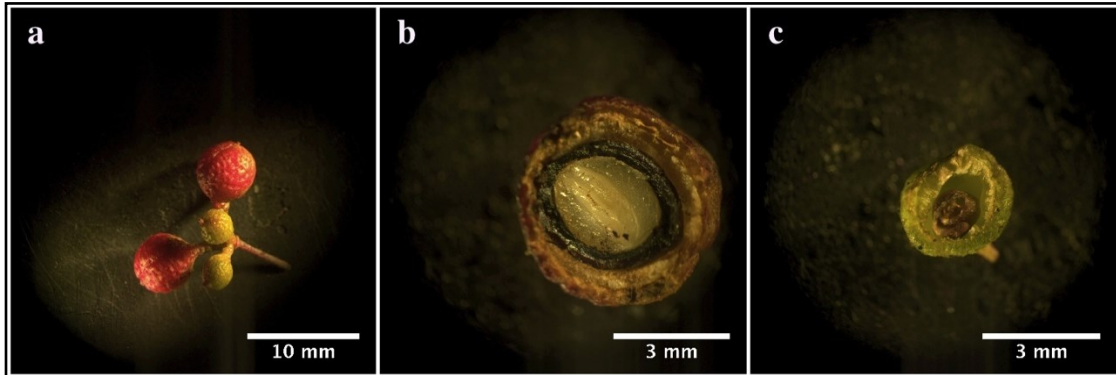


Fig. 6 Fruit clusters from pistillate *Zanthoxylum americanum* plants. (a) The clusters contain both normally developing fruits (larger fruits) and stenospermocarpic fruits (smaller fruits; arrows). (b) At ripening time, stenospermocarpic fruits (arrow) were often still green when the rest of the fruits in the same cluster were red.



Fig. 7 Fruit counts for all of the 2013 fruiting *Zanthoxylum americanum* males in the two study populations. SCB denotes plants at the St. Croix Bluffs location and CGR are those from the Cottage Grove Ravine location (see text). Fruit counts for genotype CGR023 stopped at 100; the actual fruit count on CGR023 exceeded 100.

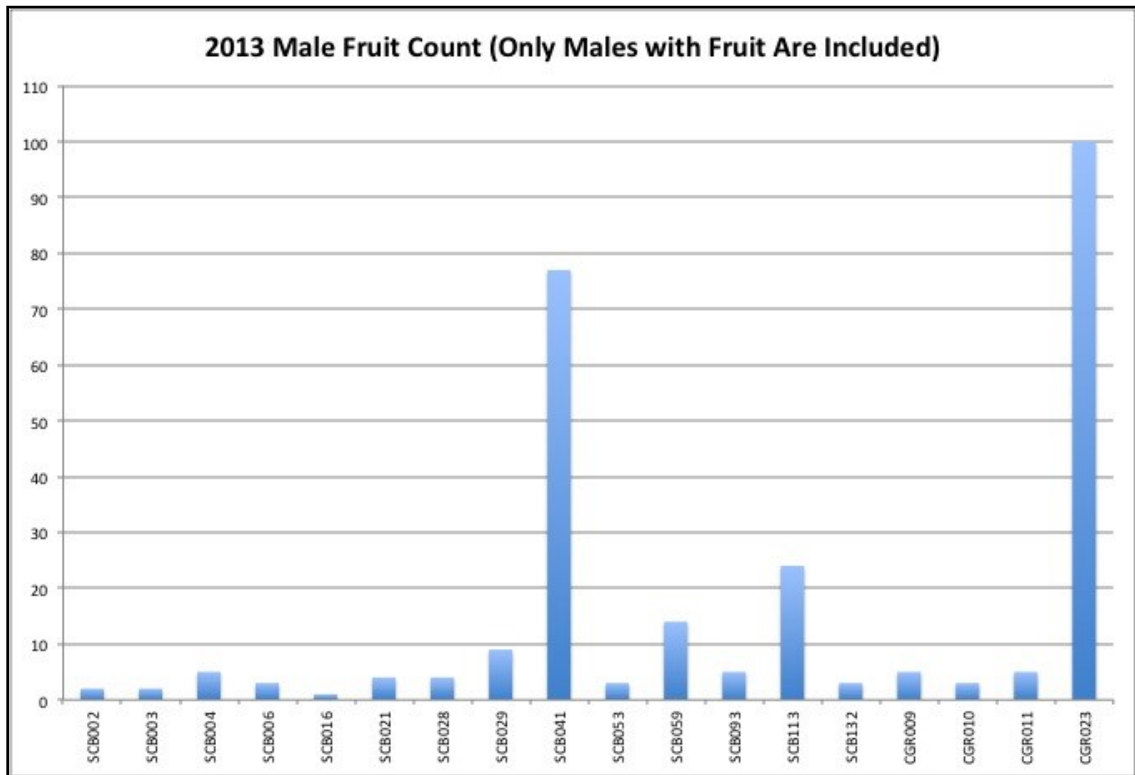


Fig. 8 Fruit cluster on a male (predominately staminate) *Zanthoxylum americanum* plant. The cluster includes both a normally developing fruit (larger fruit; dotted arrow) and stenospemocarpic fruits (smaller fruits; solid arrows).



Fig. 9 A typically sized cluster of fruits on a pistillate *Zanthoxylum americanum* plant. There are substantially more fruits in this cluster than in any male fruit cluster, which had at most five fruits/cluster.



Fig. 10 Fruits developing on a male (predominantly staminate) *Zanthoxylum americanum* plant. The fruit cluster is adjacent to the cut end of the branch (arrow).



Fig. 11 Floral remnants on *Zanthoxylum americanum* shortly after flowering. (a) Fruits developing on a female (pistillate) plant. The stigmas/styles (solid arrow) have fused and fruits are developing. (b) Staminate flowers post-anthesis (dashed arrow) are shriveled and dehiscing. (c) Remnants of hermaphroditic flowers on a predominantly male (staminate) plant; anther remnants (dashed arrow) are still attached to the flower. Remnant styles/stigmas (solid arrow) show that the stigmas had fused. Fruits have started developing (green portion of the flowers). Inset shows close-up view of remnant anthers and remnant fused styles. (d) Axillary cluster containing both hermaphroditic flower remnants (fruits have started developing – green; solid arrow) and a staminate flower remnant (flower is shriveled and beginning to dehiscence; dashed arrow).

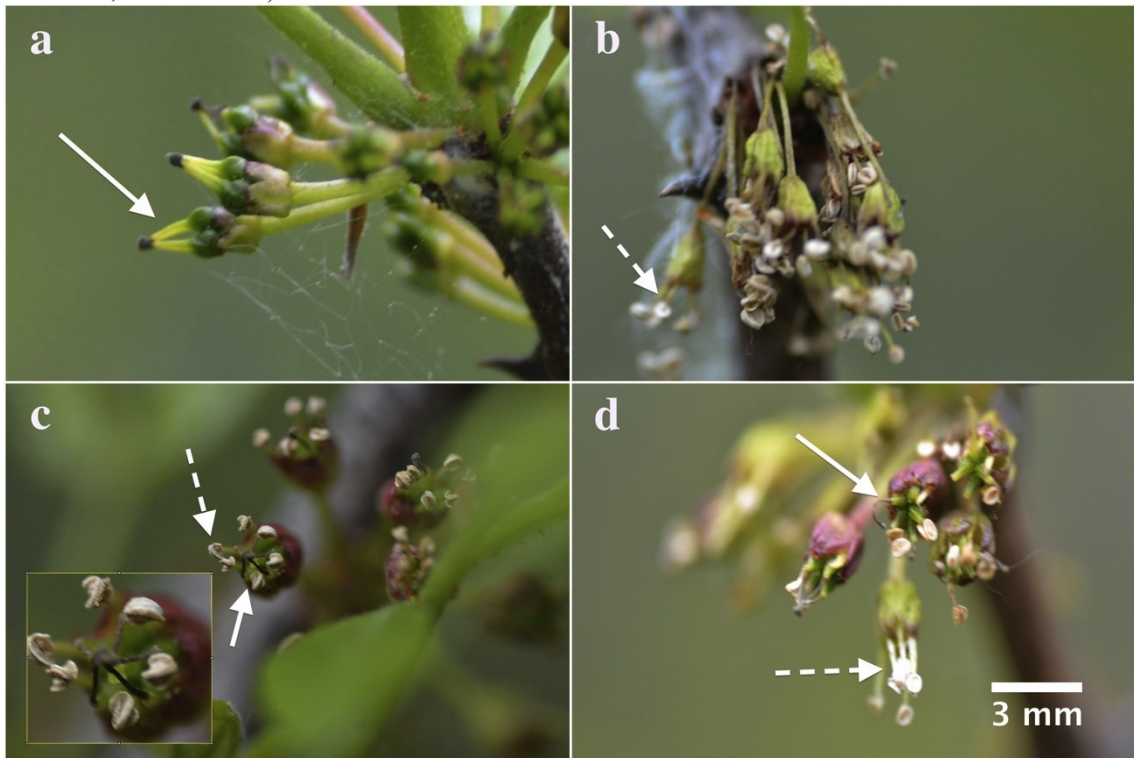


Fig. 12 Fruits on a male *Zanthoxylum americanum* developing (2014) on a branch that had been double-bagged prior to bud break. The branch cut (arrow) was made in early 2013.



Fig. 13 Pollen germinating on stigmas and styles of *Zanthoxylum americanum*: (a) Stigma and style from a female (pistillate) plant on a pistillate flower (*Zanthoxylum americanum* pollen). (b) Stigma and style from a hermaphroditic flower (the pollen is assumed to be *Zanthoxylum americanum* pollen). Arrows indicate germinating pollen/pollen tubes on stigmas/styles.

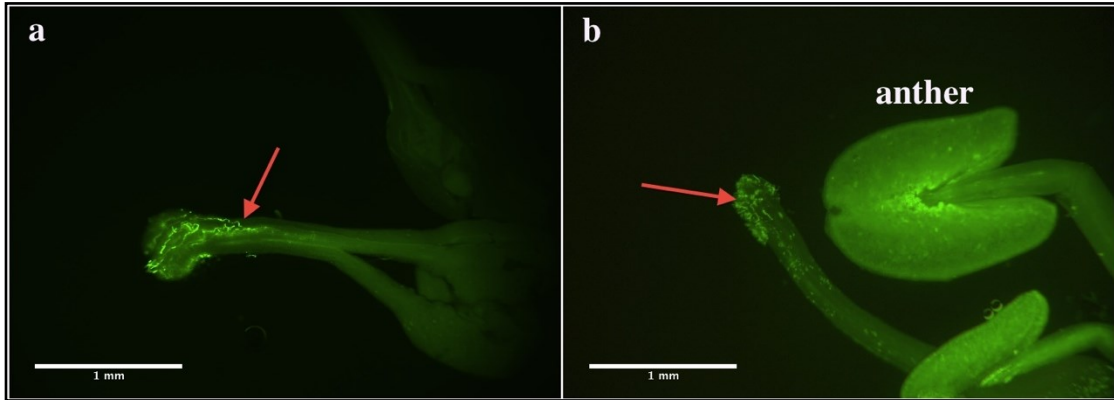


Fig. 14 Pollen germinating (arrows) on *Zanthoxylum americanum* stigmas: (a) *Zanthoxylum americanum* pollen; (b) Ponderosa lemon pollen; (c) Meyer lemon pollen (with pollen tubes growing down the styles) and (d) Clementine mandarin pollen.

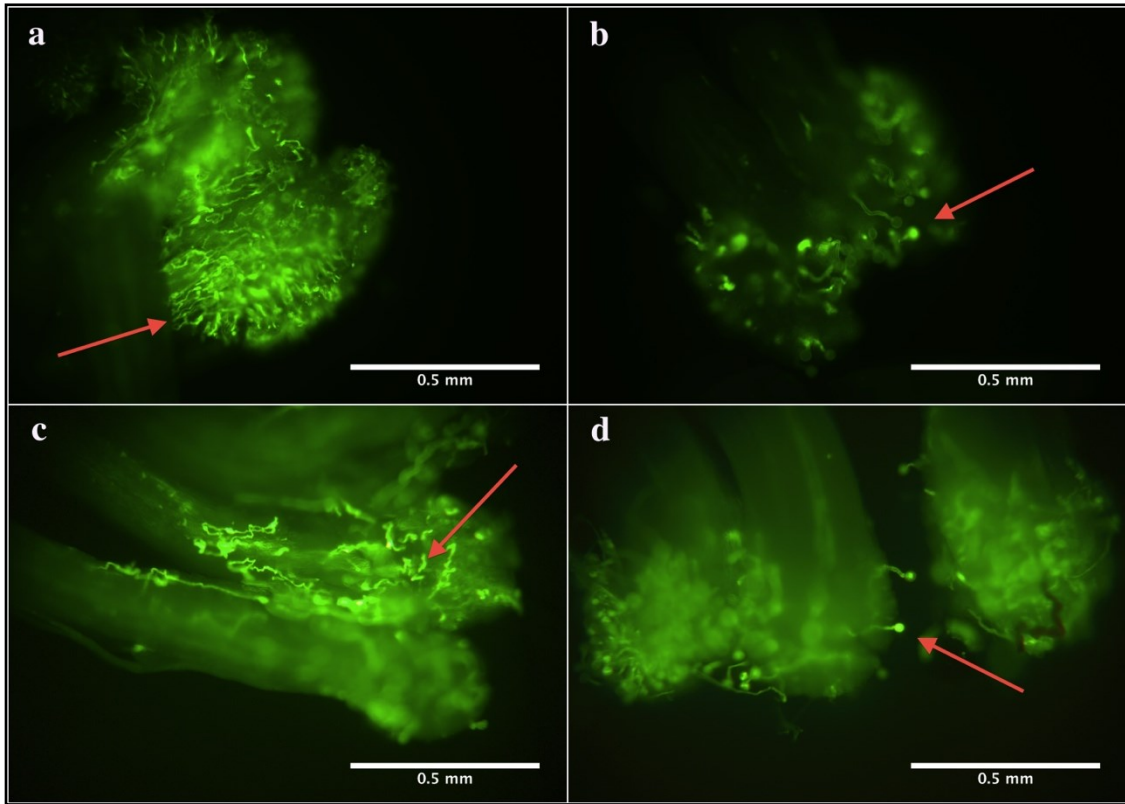


Fig. 15 Ponderosa lemon pollen germination and tube growth on/in *Zanthoxylum americanum* stigmas, styles, and ovaries: (a) Germination and growth down style (arrow); (b) Pollen tube growing into ovary (arrow).

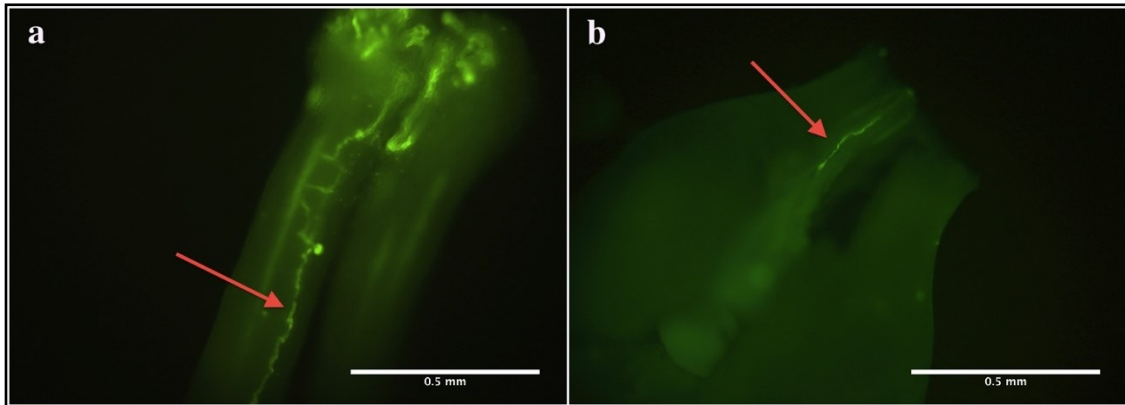


Fig. 16 *Zanthoxylum americanum* seedlings. (a) Seedling resulting from a purported *Z. americanum* x citrus cross. (b) Seedling resulting from a *Z. americanum* x *Z. americanum* cross. Both seedlings have the same maternal *Z. americanum* parent.



Fig. 17 Some possible *Zanthoxylum americanum* pathways to dioecy

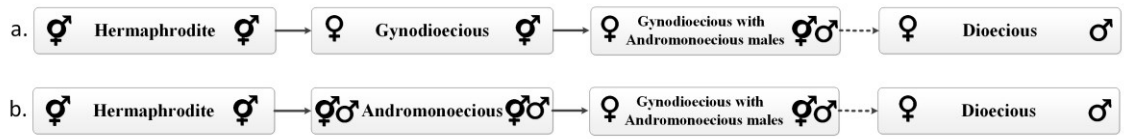


Table 1 Selected Terms Used in This Document

Term	Definition	References
Sexual Variants Identified in Evolutionary Pathways to Dioecy		
Androdioecious	Separate male (staminate) and hermaphroditic plants.	Pannell 2002
Andromonoecious	Male (staminate) and hermaphroditic flowers on the same individuals.	Ross 1982
Dioecious	Having male (staminate) and female (pistillate) flowers carried on separate male (staminate) or female (pistillate) individuals.	Ainsworth 2000
Distyly	Long styled plants are functionally females; short styled plants are functionally males.	Ross 1982
Gynodioecious	Separate female (pistillate) and hermaphroditic plants.	Ainsworth 2000
Hermaphrodite	All individuals have hermaphroditic flowers (flowers that have both functional stamens and functional pistils).	Darwin 1862 Ross 1982 Ainsworth 2000
Monoecious	All individuals have both male (staminate) and female (pistillate) flowers.	Ross 1982
Subdioecious	Dioecious species that are not completely dioecious, such as those that sometimes produce hermaphroditic flowers.	Ross 1982
Apomixis Terms		
Apomixis	Asexual reproduction through seeds.	Nogler 1984 Savidan 1999
Autonomous Apomixis	A form of apomixis in which both the embryo and the endosperm develop without fertilization.	Vinkenoog and Scott 2001 Spielman et al. 2003
Facultative Apomicts	Apomictic species or apomictic genotypes that sometimes or usually also reproduce sexually.	Asker 1980 Nogler 1984 Bicknell and Koltunow 2004
Nucellar Embryony	A type of apomixis called adventive embryony where the embryo forms from nucellus cells in the ovary.	Naumova 1992
Obligate Apomicts	Apomictic species or apomictic genotypes whose progeny are all or nearly all maternal clones.	Hearn 1994 Savidan 1999

Other Terms Used

Incongruity	Incomplete matching of the gene systems between two partners.	De Nettancourt 2001
Parthenocarpy	Development of fruits without pollination or fertilization.	Stout 1936 Gustafson 1942 Spena and Rotino 2001
Pistillate	Female flowers; flowers that have pistils but no stamens or non-functional stamens.	Darwin 1862 Ross 1982 Ainsworth 2000
Protoandry	Flowers in which the anthers develop first or reach anthesis before the pistils are fully developed.	Griffin et al. 2000
Protogyny	Flowers in which the pistils develop first; the pistils reach maturity before the anthers reach anthesis.	Griffin et al. 2000
Pseudogamy	A form of apomixis in which the polar nucleus or nuclei are fertilized by a male gamete.	Nogler 1984 Savidan 1999
Self-Incompatible (SI)	Self-incompatible species are those that have anti-selfing mechanisms that prevent pollen tube growth in the pistil, thereby preventing self-fertilization.	Dixit and Nasrallah 2001 Eberle 2012
Staminate	Male flowers; flowers that have stamens but no pistils or non-functional pistils.	Darwin 1862 Ross 1982 Ainsworth 2000
Stenospermocarpy	Seedless fruits produced as a result of fertilizations but with early abortion of the embryos.	Gustafson 1942
Sympatric	Being within the same geographic area.	Mallet et al. 2009
Unilateral Incompatibility	Self-incompatible species reject pollen from self-compatible species, while pollen is not rejected in the reciprocal cross.	Beecher et al. 2001 Eberle 2012

Table 2 Citrus Pollen Sources Used

Citrus Cultivar	Group	Species (Khan, 2007)	Source	Source ID	Fresh or Stored Pollen Used
Brown Select Satsuma	Mandarin	<i>C. reticulata</i>	Lemontree Citrus (lemontreecitrus.com)	3-4 Year Old	Stored
Calamondin	Mandarin X Kumquat	<i>C. madurensis</i>	Lemontree Citrus (lemontreecitrus.com)	3-4 Year Old	Fresh and Stored
Clementine	Mandarin	<i>C. clementina</i>	Lemontree Citrus (lemontreecitrus.com)	4-5 Year Old	Fresh and Stored
Chandler	Pummelo	<i>C. maxima</i>	Lemontree Citrus (lemontreecitrus.com)	2-3 Year Old	Fresh and Stored
Ethrog	Citron	<i>C. medica</i>	USDA Germplasm Repository, Riverside, California	RIV 6378 PL PI 508265	Stored
Fortunella hindsii	Kumquat	<i>F. hindsii</i>	USDA Germplasm Repository, Riverside, California	RIV 6292 PL PI 539724	Stored
Kao Ruan Tia	Pummelo	<i>C. maxima</i>	USDA Germplasm Repository, Riverside, California	RIV 5518 PL PI 539374	Stored
Lisbon (4N)	Lemon	<i>C. limon</i>	USDA Germplasm Repository, Riverside, California	6A-08-03 RRUT 239	Stored
Meyer	Lemon	<i>C. meyeri</i>	Logee's (logees.com)		Fresh
Ponderosa	Lemon hybrid	<i>C. pyriformis</i>	USDA Germplasm Repository, Riverside, California	RIV 6658 PL PI 539491	Stored
Ponderosa	Lemon hybrid	<i>C. pyriformis</i> or <i>C. limon x medica</i>	Lemontree Citrus (lemontreecitrus.com)	3-4 Year Old	Fresh and Stored
Tachibana	Mandarin	<i>C. tachibana</i>	USDA Germplasm Repository, Riverside, California	RIV 6099 PL PI 539679	Stored

Table 3 2013 *In Situ* Pollen Treatments

Treatment	Plant IDs SCB034, SCB035, SCB039, SCB042, SCB043, SCB044, SCB045, SCB048, SCB054, SCB057, SCB060	All Other <i>In Situ</i> Plants
No pollen, bag not removed during flowering period	No	Yes
No pollen, bag briefly removed during flowering period	Yes	Yes
<i>Z. americanum</i> pollen	Yes	Yes
Citrus pollen mixture	Yes	Yes
Mentor pollen plus citrus pollen mixture	Yes	No

Table 4 *Zanthoxylum americanum* populations and males sampled for % pollen stainability (mean +/-S.E.) using freshly collected pollen; stained with 0.1% aniline blue in 80% propionic acid

Accession Code	Percent Viable	Tukey HSD Mean Genotype Groupings ($\alpha = 0.05$)
Cottage Grove Ravine Population		
CGR001	96.1 +/-1.1	d,e,f,g
CGR002	97.8 +/-0.5	d,e,f,g
CGR003	93.2 +/-1.3	b,c,d,e,f
CGR005	98.5 +/-0.6	d,e,f,g
CGR009	95.9 +/-0.8	c,d,e,f
CGR010	95.2 +/-0.8	c,d,e
CGR011	92.4 +/-1.5	a,b,c,d
CGR015	85.8 +/-2.5	a,b
CGR016	97.3 +/-1.1	d,e,f,g
CGR023	98.3 +/-0.5	e,f,g
CGR025	95.9 +/-0.7	c,d,e,f,g
CGR026	95.5 +/-1.4	d,e,f,g
CGR027	99.0 +/-0.6	f,g
CGR029	97.9 +/-0.6	d,e,f,g
CGR Pooled	95.7 +/-0.4	
St. Croix Bluffs Population		
SCB001	99.1 +/-0.4	f,g
SCB002	98.4 +/-0.4	d,e,f,g
SCB003	97.6 +/-0.7	d,e,f,g
SCB004	97.1 +/-0.8	d,e,f,g
SCB005	95.6 +/-1.1	d,e,f,g
SCB006	98.2 +/-0.6	d,e,f,g
SCB015	95.7 +/-1.2	c,d,e,f,g
SCB016	94.7 +/-1.2	d,e,f,g
SCB027	81.5 +/-1.8	a
SCB053	90.5 +/-1.5	a,b,c
SCB058	97.0 +/-0.7	d,e,f,g
SCB095	92.3 +/-1.6	b,c,d,e
SCB097	97.3 +/-0.6	d,e,f,g
SCB104	98.5 +/-0.5	d,e,f,g
SCB112	99.9 +/-0.1	g
SCB113	96.5 +/-1.1	d,e,f,g
SCB Pooled	96.0 +/-0.4	
All Males Sampled	95.8 +/-0.3	

Table 5 *Zanthoxylum americanum* males and hermaphrodites sampled for % pollen stainability (mean +/- S.E.) using freshly collected pollen; stained with 0.1% aniline blue in 80% propionic acid

Plant Type Sampled	Percent Viable	Tukey HSD Plant Type Means Groupings ($\alpha = 0.05$)
All Males Sampled	95.8 +/-0.3	
Non-fruiting Males Sampled	95.6 +/-0.4	a
Fruiting Males Sampled	96.1 +/-0.4	a
Hermaphroditic Group	98.7 +/-0.4	b

Table 6 Stored *Zanthoxylum americanum* pollen (bulked) % pollen stainability (mean +/-S.E.) using pollen stored at -12° C; stained with 0.1% aniline blue in 80% propionic acid

Date	Percent Viable
2013 Pollen (collected April 2013 ; stored at -12° C)	
2013-May	87.7 +/-1.6
2013-June	79.3 +/-3.9
2013-July	86.1 +/-0.9
2013-August	76.4 +/-2.1
2013-September	75.9 +/-1.7
2013-October	86.7 +/-1.7
2013-November	80.2 +/-1.8
2013-December	74.8 +/-1.7
2014-January	76.5 +/-1.1
2014-February	80.1 +/-1.4
2014-March	79.8 +/-1.2
2014-April	80.4 +/-1.2
2014-May	81.9 +/-1.4
2014-June	78.3 +/-1.7
2014-July	80.5 +/-1.8
2014-August	81.7 +/-1.4
2014-September	77.1 +/-1.6
2014-October	78.0 +/-1.7
2014-November	78.6 +/-1.1
2012 Pollen (Stored at -18° C for 30 months)	
2014-December	87.5 +/-1.6

Table 7 *In situ* 2013 and 2014 Pollination Treatments

2013 and 2014 Treatments	No. Bagged Branches	No. Bagged Branches With Fruits	No. Flowers	No. Fruits	% Bagged Branches with Fruits	Fruit:Flower Ratio *100 (%+/- S.E.)
2013						
Citrus Mix ^a	67	34	4761	248	50.7	5.2 +/-1.2
Citrus Mix + Mentor	11	5	853	32	45.5	3.8 +/-1.5
Mentor Control (Citrus Mix)	23	6	1300	58	26.1	4.5 +/-5.8
Not Pollinated, Bag Removed During Flowering	90	35	6759	417	38.9	6.2 +/-1.7
Not Pollinated, Bag Not Removed During Flowering	44	26	N/A	296	59.1	N/A ^b
<i>Z. americanum</i>	90	77	5460	2538	85.6	46.5 +/-5.3
2013 Total ^b	324	183	19133	3589	56.5	
2014						
Citron, Ethrog	9	1	353	2	11	0.6 +/-0.6
Citrus Mix ^c	15	7	1382	102	46.7	7.4 +/-2.2
Citrus Mix + Mentor	12	3	1107	58	25	5.2 +/-5.9
Lemon, Lisbon (Tetraploid)	13	0	951	0	0.0	0.0
Lemon, Ponderosa	11	2	918	72	18.2	7.8 +/-4.9
Mandarin, Clementine	21	8	1991	16	38.1	0.8 +/-0
Mandarin, Clementine + Mentor	18	5	1529	65	27.8	4.3 +/-1.7
Mandarin, Tachibana	5	0	338	0	0.0	0.0
Not Pollinated	59	9	4110	14	15.3	0.3 +/-0
Not Pollinated, Cropped	6	0	374	0	0.0	0.0
Pummelo, Chandler	5	0	262	0	0.0	0.0
Pummelo, Kao Ruan Tia	3	0	138	0	0.0	0.0
<i>Z. americanum</i>	23	21	1476	829	91.3	56.2 +/-11.5
2014 Total	200	56	14929	1158	28.0	7.8

^a The 2013 citrus mix included Ethrog citron; mandarins Brown Select Satsuma, Clementine, and Tachibana; lemons Ponderosa and Meyer; Calamondin mandarin/kumquat; and kumquat (*Fortunella hindsii*).

^b The 2013 “Not Pollinated, Bag Not Removed During Flowering” entry does not include a flower count or fruit:flower ratio because the flowers were not counted on those branches.

^c The 2014 citrus mix included Ethrog citron; lemons Lisbon (tetraploid form) and Ponderosa; mandarins Clementine and Tachibana; and pummelos Chandler and Kao Ruan Tia.

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<https://www.google.com/maps/place/44°47'41.7%22N+92°48'04.0%22W/@44.7949167,-92.8011111,600m/data=!3m2!1e3!4b1!4m2!3m1!1s0x0:0x0>
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