BROADENING THE GENETIC BASE OF PAPAYA VIA INTERGENERIC HYBRIDIZATION WITH WILD RELATIVES

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ABSTRACT

Pre-breeding of papaya by hybridizing it with wild relatives could yield new traits of value to papaya production, both for agricultural (e.g. disease resistance) and consumer oriented (e.g. flavor components) traits. Hybrids between papaya and its closest major relatives, the *Vasconcellea*, ultimately result in sterile progeny. Here, inducing tetraploidy is proposed as a method of overcoming that sterility. Crosses were made between papaya and several *Vasconcellea* species by utilizing embryo rescue techniques. These hybrids were then treated with a mitotic inhibitor, oryzalin, to produce plants with tetraploid tissues. Thus far, flowering has occurred in interspecific hybrids, resulting in the production of larger, yet well formed, pollen grains.

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CHAPTER 1. INTRODUCTION AND OBJECTIVES

1.1 Introduction

Papaya (*Carica papaya* L.) is a widely grown fruit tree bearing large, sweet fruits. It is an important fruit crop throughout the tropics, consumed as either a fruit, if ripe, or a vegetable, if unripe. It is fast growing, with deeply lobed leaves on long petioles, bearing cauliflorous fruit on typically unbranched stems within 9 months of planting. In cultivation, papaya is often gynodioecious, producing either female or hermaphrodite plants, with the hermaphrodite fruit being the market standard due to its pyriform shape, which is preferred by consumers

Papaya is the only member of its genus. The largest genus in the papaya family, Caricaceae (order Brassicales), is the Vasconcellea genus, whose members possess a number of valuable traits. It would therefore be desirable to allow for the transfer of genetic material between the species. Hybridization of papaya and Vasconcellea is a wide cross, however, meaning that the species are rather distantly related. As such, the technique of embryo rescue was required for the production of hybrids. In this technique, tissue culture methods are employed to retrieve embryonic material from otherwise inviable seeds, and from this material, generate complete plants.

However, even when hybrids are produced between papaya and Vasconcellea, they tend to be infertile. This sterility is the second problem which much be addressed in order to make full use of the genetic resources found in the Vasconcellea. One possible method of overcoming this challenge is the use of induced polyploidy. If the problem resulting in infertility is due to mispairing of the homeologous chromosomes of the two species, than doubling the chromosomes, thereby forming amphidiploid plants in which each set chromosome functions independently of the others, with a similar pairing partner during meiosis, may restore some of the fertility of the hybrids. This was done by treating the hybrid meristems with oryzalin, a mitotic inhibitor which prevents the cells from properly dividing chromosomes between daughter cells, resulting in genome doubling.

1.2 Research Objectives

The objective of this research is to develop interspecific hybrids within *Vasconcellea* and intergeneric hybrids between *Carica* and *Vasconcellea*, and to raise these hybrid materials to the tetraploid level via oryzalin treatment. With such a hybrid produced, the fertility of an intergeneric amphidiploid hybrid of papaya and a wild relative of the *Vasconcellea* genus can be evaluated. The significance of this work is to unlock the potential of using wild genetic resources in papaya improvement. This could lead to the introduction of new desirable traits, including tolerance of abiotic stresses, resistance to diseases like *Phytophthora* or papaya ringspot virus, and novel flavor components. This work will also demonstrate the possibility of using induced polyploidy as method to restore fertility in *Carica* x *Vasconcellea* wide crosses and elucidate the nature of the hybrid sterility of these intergeneric hybrids.

Summary of objectives:

- Produce intergeneric Carica x Vasconcellea hybrids and interspecific Vasconcellea hybrids
- Confirm hybrids using isozyme markers and characterize morphology & fertility of hybrids
- Produce allotetraploid hybrids via oryzalin induced chromosome doubling and determine effects on fertility

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Along with banana, pineapple, and mango, the papaya is one of the most important of the tropical fruit crops. The world production of papaya was 12.4 million tonnes in 2013 (FAO, 2016). It is cultivated throughout the tropics, with the top five producers being India, Brazil, Indonesia, Nigeria, and Mexico. In the United States, which ranks 30th in world papaya production producing 12515 tonnes, papaya production is based principally in the states of Hawai‘i and Florida, with some minor production occurring in California (Warnert, 2004) and Texas (Sauls, [date unknown]). In Hawai‘i, there is 28.6 million pounds of papaya production on 2000 acres worth $9.7 million (NASS, 2011)

Papaya is commonly consumed as a sweet fruit, in which manner it can be consumed fresh, dry, or canned. The skin color is yellow when ripe, although the flesh color can be either red or yellow, with the yellow flesh color being dominant. There is great size variation in the fruits, with smaller, single serving varieties, commonly referred to as ‘Solo papayas’ being the standard in some parts of the world, and large, several pound varieties being the standard elsewhere. The most well-known Hawaiian varieties, including the ‘Rainbow’, ‘Sunset’, ‘Laie Gold’, and ‘Kapoho’ varieties, are solo types. The fruit can also be consumed under ripe as a savory vegetable in the form of green papaya salad, a common custom in southeast Asian countries, whereby the papaya is shredded and mixed with ingredients such as chili, lime, peanut, and shrimp. Additionally, its seeds possess a peppery taste reminiscent of other pungent flavors found in the Brassicales order, which can be crushed and used to make papaya seed dressing.

However, the genetic base of papaya is narrow (Kim et al., 2002), with its closest relatives existing only in other genera, and has been further selected to have further reduced diversity (Moore, 2013). This limits access to useful genetics, and required less conventional methods of solving problems, for example, to counter ringspot virus required transgenic methods (Gonsalves et al., 2004).

Genepools can be thought of as existing in three pools: primary, secondary, and tertiary (Harlan and de Wet, 1971). Primary genepools are easily accessible, and commonly found within the same species. Secondary genepools are those which are more difficult to access, such as traits found in other closely related species. In other crop species, tapping into the secondary genepools has yielded beneficial traits. In lettuce (*Lactuca sativa*), black aphid resistance has been brought in from *L. virosa* via the bridge species *L. serriola* (Dieleman and Eenink, 1980). In tomato (*Solanum lycopersicum*), resistance to late blight has been brought in from *S. pimpinellifolium* (Gardner and Panthee, 2010). In common bean (*Phaseolus vulgaris*), work is being done to bring abiotic stress resistance to common bean from *P. acutifolius* (Porch, 2013). In broccoli (*Brassica oleracea* var. *Italica*) and cabbage (*B. oleracea* var. *capitata*) work has been done to bring in resistance to powdery mildew from *B. carinata* (Tonguç 2004). In squash (*Cucurbita pepo)* resistance to powdery mildew has been introgressed from C. *martinezii* (Whitaker and Robinson, 1980).

However, in all these cases, the hybrids are interspecific. Such crosses are not possible with papaya, as *Carica* is a monospecific genus. In the case of papaya, the only source of novel genetic material must be acquired from the tertiary genepool, the most distantly related and hardest to access possible source of genes. While breeders have long made attempts to work with the existing gene pool within the papaya family in the *Vasconcellea* genus, these efforts have been met with limited success due to the infertility of the hybrid progeny.

2.2 Overview of Relevant Species and Attempts to Hybridize Them

Papaya is the sole member of the *Carica* genus of the family Caricaceae. The family contains five other genera: *Cylicomorpha*, native to Africa, and the most basal of the family; *Jarilla*; *Jacaratia*; *Horovitzia*, a monotypic genus; and *Vasconcellea*, the largest genus of the family which was once classified as *Carica*. The family is thought to have origins in Africa and drifted into South America (Carvahlo and Renner, 2012). One point of distinction between the *Vasconcellea* and *Carica* is that *Carica* possess a single, hollow seed cavity within the fruit, while *Vasconcellea* has a seed cavity filled

with placental tissue. Both *Carica* and *Vasconcellea* have nine chromosomes. In this project, there were nine species of *Vasconcellea* available.

Vasconcellea pubescens A. DC. (syn. *V. cundinamarcensis*), sometimes called the mountain papaya, is so named for its fuzzy, pubescent leaves. Along with virus resistance, it is suitable for cultivation due to its aromatic qualities, and is grown on a small scale in South America (NAP, 1989). The species is also able to tolerate light frost. As the current genetically engineered variety was developed using a specific strain of PRSV, it does not always display resistance to other strains of the virus (Tennant et al., 1994). Although there is no present evidence of resistance breakdown in transgenic papaya, this is a concern, and there exists the potential for resistance breakdown if ever a virulent strain of the virus by some means comes to the growing regions.

Hybridization with a naturally resistant species could lead to a more durable resistance, and as such, species such as *V. pubescens* are desirable targets from which to produce a successful hybrid. Additionally, this species has favorable eating quality when processed. Although lacking in sweetness when fresh, sweetened canned products are produced in Chile from this species. These canned Chilean papaya products are highly delectable, with a firm, pleasant mouthfeel, and a flavor somewhat reminiscent of jackfruit or mango. The leaves (Fig. 1) and fruit (Fig. 2) appear similar to that of a papaya, although the stems and veins of the leaves possess pubescence. The fruits are smaller, more heavily ridged, and very aromatic.

Figure 1. *Vasconcellea pubescens* growing at Lālāmilo, Hawai‘i

Figure 2. Ripe *Vasconcellea pubescens* fruits

Vasconcellea quercifolia A. St.-Hil. is the second species with virus resistance. It is sometimes called the oak-leafed papaya. This species is arborescent. The fruit of this species is small and yellow, with a high seed to flesh ratio, and is lacking in flavor, however, it has a high brix. Flowers are a greenish color.

Vasconcellea stipulata (V.M. Badillo) is the last species with virus resistance. It is so named for the stipules on the trunk of the plant, which are modified to form spines. The fruit possess a desirable, lime-like aroma. It is a mountainous species, which gives it suitability to high altitude cultivation (NAP, 1989). *V. pubescens*, *V. quercifolia*, and *V. stipulata* were the species of primary interest, as these three possess the most desirable characteristics. The flower color is yellow.

Vasconcellea parviflora A. DC. is a small, pachycaul plant. The small flowers are an ornamental shade of pink. The fruit is described as edible although it is too small be of value. A short plant, it is deciduous, and displays drought resistance. The fruit of the plants utilized in this project were mildly palatable, with a taste reminiscent of dried tomato paste.

Vasconcellea monoica (Desf.) A. DC., sometimes called "*col de monte"*, has the unique trait of being monoecious, with a central female blossom surrounded by a number of male blossoms on each inflorescence (Badillo, 1993). In places where it is cultivated, both the fruit and the leaves are consumed, as is indicated by its name, which means "cabbage of the mountain" (NAP, 1989). It has some resistance to papaya bunchytop disease, a disease of papaya in the Caribbean. Fruits are egg-shaped, with yellow skin, and yellow flesh. The fruit of the plants utilized in this project were not palatable. Flowers are white.

Vasconcellea goudotiana Triana & Planch is a tall, branching plant. While *V. goudotiana* is not resistant the ringspot virus, the plant displays resistance to *Phytophthora*, another disease of papaya. Fruits are described as having variable quality, with the best described as being 'apple like' in their flavor. Fruits can be either yellow, or a deep red wine color, both with pale yellow flesh. Flowers are white, with some red pigmentation on the calyx.

V. × *heilbornii* (V.M. Badillo) is assumed to be a hybrid complex of other *Vasconcellea* species. It is commonly called a '*babaco'*, and is known for having mild,

pleasant fruits. It is seedless in female plants and as such requires asexual propagation through cuttings. It is cultivated on a small scale. As it is assumed to be a hybrid of PRSV resistant species, it too should have resistance. It potentially may also give flavor attributes to its progeny, however the fruit quality of this plant could not be evaluated as only a male was available, producing small white flowers.

Vasconcellea glandulosa (V.M. Badillo) and *V. pulchra* (V.M.Badillo) were also available for hybridization attempts, however, these are fairly unremarkable species. The practical use of hybrids derived from any these species remains to be seen. A female *V. horovitziana* (V.M. Badillo), which has the unique feature among the family of being a liana, was also established, however, it never flowered and could not be utilized in any hybridization attempts.

Additionally, a Caricaceae outside of the *Vasconcellea* genus was used when available: *Horovitzia cnidoscoloides* (Lorence & R. Torres). *H. cnidoscoloides* is a recently named species. Like *Carica*, *Horovitzia* is monotypic. The species name means nettle-like, in reference to the nettle like trichomes on the leaves. Little has been reported on this species, and it is not known if it is resistant to the ringspot virus. There are no known reports of attempted hybridization of *Carica* and *Horovitzia*, although genetic studies have indicated that it has more recently diverged from *Carica* than *Vasconcellea* (Carvalho and Renner, 2012), which suggest that it may be more apt to successfully hybridize with *Carica*.

Hybridization attempts within the Caricaceae have been made for over a century. Horovitz and Jimenez report early extensive work in hybridization among multiple *Vasconcellea*, as well as *C. papaya* x *V. pubescens* (Horovitz and Jiménez, 1967). Mekako also made numerous crosses within the *Vasconcellea*, however reported *Vasconcellea*'s incompatibility with papaya (Mekako and Nakasone, 1975). Wenslaff did produce hybrids between *Carica* and *Vasconcellea*, however, with limited fertility (Manshardt and Wenslaff,1989). Numerous hybrids have been produced at Griffith University in Australia between papaya and *Vasconcellea*, including hybrids with *V. goudotiana*, *V.parviflora*, and *V. quercifolia* (Drew, 1998). Backcrossing has been attempted with some of the resulting progeny with some success (Drew, 2011). Despite the effort, there has yet to be an example of a commercial improvement in papaya

through transfer of genes from wild relatives, due to the resulting sterility of the hybrids, which consequently prevents the use of *Vasconcellea* genes in papaya improvement.

2.3 Induced polyploidy and fertility

Although they possess the same number of chromosomes (2n=2x=18), a lack of meiotic pairing may be a cause of the sterility of intergeneric *Carica* x *Vasconcellea* hybrids; a doubling of the chromosomes may allow each set a partner for proper pairing and division, resulting in restored fertility. Chromosome doubling has been used to restore fertility in some intergeneric hybrids, such as in the intergeneric ornamental tree x*Chitalpa* (Olsen, 2006) and in okra interspecific hybrids (Reddy, 2015). In the well-known case of the intergeneric hybrid grain triticale, attempts had been made for almost a century to hybridize wheat and rye, however the hybrids had little to no fertility. It was not until the hybrids underwent chromosome doubling that the fertility was restored and triticale could become a viable crop (Ammar, 2004).

CHAPTER 3: WIDE CROSSING OF *CARICA PAPAYA* L. AND *VASCONCELLEA* SPECIES AND RESCUE OF HYBRID EMBRYOS

3.1 Introduction

Embryo rescue is a tissue culture technique used to establish otherwise nonviable hybrid offspring. In the normal development of a seed, endosperm develops in the seed to serve as a reserve of nutrients for the embryo, to give it sufficient energy to produce photosynthetic organs and survive independently. However, in cases where normal endosperm development does not occur, the only means of allowing an embryo further growth is to grow it in vitro. In a wide cross between species separated by diverging evolutionary pathways, such developmental abnormalities as endosperm failure are common. Therefore, it may be that the only way to produce wide crosses is to make use of embryo rescue.

In this process, fully formed embryos or partially developed embryonic material have potential to develop into fully formed plants. A fully formed or nearly fully formed embryo may be able to germinate normally when given a source of energy to substitute for the endosperm it naturally lacks. An immature or poorly formed embryo may be cultured on media designed to encourage further development, or to undergo alternative routes of plant development, such as the production of somatic embryos, which in turn are able to develop and germinate to form plants.

Embryo rescue has been utilized to facilitate the hybridization among numerous crop plants and their relatives, including papaya (Drew, 1998). While some interspecific hybrids in the Caricaceae are able to be produced without the use of embryo rescue (Horovitz and Jiménez, 1967, Mekako and Nakasone, 1975), or occur spontaneously in the wild (Badillo, 1993), there has been no report of successful hybridization between papaya and any other species without the use of embryo rescue.

3.2 Materials and Methods

3.2.1 Plant material origin and establishment

Plant material of wild species was obtained from 2014-2016 from the USDA Tropical Plant Genetic Resources and Disease Research Unit of the Daniel K. Inouye

Pacific Basin Agricultural Research Center in Hilo, Hawai‘i, as either woody cuttings, seeds, or immature seedlings (Table 1). Woody cuttings 3-4 inches in length of both terminal and non-terminal sections were rooted in Oasis™ Rootcubes (Smithers-Oasis, Kent, OH) after being dipped in Hormex Rooting Powder #3 (IBA 0.3%) (Maia Products – Hormex, Westlake Village, CA), and placed in plastic boxes to maintain high humidity on a south facing windowsill. Non-terminal cuttings had the apical end wrapped in Parafilm (Bemis, Oshkosh, WI). Cuttings of *V. pubescens*, *V. stipulata*, *V. quercifolia* failed to root, but *V. x heilbornii*, *V. glandulosa*, and *V. pulchra* rooted readily.

Seeds of *V. stipulata*, *V. goudotiana*, *V. monoica*, and *V. parviflora* were soaked overnight in water, and placed in vermiculite, which was kept moist, in a greenhouse. Germination typically occurred within a month. In the case of *V. stipulata*, germination was only rapid when using fresh seed taken right out of a fruit. In contrast, a long dormancy period prevented ready germination of *V. quercifolia* and *V. pubescens*. This was overcome by removing and growing the embryos in culture.

To overcome the dormancy problem, seeds were placed in a solution of 1.05% sodium hypochlorite with liquid detergent as a surfactant, and kept submerged for an hour. Afterward, they were removed in a sterile flow hood, rinsed with sterile water, dissected, and embryos placed on the charcoal media (see Chapter 3.2.3. Embryo Rescue). Germination of these was greatly expedited to less than two months, and within three months seedlings were established out of culture. The germination rate of these cultured embryos was only around 25%, with most embryos, even those undamaged by the extraction, failing progress; this was nonetheless an improvement over the total lack of germination when attempting standard seeding procedures.

Material from *Horovitzia cnidoscoloides*, a closely related species with leaves resembling nettle leaves, was also obtained, however, cuttings did not root and seeds died of fungal pathogens shortly after germination.

Plants which were established were grown in greenhouse conditions or field planted, with the exception of *V. pubescens*. *V. pubescens* did not tolerate the warm (>30 C°) conditions of the greenhouse, and required cooler (23 C°) indoor conditions on a south facing windowsill in an air-conditioned lab to survive.

Multiple inbred (Table 2) and hybrid papaya genotypes were used as parents in cross-pollinations with wild relatives. Papaya were grown at the Waimānalo Experiment Station in Waimānalo, Oʻahu, and at the Magoon Horticulture Facility in Mānoa, Oʻahu. Additionally, several feral papaya plants of unknown origin, which were growing at the University of Hawaiʻi at Mānoa Campus in Honolulu, Hawaiʻi, were used.

Table 1. List of species and type of material received

Species	**Propagative material**	**Plants established**
Vasconcellea pubescens	Cuttings, seeds, pollen	2
Vasconcellea stipulata	Cuttings, seeds	7
Vasconcellea quercifolia	Plants, cuttings	16
Vasconcellea goudotiana	Seeds	8
Vasconcellea monoica	Plants, seeds	5
Vasconcellea parviflora	Plants, seeds	14
Vasconcellea xheilbornii	Cuttings	2
Vasconcellea glandulosa	Cuttings	3
Vasconcellea pulchra	Cuttings	3
Horovitzia cnidoscoloides	Cuttings, seeds, pollen	0

Table 2. Papaya inbred and hybrid genotypes used as parents in cross-pollinations with wild relatives

Big Island x Sekaki	Big Island x Laie Gold	Sekaki x Kapoho Line 8
Sekaki x Kapoho	Sekaki x SunSet	(Line 34 x Sekaki) x Laie Gold
SunUp x Rainbow	Big Island x SunSet	Sekaki
Big Island	Kapoho	Kapoho Line 8
Line 58	Line 34	Laie Gold F_2
SunUp	Sunrise	Sekaki x Laie Gold

3.2.2 Crossing procedure

Papaya was typically used as the female parent owing to a lack of flowering female *Vasconcellea* plants. In some instances, however, female *Vasconcellea* flowers were readily available, and in these cases hermaphrodite papaya pollen was used both due to lack of male papaya flowers and in order to carry the desirable hermaphrodite gene into the interspecific progeny. Male *Vasconcellea* flowers were removed from their inflorescences after anthesis, and their five petals were removed to expose the anthers. Female papaya flowers were manually opened just prior to anthesis to ensure receptivity and pollinated immediately to avoid unintended cross-pollination. Multiple male or hermaphrodite flowers were used for pollinations in all instances where material was available, typically between 5-10 flowers. The flowers were gathered into bundles, and anthers were rubbed on the stigma of the papaya flower to encourage pollen shedding. Pollen-bearing flowers were left in contact with the stigmatic surface within the corolla of the female flower. After pollination, papaya flowers were covered with glassine paper bags (Brown Paper Goods, Waukegan, IL) and tied around the floral pedicel to prevent pollen contamination from other sources (Fig. 3). Ties with pre-attached metal rimmed paper tags (Avery-Dennison 14313, Brea, CA) were used for identification purposes. Pencil was used to mark the tags, as it is less prone to fading in strong light than ink.

Figure 3. Bagged and tagged pollinated female papaya flowers

3.2.3 Embryo rescue

Fruits were allowed to develop on the tree for a minimum of three months, but sometimes until maturity at five months. To prepare the fruits for embryo rescue, they were surface sterilized before being introduced into the sterilized flow hood. Fruits were immersed in 1.05% sodium hypochlorite, with the addition of several drops of liquid detergent as a surfactant, for a minimum of 30 minutes. The fruits were rinsed with 95% ethanol, flamed, and placed on a sterile petri dish within the laminar flow hood. Autoclaved forceps and scalpels were used to open the fruits.

Concerning sterility of the culture, it should be noted that papaya has been reported to harbor endophytic bacteria which sometimes proliferate in tissue culture. A bacterial contaminant, was recurrently encountered among rescued embryos and in vitro transfers for a period of time. This problem was eliminated by taking additional sterility precautions, including autoclaving tools for 40 minutes (at 120°C and 20 psi) before every use, and bleaching and changing the alcohol in the tool holder. Autoclaving for 20 minutes was unsuccessful at eliminating the contaminant.

Ovules were opened in a sterile dish with the aid of a dissecting microscope, and any embryonic material was removed and placed in tissue culture. Media were contained in Magenta boxes which had been vented by drilling holes in the lid and covering with Micropore tape (3M Health Care, St.Paul, MN). Three types of media were used, depending on the degree of development of the embryo obtained (Table 3). All formulations were based on Murashige & Skoog medium with organics (Murashige & Skoog, 1962).

The standard embryo rescue medium contained activated charcoal. This was used as the general, all-purpose media. Activated charcoal is used in tissue culture to adsorb secondary metabolites, which are secreted by the plant and which could hinder growth if an excess build-up were to form, as well as to shade the roots (Pan and van Staden, 1998). The second type contained coconut water, which contains a natural mixture of hormones, including cytokinins, which are used to promote embryo development. The third type contained the synthetic auxin 2,4-D, which is used to induce somatic embryogenesis, and has been used in papaya tissue culture for that purpose at 5 ppm (Fitch, 1993). Each type could be used for a different potential

embryonic material: the charcoal media for normal, well developed embryos, to promote normal germination; the coconut water media for less mature embryos, which may benefit from further development; the 2,4-D media for poorly formed embryonic masses, which may produce somatic embryos rather than be directly germinated.

Table 3. Tissue culture media ingredients

Media type	**Sucrose**	**Gelrite**	**MS Strength**	**Additives**
Standard	30 g/L	2.8 g/L	1.0 x	Charcoal, 10 g/L
Coconut	60 g/L	2.8 g/L	0.5 x	Coconut water, 100 ml/L
2,4-D	60 g/L	2.8 g/L	0.5 x	2,4-D, 5 ppm; Glutamine, 400 mg/L

Embryos were kept in the dark until they had grown upright and begun to open their cotyledons, at which time they were placed on shelves under four 40-watt fluorescent bulbs. When they appeared to have developed sufficient root mass and leaf area to sustain themselves outside of culture, they were lifted from their Magenta boxes, placed in dishes of water, and the media was scraped away using forceps and a spatula. Plants were then potted in sterilized vermiculite which had been watered with full strength MS solution, enclosed in clear plastic bags, and placed in indirect natural light on a south facing windowsill (Fig. 4). After several days, the bags were cut open to allow for a gradual decrease in humidity and an adjustment to normal conditions, with the cuts being further widened every 2-3 days, until no moisture condensation was visible inside the bag.

Figure 4. *C. papaya* x *V. goudotiana* being acclimated from culture

Plants remained indoors and in vermiculite until they had begun to develop new growth, and typically until root growth could be observed at the bottom of the container.

At this point, they were transferred to Sunshine Mix #4 (Sun Gro Horticulture, Agawam, MA) and vermiculite and fertilized as needed with soluble fertilizer (Miracle-Gro® Water Soluble All Purpose Plant Food, 24-8-16) (Scotts, Marysville, OH) or slow release fertilizer (Osmocote, 14-14-14) (Scotts, Marysville, OH).

3.3 Results

3.3.1 Intergeneric *Carica* x *Vasconcellea* hybridization

No intergeneric crosses produced endosperm within the ovules. Fruit size and ovule development were not necessarily correlated with embryo development, as some crosses produced an abundance of ovules which were lacking embryonic material. Ovules that did develop, particularly in papaya, tended to be located primarily toward the stigma end of the fruit (Fig. 5). Particularly on the crosses made onto *Vasconcellea*, fruit abortion was common. Embryos displayed a wide range of variation, with some crosses yielding polyembryonic masses, others yielding reasonably well formed single embryos of differing stages of development, and still others producing small, solid white masses of embryonic material.

Figure 5. Papaya fruit showing typical ovule placement

C. papaya x *V. monoica*

The female papaya parent was a vigorously growing feral tree. Crosses produced well developed embryos which were commonly polyembryonic. Despite the relative ease of making the hybrid, the embryos which germinated in culture inevitably senesced shortly after the production of true leaves, typically appearing to melt from the apex, commonly producing somatic embryos from the roots (Fig. 6). This happened in almost all instances, regardless of how healthy plants appeared when younger.

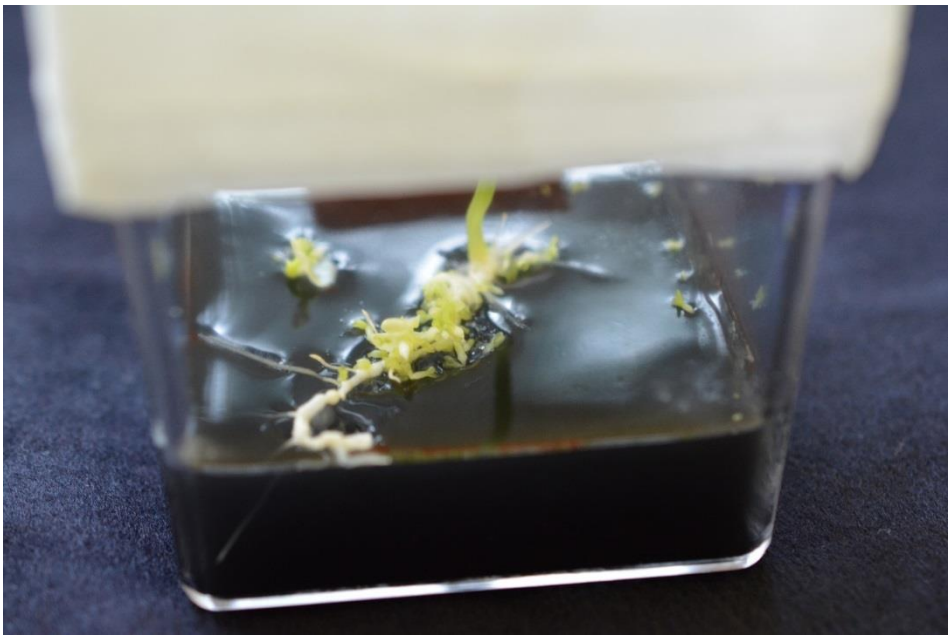


Figure 6. *C. papaya* x *V. monoica* hybrid displaying somatic embryogenesis from the roots

C. papaya x *V. parviflora*

Despite the production of embryos, which tended to be small and immature, and which could be either solitary or polyembryonic, most of the retrieved material failed to produce anything similar to a well-developed plant, even in cases where more developed embryos were produced. Although large masses of callus were produced by the embryonic materials (Fig. 7), all of it failed to develop into organized tissues. A small number of embryos germinated, but none produced any leaves beyond the cotyledons. One exception was able to produce a single, small, green leaf; however, it undertook no further development, and later senesced. Six crosses were made onto

papaya, and 94 were made onto *V. parviflora* using hermaphrodite papaya pollen. While the former cross was able to generate embryos, the reciprocal yielded no embryonic material.

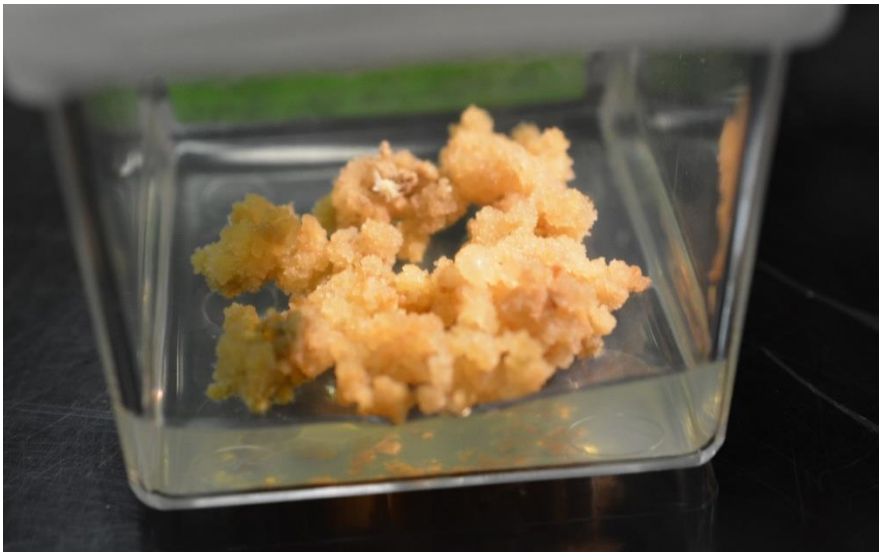


Figure 7. *C. papaya* x *V. parviflora*, typical callus formation

C. papaya x *V. goudotiana*

Plants were generated by removing embryos, which were well formed and often polyembryonic, from generally well formed ovules. Media transfers were dependent on the growth and progress of individual plantlets, however, the general process was to place the embryos on coconut water for 5 months, during which growth was commonly disorganized and resulted in the production of a small amount of somatic embryos. Well developed, germinated embryos, when placed on charcoal media, quickly developed, and within 3-4 months were ready to be transferred into light. Although there were some instances of root rot upon being taken out of culture, most plants continued to display hearty growth.

Reciprocal crosses were also made, using pollen from ‘Kapoho’ hermaphrodite papaya flowers, with two plants resulting. In contrast to the crosses using papaya as the female parent, the fruit of the *V. goudotiana* maternal parent for this cross was opened only 25 days following pollination, following the early abortion of the fruit. These crosses were grown via ovule culture, without the removal of embryos, exclusively on charcoal media. Germination was slow, taking 6 months before change was observed,

and growth in culture was also slow, taking about a year before it was removed from culture.

C. papaya x *V. pubescens*

Of 59 attempted cross-pollinations, in one fruit small, white, globular stage embryos were found within well-developed ovules. The embryonic material was placed on 2,4-D media, upon which the embryos gave rise to multiple plants, callus, and somatic embryos. After two months, material which had developed into organized plants were placed on the charcoal media. Four months later they were transferred into the light (Fig. 8). Plants placed in light tended to quickly develop strong root and shoot growth, so that after one additional month, they could be taken out of sterile culture and adapted well to autonomous survival.

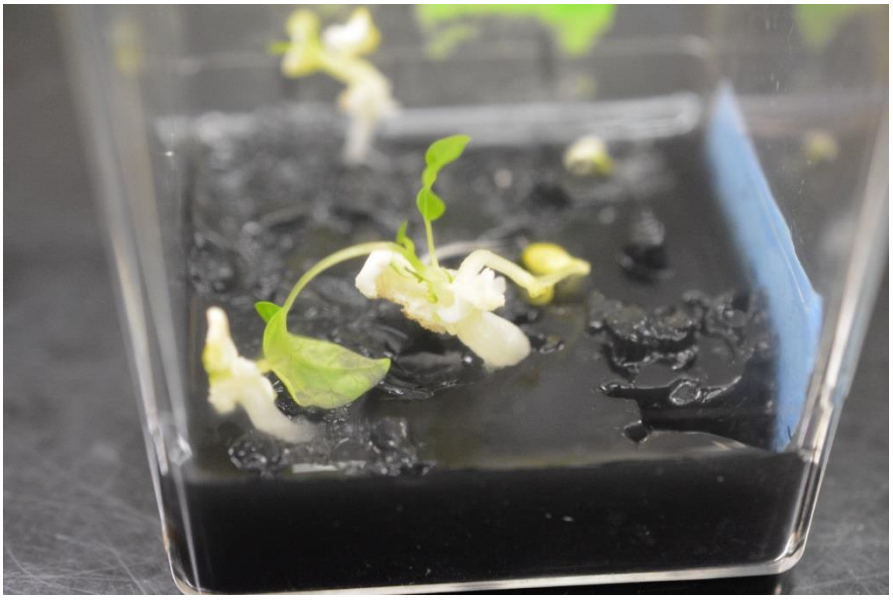


Figure 8. *C. papaya* x *V. pubescens*. This plant survived to grow vigorously out of culture.

C. papaya x *V. stipulata*

Crosses produced generally well formed embryos, which were able to germinate into well-formed plants when placed on charcoal media. Germination was rapid, and plants were transferred to the light in two months, and into vermiculite in another two months.

C. papaya x *V. quercifolia*

There were two successful crosses out of 71 attempts. These were well formed embryos, which germinated in charcoal media. The first of which displayed callus production and growth abnormalities in early development, but later growth appeared normal. The second germinated rapidly, within two weeks, and upon being placed in a lighted environment, developed normally. These are currently still in culture.

C. papaya x *V. x heilbornii*

Embryonic material, which was not distinguishable from ovule wall at the time of removal, was placed on charcoal media for five months, after which developing plantlets were transferred to the light, where they remained for another month before being moved out of sterile culture.

Minor crosses

Crosses were made using *V. glandulosa* as the female parent with hermaphrodite papaya pollen. This cross was only made onto the Vasconcellea parent, as no male of the species was present. Of 99 crosses, none yielded embryonic material or hybrid plants (Table 4). Two crosses were made using *V. pulchra* as a male parent onto papaya; both failed. Eight crosses were made using *Horovitzia cnidoscoloides* pollen, but these also failed completely (Table 4).

Table 4. Intergeneric crosses and successes*

Female parent	Pollen parent	Crosses	Successes	Success rate
C. papaya	*V. goudotiana*	8	4	50%
C. papaya	*V. monoica*	7	2	22%
C. papaya	*V. parviflora*	6	0	0%
C. papaya	*V. pubescens*	59	1	2%
C. papaya	*V. pulchra*	2	0	0%
C. papaya	*V. quercifolia*	71	2	3%
C. papaya	*V. stipulata*	33	2	6%
C. papaya	*V. x heilbornii*	17	1	6%
C. papaya	H. cnidoscoloides	8	0	0%
V. glandulosa	*C. papaya*	99	0	0%
V. goudotiana	*C. papaya*	10	1	14%
V. monoica	*C. papaya*	4	0	0%
V. parviflora	*C. papaya*	94	0	0%
V. quercifolia	*C. papaya*	16	0	0%

*Successes defined by number of unique hybrid yielding fruits

3.3.2. Interspecific *Vasconcellea* hybridization

V. parviflora x *V. pubescens*

Ovules were well developed, however, did not appear to have embryos, only large amounts of what seemed to be endosperm. This was placed on charcoal and coconut water media. Two months later, developing plantlets were present on the coconut water media. These were transferred to charcoal media, before being placed in the light, as development progressed, and ultimately into vermiculite.

V. glandulosa x *V. pulchra*

Ovules contained fully formed embryos with endosperm. Several excised embryos rapidly germinated in culture in charcoal media. One which was seeded in vermiculite also germinated under greenhouse conditions, however much more slowly, possibly owing to the dormancy period common in the wild species.

V. monoica x *V. stipulata*

Ovules of this cross contained endosperm and well developed embryos, such that they were very similar to non-hybrid seed, but the ovules were smaller. Embryos germinated readily on charcoal media, within two weeks.

V. monoica x *V. parviflora*

Ovules contained polyembryonic clusters, which were able to germinate on charcoal media.

V. parviflora x *V. monoica*

Ovules contained undifferentiated tissue, which was grown on 2,4-D media, and later transferred to charcoal media to germinate.

V. parviflora x *V. goudotiana*

Development of ovules appeared normal, with endosperm, and these were placed in charcoal media. Germination was slow, taking 4 months.

V. quercifolia x *V. parviflora*

These hybrids were produced in 2012-2013 by previous researchers. Ovules were plated and subcultured on charcoal medium. Plantlets were ready for transfer to potting mix in about 6 months.

V. quercifolia x *V. parviflora* F2

This cross was made by sibbing male and female *V. quercifolia* x *V. parviflora* F1 plants. Placed on the charcoal media, this hybrid took seven months to develop in darkness.

3.4 Discussion

Crosses were made using a variety of materials within the Caricaceae, focusing predominantly on intergeneric crosses between *Carica* and *Vasconcellea*. Successful crosses yielded embryonic material which was able to generate an autotrophic plant. Due to the partial incompatibility of some of the interspecific crosses, and all of the intergeneric crosses, embryo rescue was employed in order to generate hybrids. Ultimately, a variety of successful interspecific crosses, and five viable types of intergeneric hybrids were produced. The intergeneric hybrids include: *C. papaya* x *V. pubescens*, *C. papaya* x *V. goudotiana*, *C. papaya* x *V. x heilbornii*, *C. papaya* x *V. stipulata*, and *C. papaya* x *V. quercifolia*. Of these, *C. papaya* x *V. x heilbornii* has not been reported previously in the literature. Two additional interspecific crosses, *V. goudotiana* x *C. papaya* and *C. papaya* x *V. monoica*, were established outside of culture; however, these lacked vigor and subsequently died.

Crosses displayed varying degrees of cross compatibility. In the interspecific crosses, *V. glandulosa* x *V. pulchra*, *V. monoica* x *V. stipulata*, and *V. parviflora* x *V. goudotiana*, fruit and ovule development appeared normal, and largely indistinguishable from intraspecific crossing, with embryo and endosperm development being relatively normal. In others, compatibility was lower, as was the case of *V. parviflora* x *V. pubescens*, where the fruit appeared normal but embryo development was not, and embryo growth was slow and sporadic.

In intergeneric crosses, while all *Carica* x *Vasconcellea* attempts displayed a lower degree of cross compatibility, there was still variation. The ability of crosses to produce well developed embryos did not necessarily ensure the success of the cross. Hybrids between *C. papaya* and *V. parviflora* and *V. monoica* were able to produce torpedo stage embryos, but, these crosses were generally not successful in producing viable hybrid plants. In the case of the *C. papaya* x *V. monoica* material, although embryos were able to proliferate and germinate in culture, they ultimately had the habit of dying back, occasionally showing further production of embryos afterwards along the roots. Development rarely occurred beyond the cotyledons, and only two plants capable of being removed from culture were produced. In *C. papaya* x *V. parviflora*, almost no organized tissues, were produced at all, only callus. The few examples of organized tissue which were produced inevitably died. This is in stark contrast to other reports of *C. papaya* x *V. parviflora*, which has produced well-formed flowering plants (O'Brien, 2009) and has been described as being sufficiently fertile to be used as a bridging species for the introgression of traits from other *Vasconcellea* into papaya (O'Brien and Drew, 2009).

Conversely, *C. papaya* x *V. pubescens* produced only globular stage embryos, and many of the fruits of this cross aborted before substantial fruit development, yet these hybrids proved to be vigorous and healthy. Additionally, fruit development also did not necessarily correlate with the development of embryonic material; papaya in particular was commonly able to produce mature, ripened fruits without any development of ovules.

CHAPTER 4: CONFIRMATION OF HYBRIDS AND DESCRIPTION OF MORPHOLOGY AND FERTILITY

4.1 Introduction

Although morphological differences could oftentimes be readily observed in the hybrids, such as the pink flower color of *V. parviflora* or the pubescence of *V. pubescens*, hybrid phenotypes are not always predictable from parent characteristics or diagnostic of hybrid status. Reproductive sterility may be used to support morphological identification of hybrids. Several potential sources of error, such as accidental cross pollination, mislabeling, or potential apomictic seed development could lead to obfuscation of putative hybrids, thus necessitating more objective measures of parentage. Isozyme markers were used to acquire objective proof of the hybrid origin of reported crosses.

Isozymes are enzymes which show molecular variation across, and even within, taxa. This variation allows taxa to be distinguished as separate, or in this case, offering molecular confirmation of the parentage of hybrids. By extracting proteins and separating isozymes by gel electrophoresis, the true origin of a putative hybrid can be confirmed in isozyme banding phenotypes containing species-specific alleles of both parents. This method was used to acquire objective proof of the hybrid origin of reported crosses.

4.2 Materials and Methods

4.2.1 Greenhouse acclimation of hybrids

Plants were acclimated to the greenhouse by first placing them under a dual layer of shadecloth (2x 50% reflective shade, or 25% ambient light) for a period of a week, and then moving them under a single layer. The greenhouse was located at the University of Hawai‘i Magoon Horticulture Facility in Mānoa, O‘ahu.

4.2.2 Isozyme testing of hybrids

The histidine-citrate buffer (700 ml) for the electrophoresis gel and trays was prepared containing 65 mM L-histidine and 7 mM citric acid and adjusted to pH 6.5. A potato starch gel (13.3% wt:vol) was prepared by mixing 53.2 g potato starch and 400 ml of a 1:3 of dilution of the electrophoresis buffer and water (100 ml buffer stock and 300 ml water). This was brought to a boil, while being mixed well using a mechanical stirrer, and air bubbles were removed in a vacuum flask. This was then poured into the gel mold and left overnight to cool in a refrigerator at 4 C°.

Isozyme extraction buffer was prepared according to Aradhya et al. 1998. This slurry was mixed well. A 30-well Plexiglas grinding block was prepared for the extraction by pre-cooling it in the -20 C freezer and loading each well with 0.5 ml of extraction mixture and two, 6-mm dia. leaf discs, punched out with a hole punch. The grinding block was kept on ice, while the discs were ground with a glass rod until the contents of the well appeared green throughout and the leaf material was completely pulverized. Wicks of paper (Whatman Grade 3MM Chr Chromatography Paper) were cut into thin rectangles and inserted in the wells for 10 minutes to absorb the mixture. Two wicks were used per well.

Wicks were inserted into the gel by making a cut with a scalpel along the long side of the gel, and wicks were inserted into the gel using forceps, making sure that the wicks reached the bottom of the gel. Gels were run for 5 hours at 225 volts and 65 milliamps, keep cool in a refrigerator, wrapped in plastic wrap with a plastic box of ice on top. After 10 minutes, the current was momentarily turned off and the wicks were removed to allow for better current flow.

After stopping the current, the gel was removed and cut into sections. Placed face up on a cutting tray, nylon thread was run along removable plastic slats, such that the gel was cut into four thin horizontal slices. The top section was discarded. The remaining sections were placed in trays containing substrates appropriate to the enzyme system to be tested (Table 5). Components common to all systems were 20 ml 0.2M Tris-HCl, pH 8.0), 20 ml deionized water, 1 ml MTT (a tetrazolium dye), 1 ml phenazine methosulfate (PMS), and 1 ml nicotinamide adenine dinucleotide (NAD). After 90 minutes incubation at 37°C, gels were removed, washed lightly with DI water,

and a placed in a fixative solution of 10 parts methanol to 10 parts DI water to 1 part glacial acetic acid.

Table 5. Isoyzyme system specific ingredients

System	System specific ingredients
MDH	2.5 ml malate solution
PGM	1.0 ml $MgCl_2$, 1 ml G6PDH, 40 mg Glucose-1-phosphate
PGI	1.0 ml $MgCl_2$, 1 ml G6PDH, 20 mg Fructose-6-phosphate
IDH	1.0 ml $MgCl_2$, 40 mg Isocitric acid, Na_3 salt, 1.0 ml NADP (replaces NAD)

4.2.3 Pollen testing of hybrids

Only four hybrids produced male flowers: *C. papaya* x *V. goudotiana*, *V. goudotiana* x *C. papaya*, *V. quercifolia* x *V. parviflora*, and *V. quercifolia* x *V. parviflora* F_2. An estimate of the fertility of the hybrid pollen can be made by staining with acetocarmine and observing the amount of symmetrical, deeply red-staining pollen grains compared to the amount of small, irregularly shaped and colorless pollen grains produced. This was done by staining pollen with acetocarmine. Anthers were removed from two flowers and placed on a glass slide. Two drops of acetocarmine stain in 45% acetic acid were dripped on the anthers, and the anthers were repeatedly mashed into the dye for several seconds before being removed and covered with a glass cover slip. Nail polish (Sally Hansen Hard as Nails) was used to seal the coverslip. An Olympus BX-51 compound microscope with Optronics MacroFire camera (www.pbrc.hawaii.edu/bemf/) was used to record images of the slides at 200x or 400x magnification, and ImageJ software (https://imagej.nih.gov/ij/) was used to analyze the images. Pollen grains which appeared normal in size and shape and which were able to take up the dye, were considered to be viable pollen grains, while those which were abnormal and could not uptake the dye were considered to be inviable.

4.3 Results

4.3.1 Characterization of intergeneric *Carica* x *Vasconcellea* hybrids

C. papaya x *V. monoica*

Although an abundance of material proliferated in vitro, only two hybrid plants were able to be established outside culture. One developed three-lobed leaves (Fig. 9), while the other developed five-lobed leaves. Both displayed a marked lack of vigor, and subsequently died while still less than 10 cm tall. This hybrid has been reported previously (Malaguti, 1957), but not characterized, perhaps due to its difficulty surviving.

Figure 9. C. papaya x V. monoica

C. papaya x *V. goudotiana*

This hybrid was generally strong growing and vigorous. Leaf shape appeared intermediate to the parents, similar to papaya, but somewhat more deeply lobed, like *V. goudotiana* (Fig. 10). Petioles commonly displayed red pigmentation, which was also present on the trunks (Fig. 11). Cuttings of this hybrid were generally able to root well. Flowering occurred earlier than either parent, sometimes when the plants were less than 60 cm tall and only four months old, but these precocious flowers inevitably aborted. Flowers were a cream color (Fig. 12-13).

Sex expression in the hybrids did not conform to a truly dioecious system, such as would be expected in the instance of a heterozygous male parent pollinating a homozygous recessive female. While the female flowers were normal females (Fig. 14), the “male” plants produced almost all hermaphrodite flowers. The inflorescences were mostly normal in the male (i.e., long and pendulous), although possessing more bracts at the nodes of the inflorescence than either parent. Most flowers were only modestly hermaphroditic, with a small ovary and stigma at the base of the flower below the anthers. However, terminal flowers tended toward being more hermaphroditic, with anthers subtending the stigma, and much larger ovaries (Fig. 15-16). These flowers

also were able to produce fruit when pollinated, unlike the more male-like lateral hermaphrodite flowers. Female flowers had morphology similar to the *Vasconcellea* parent, with petals open at the base.

Fruits almost always aborted prior to ripening; ripened fruit of the female was both insipid and lacking sweetness. As these hybrid plants were pot grown, environmental factors may have played some role in this; nonetheless the complete lack of any eating quality to speak of indicates no immediate horticultural utility. Fruits were yellow skinned and yellow fleshed, with closed locules similar to the *Vasconcellea* parent (Fig. 17-20).

Reciprocal crosses were much less vigorous than hybrids made onto female papayas. Leaf morphology was similar, although appeared less deeply cut. One was moved to the field, where it suffered rot towards the base and died. The other remained in greenhouse conditions, where is lost all leaves and died. Although it began flowering before being transferred to the field, only the field-planted hybrid flowered; it possessed hermaphrodite flowers. PCR testing confirmed this plant inherited a Sequence Characterized Amplified Region (SCAR) DNA marker that has been shown to be tightly linked to a locus in the papaya genome determining hermaphrodite sex expression (Deputy et al, 2002; Qingyi Yu - personal communication). The flowers were born close to the plant on compact inflorescences, and appeared similar to the non-terminal flowers of the male plant described previously (Fig. 21). One reason for the lack of vigor in these plants may be that there exist cytoplasmic factors incompatible with the *C. papaya* nuclear genome. An alternative possibility might be that the presence of the papaya hermaphrodite gene may have deleterious effects in intergeneric hybrids.

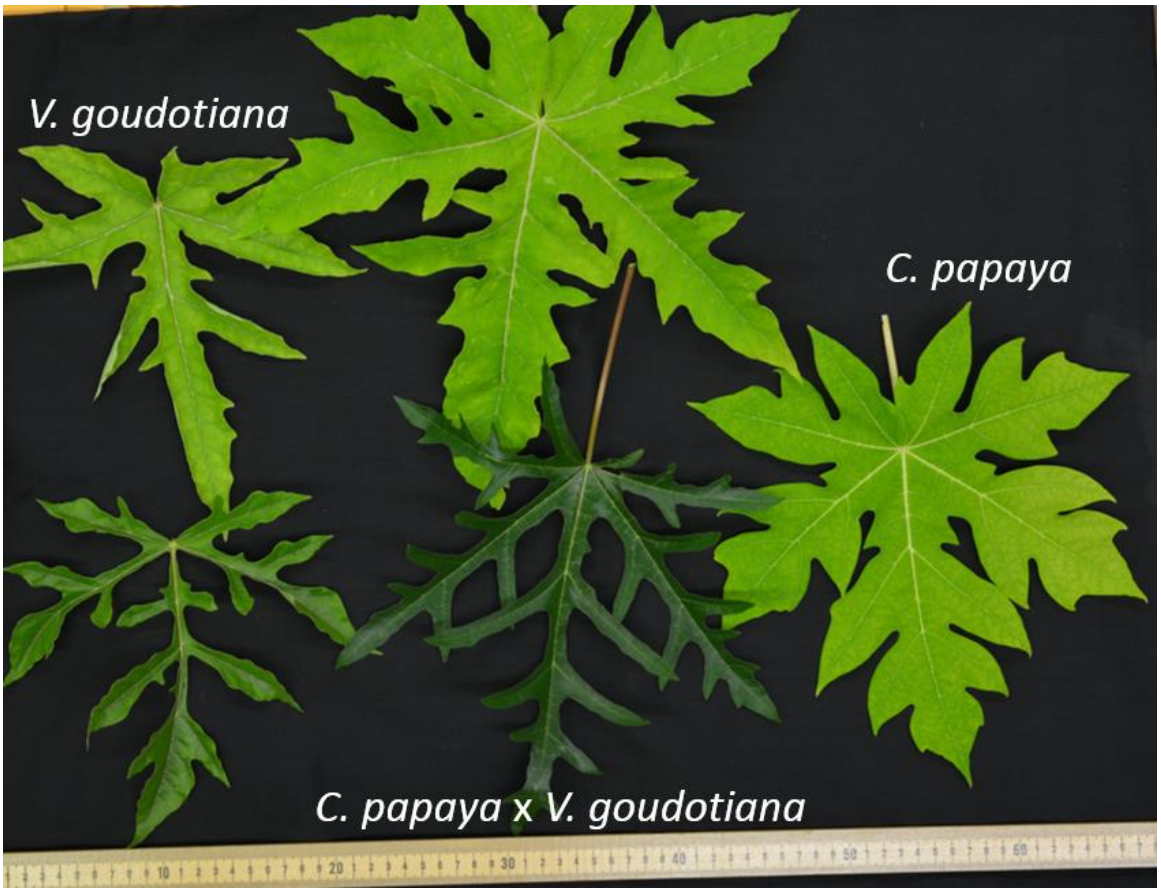


Figure 10. Leaves of parent species and hybrids. *V. goudotiana* (left two), *C. papaya* x *V. goudotiana* (center two), *C. papaya* (right)

Figure 11. *C. papaya* x *V. goudotiana* displaying lacy foliage

Figure 12. From left to right, *V. goudotiana* male flower, *C. papaya* x *V. goudotiana* male-like hermaphrodite flower, *C. papaya* hermaphrodite flower

Figure 13. From left to right, *V. goudotiana* male flower, *C. papaya* x *V. goudotiana* male-like hermaphrodite flower, *C. papaya* hermaphrodite flower

Figure 14. *C. papaya* x *V. goudotiana* female flower

Figure 15. Terminal flower of *C. papaya* x *V. goudotiana* male-like hermaphrodite inflorescence, displaying characteristic hermaphrodite flower

Figure 16. *C. papaya* x *V. goudotiana* male-like hermaphrodite terminal flower cut open

Figure 17. Fruit of *C. papaya* x *V. goudotiana* male-like hermaphrodite

Figure 18. Cross section of ripe fruit of *C. papaya* x *V. goudotiana* male-like hermaphrodite

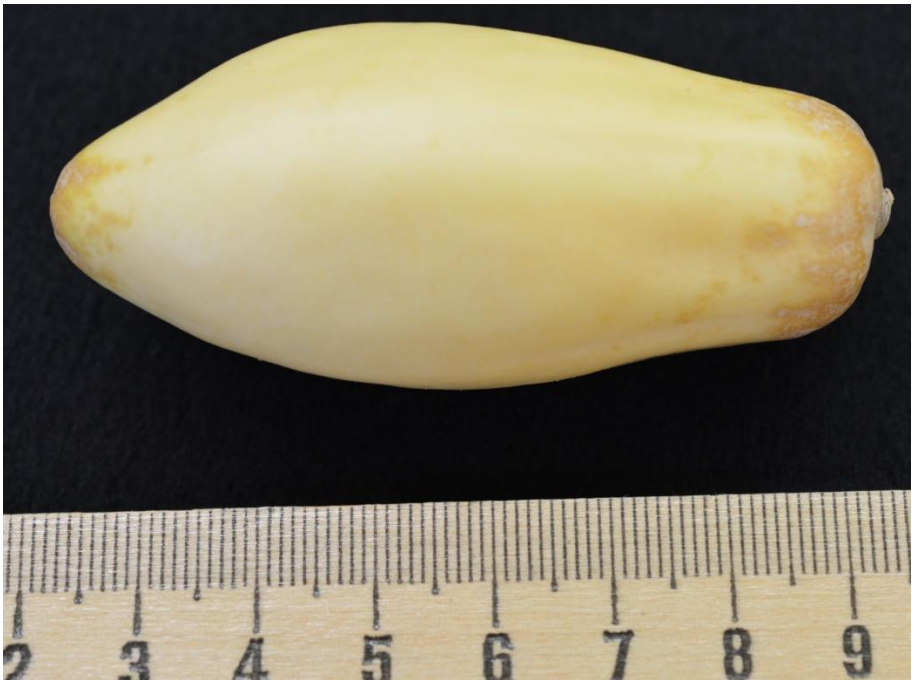


Figure 19. Ripe fruit of *C. papaya* x *V. goudotiana* female

Figure 20. Fruit of *C. papaya* x *V. goudotiana* female, halved

Figure 21. *V. goudotiana* x *C. papaya* hermaphrodite inflorescence

C. papaya x *V. pubescens*

Upon being planted out, these hybrids continued to displayed vigorous growth. Leaf morphology appeared intermediate between the species, with light colored venation similar to that of *V. pubescens* (Fig. 22-23). The leaves, although not the stem, possessed small amounts of the pubescence characteristic of *V. pubescens*.

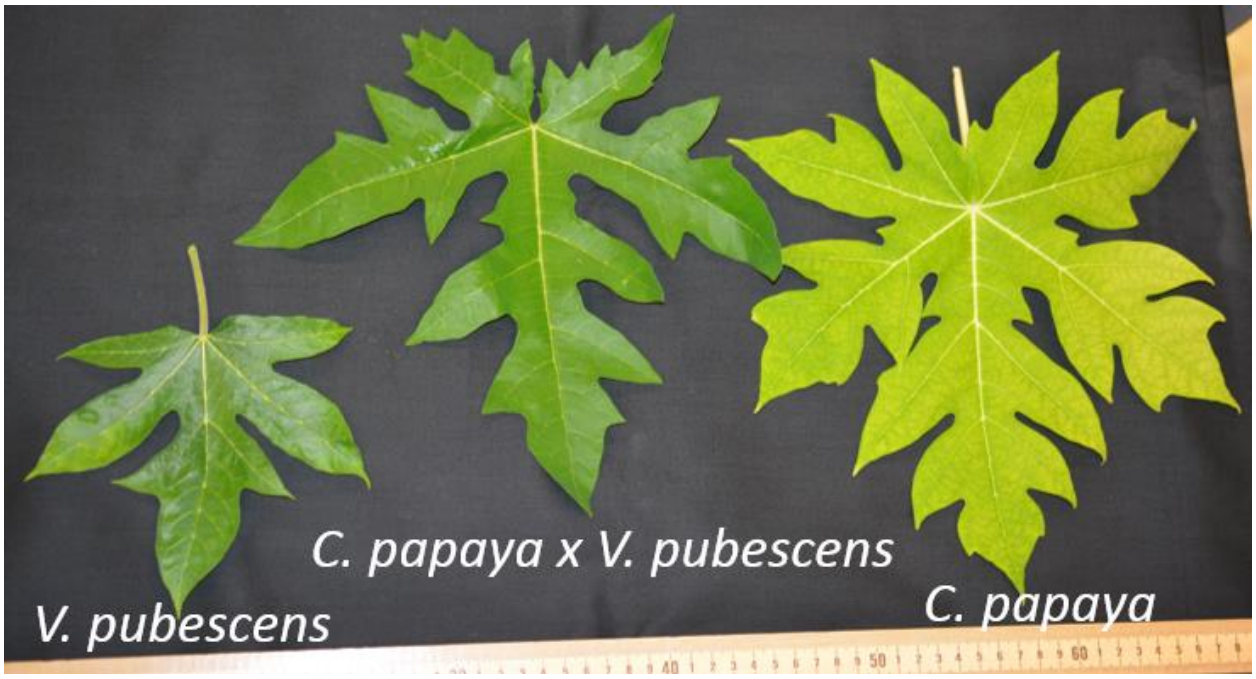


Figure 22. Leaves of parent species and hybrid. *V. pubescens* (left), *C. papaya* x *V. pubescens* (center), *C. papaya* (right)

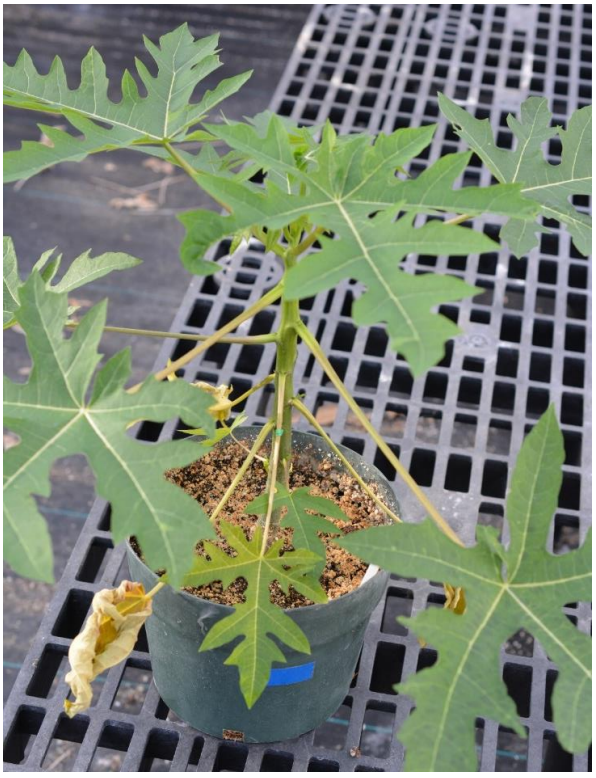


Figure 23. *C. papaya* x *V. pubescens*

C. papaya x *V. stipulata*

Plants displayed generally poor vigor upon removal from culture. Leaves had five lobes (Fig. 24). The eponymous stipules of the male parent could not be observed in the hybrid progeny. Plants tended to senesce from the apex and produce lateral shoots, which also lacked vigor.

Figure 24. *C. papaya* x *V. stipulata*

C. papaya x *V. x heilbornii*

Plants displayed good vigor. Leaf morphology was more heavily serrated along the margins than papaya leaves, a trait of the male parent (Fig. 25).

Figure 25. *C. papaya* x *V. x heilbornii*

4.3.2 Characterization of interspecific *Vasconcellea* hybrids

V. parviflora x *V. pubescens*

Leaves appear similar to *V. pubescens*, however possessing less pubescence, and less deeply lobed. The relative thickness of the roots and base of the plant seems to indicate the plant has the potential for developing a pachycaul trunk, like the *V. parviflora* parent. Additionally, the plant appears to display the heat intolerance of the *V. pubescens* species, since it lost all leaves when transferred to a greenhouse in the summer months, but rapidly recovered when brought back to a cooler indoor environment.

V. glandulosa x *V. pulchra*

Four plants were produced. Leaf morphology did not display intermediate characteristics, but rather, took the form of one parent or the other. One flowered, a female, possessing flowers which were not distinguishable from the female parent.

V. monoica x *V. stipulata*

Healthy, vigorous plant with five lobed leaves, like the *V. stipulata* parent, and small stipules.

V. monoica x *V. parviflora*

Leaves appeared as an intermediate between the two parents. A single plant flowered, a male, which had pink, *V. parviflora*-like flowers.

V. parviflora x *V. monoica*

This cross displays good health, but is not yet producing mature leaves.

V. parviflora x *V. goudotiana*

This cross displays good health, but is not yet producing mature leaves. However, it does appear to produce the red tinted petioles of *V. goudotiana*.

V. quercifolia x *V. parviflora*

Plants had large, arborescent growth similar to the *V. quercifolia* parent, while the flowers were a bright pink, similar to the parviflora parent. Leaves appeared as an intermediate of the two parents (Fig. 26). Male and female plants were present.

Figure 26. *V. quercifolia* x *V. parviflora* female (left) and male (right)

4.3.3 Fertility of hybrids

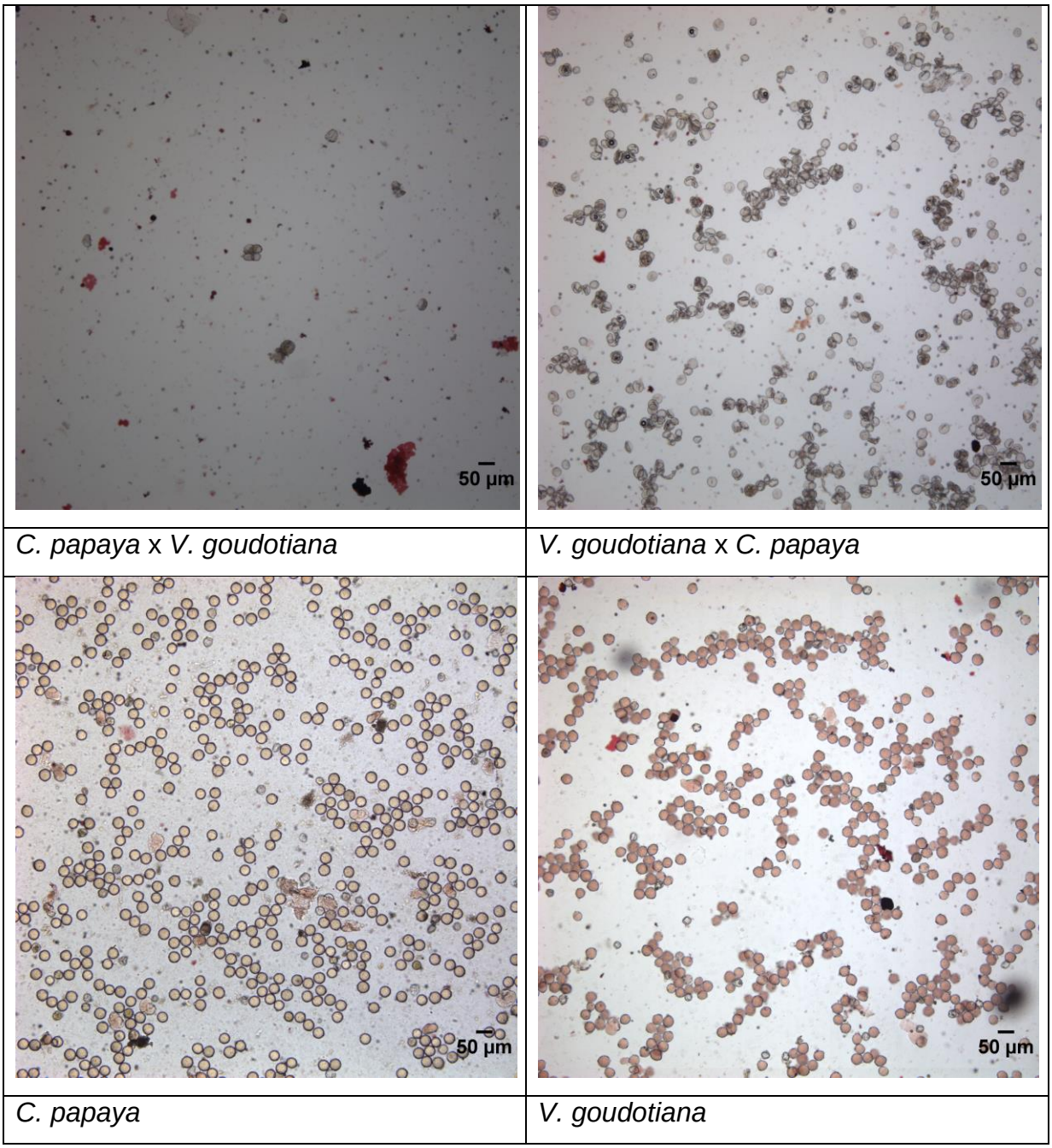
4.3.3.1 Intergeneric hybrid backcrosses

Using *C. papaya* x *V. goudotiana* F_1s and reciprocals as female parents, attempts were made to backcross the pollen of papaya or *V. goudotiana* onto the F_1 hybrids. In all cases, this failed (Table 6), and typically no ovule growth was present, but there were two exceptions. In one case, a single black ovule was found in a backcross on the male-like hermaphrodite plant; however, it was empty. In another, a large number of ovules were found in a backcross on the hybrid female plant. Placed in charcoal media, these ovules continued to develop into large, white ovules. However, upon dissection, no embryonic material could be found inside.

4.3.3.2 Intergeneric hybrid pollen stainability

In general, pollen quality of all intergeneric hybrids was poor. Hybrid plants showed reduced pollen production, and the pollen which was observed microscopically was frequently smaller, abnormal, and clearly inviable (Fig. 27). The male *C. papaya* x *V. goudotiana* plant did not appear to produce any pollen grains which developed

beyond the early stages of development. The reciprocal cross did produce distinct pollen grains, however, these were severely misshapen and did not stain.



C. papaya x *V. goudotiana*	*V. goudotiana* x *C. papaya*
C. papaya	*V. goudotiana*

Figure 27. Pollen stains of intergeneric hybrids and parents, 200x

Table 6. Intergeneric backcrossing attempts and successes

Female parent	Pollen parent	Crosses	Successes	Success rate
C. papaya x *V. goudotiana* F_1 ♀	*C. papaya*	13	0	0%
C. papaya x *V. goudotiana* F_1 ♀	*V. goudotiana*	1	0	0%
C. papaya x *V. goudotiana* F_1 ♂*	*C. papaya*	27	0	0%
C. papaya x *V. goudotiana* F_1 ♂*	*V. goudotiana*	3	0	0%

*Refers to the male-like hermaphrodite plant

4.3.3.3 Interspecific hybrid backcrosses and F_2 generations

Using *V. quercifolia* x *V. parviflora* F_1s, attempts were made in several ways to produce progeny. This cross had both flowering males and females, allowing crosses to be made with and onto F_1 plants. In both cases, neither was successful (Table 7). However, although there were fewer attempts due to the seeming unlikelihood of success, one sibling cross did produce a viable embryo (see chapter 3).

Vegetative growth of the *V. quercifolia* x *V. parviflora* F_2 was very similar to the F_1, and flowers were also a bright pink, although they seemed to possess some lighter streaking when closely observed. Flowers were able to visibly shed pollen, unlike the F_1 parents. One note of interest is that the flowers are potentially rich in papain, a protease found both *Carica* and *Vasconcellea* which is commonly used as a meat tenderizer. Contact with the sap of the flowers of this plant, unlike the flowers of the parents plant or pure species, resulted in irritation lasting several days. When crossed onto *V. parviflora*, ovules produced endosperm, and backcross plants resulted from embryo rescue (Fig. 28).

4.3.3.4 Interspecific hybrid pollen stainability

V. quercifolia x *V. parviflora* displayed greater fertility than the intergeneric hybrids, however, fertility was still poor, as demonstrated by both pollen staining and backcrossing attempts. The single resulting progeny, the *V. quercifolia* x *V. parviflora* F_2 plant, showed a partial recovery of fertility (Table 8, Fig. 29). In addition, it displayed visible pollen shedding, unlike the F_1 parents.

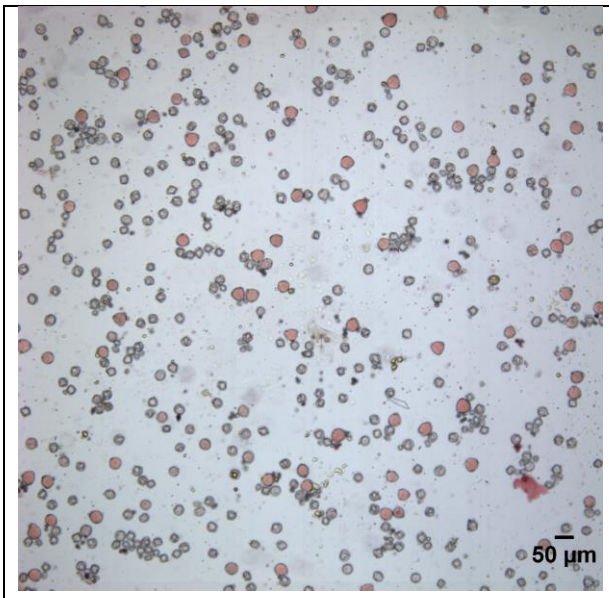
Figure 28. *V. quercifolia* x *V. parviflora* F_1 (left), F_2 (center), and backcross (right) plants

Table 7. Interspecific backcross and sib attempts and successes

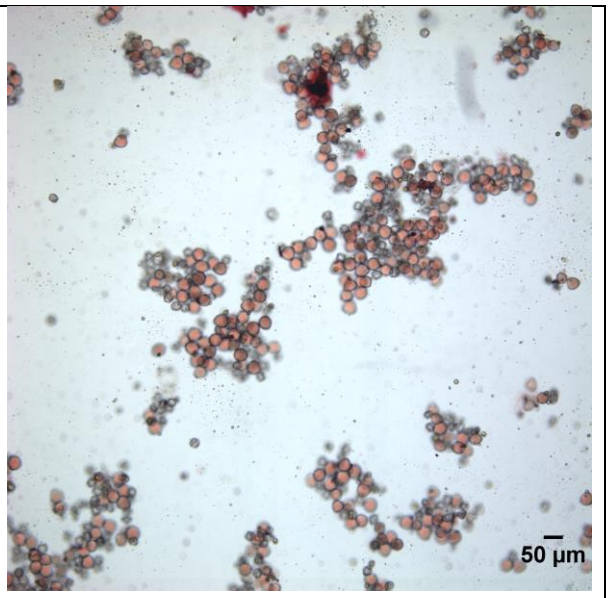
Female parent	Pollen parent	Crosses	Successes	Success rate
V. quercifolia x *V. parviflora* F1	*V. parviflora*	11	0	0%
V. parviflora	*V. quercifolia* x *V. parviflora* F1	81	0	0%
V. quercifolia x *V. parviflora* F1	*V. quercifolia* x *V. parviflora* F1	3	1	33%

Table 8. Interspecific hybrid stainable pollen

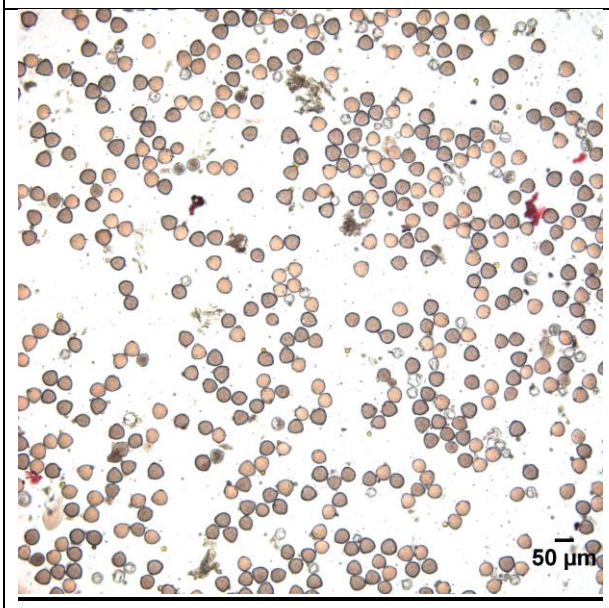
Species	Stainable pollen grains	Non-Stainable pollen grains	Percent stainable
F_1	157	1353	10.40%
F_2	1095	648	62.82%
V. parviflora	1109	147	88.30%
V. quercifolia	888	25	97.26%



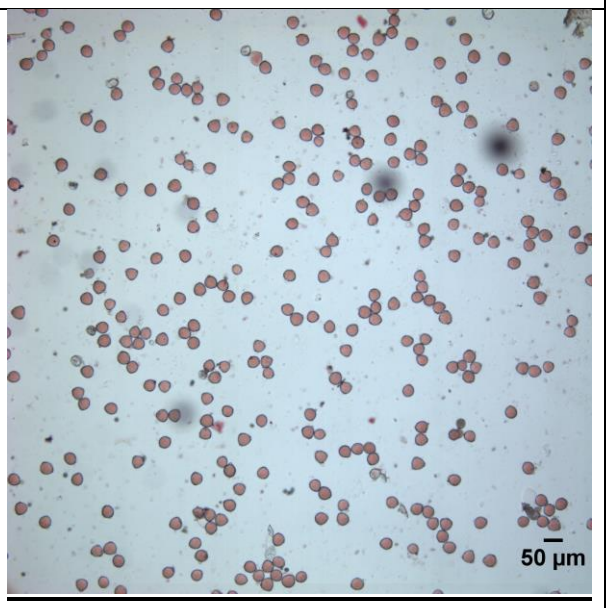
V. quercifolia x *V. parviflora* F_1 pollen



V. quercifolia x *V. parviflora* F_2 pollen



V. parviflora pollen



V. quercifolia pollen

Figure 29. Pollen stains of interspecific parents and hybrids, 200x

4.3.4 Hybrid confirmation by isozyme analysis:

Isozyme testing gives clear confirmation that hybrid plants are what they are claimed to be. Several isozyme systems were used. The best, most consistent results came from malate dehydrogenase, phosphoglucose isomerase, and phosphoglucomutase systems. The isocitrate dehydrogenase system did not produce distinctions.

Malate dehydrogenase (MDH) is a dimeric system, i.e., the enzyme is a dimer, produced by two subunits. This is useful for isozyme tests as it can result in bands indicating the presence of both homo-dimers and an intermediate hybrid hetero-dimer. In the case of the intergeneric *Carica* x *Vasconcellea* hybrids, MDH hetero-dimer bands appeared in all tested individuals, indicating clear hybrid origins. The *Vasconcellea* isozyme variant appeared nearly identical in *V. goudotiana*, *V. pubescens*, *V. stipulata*, and *V. x heilbornii* (Fig. 30).

In the interspecific hybrids, *V. quercifolia* and *V. parviflora* had differing MDH bands, which allowed for the *V. quercifolia* x *V. parviflora* plants to display a hetero-dimer band. The *V. quercifolia* x *V. parviflora* F_2 plant had MDH banding identical to that of the F_1, as did the *V. parviflora* x [*V. quercifolia* x *V. parviflora* F_2] backcross plant (Fig. 31).

Phosphoglucose isomerase (PGI) is also a dimeric system. In *C. papaya* and *V. pubescens*, tested plants were homozygous, with the *C. papaya* and *V. pubescens* alleles being indistinguishable, while in *V. goudotiana* and *V. stipulata*, tested plants were heterozygous. Consequently, the tested *C. papaya* x *V. pubescens* plants were unable to be confirmed as hybrids using this system. *C. papaya* x *V. goudotiana* and *C. papaya* x *V. stipulata* plants may have been able to be identified, however, tested hybrids appear to have received the same allele from both parents, making them also unverifiable with this system. In *C. papaya* x *V. x heilbornii*, however, the parents were homozygous for different PGI alleles, both of which appear in the putative F1, demonstrating a hybrid origin (Fig. 32).

Phosphoglucomutase (PGM) is a monomeric enzyme system. In tested intergeneric hybrids, bands from both species could be found. Tested *C. papaya* plants displayed different bands, indicating differing alleles for this enzyme. *C. papaya* x *V.*

goudotiana possessed two bands from each parent species. In *C. papaya* x *V. pubescens*, only three bands appeared, as one allele from *V. pubescens* was identical to one that appeared in *C. papaya*. In *C. papaya* x *V. x heilbornii*, four bands appear, two from each parent, however, not all papaya bands appear, indicating that the papaya parent used in that cross was heterozygous for that PGM. The *C. papaya* x *V. stipulata* hybrid displays one band from *V. stipulata* and two from *C. papaya*, and lacks two from *V. stipulata* and one from *C. papaya*, however, the plants used were not the parents of the hybrids. Due to this intraspecific diversity of alleles, this particular enzyme system does not confirm that the C. papaya x V. stipulata plants are hybrids (Fig. 30).

In the interspecific hybrids, the PGM system shows that the *V. quercifolia* x *V. parviflora* F_1 hybrids possess all three bands indicated in the parents. However, the *V. quercifolia* x *V. parviflora* F_2 and *V. parviflora* x [*V. quercifolia* x *V. parviflora* F_2] backcross display only the *V. parviflora* bands (Fig. 31)

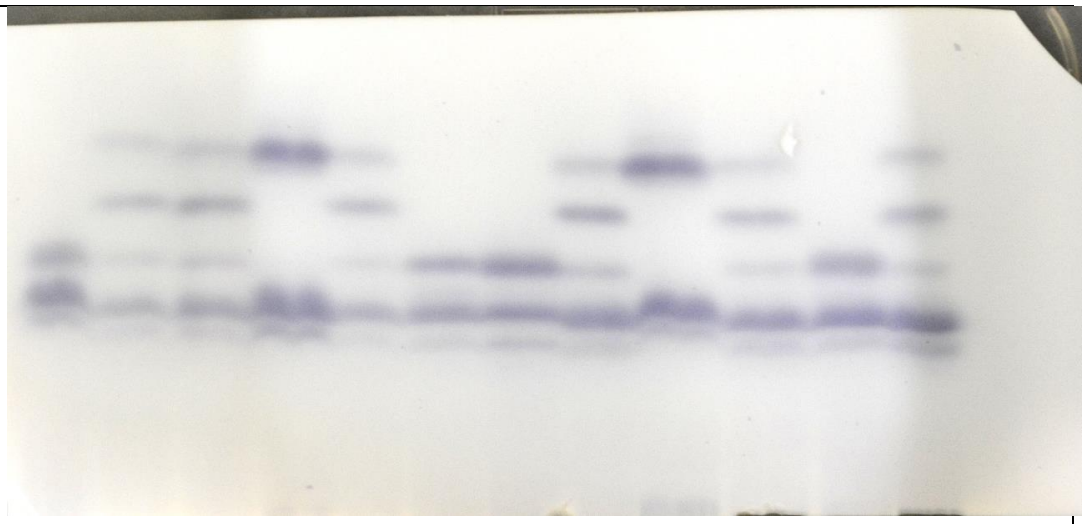
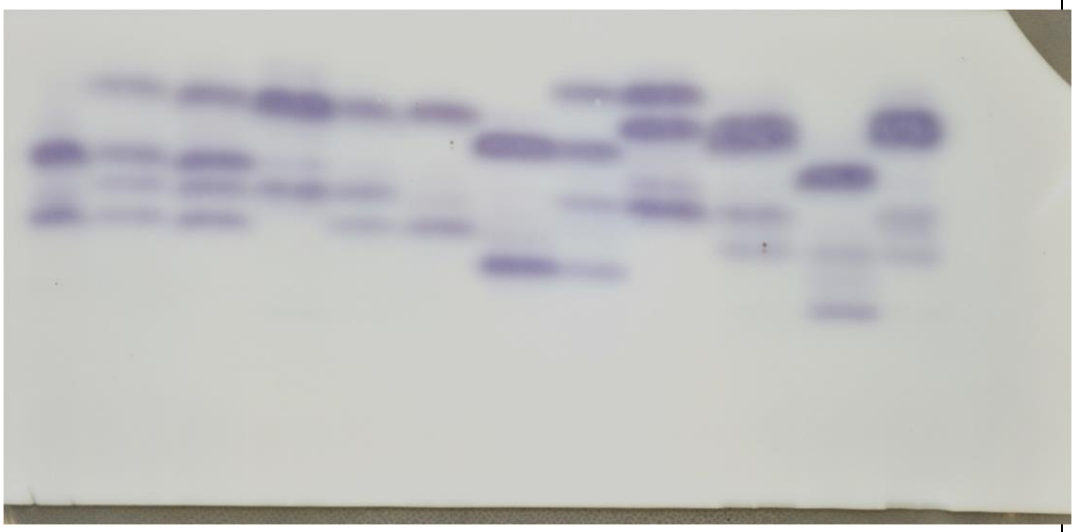
MDH	
PGM	
Lanes	1. *V. goudotiana* 2. *C. papaya* x *V. goudotiana* 3. *C. papaya* x *V. goudotiana* 4. *C. papaya* 5. *C. papaya* x *V. pubescens* 6. *V. pubescens* 7. *V. x heilbornii* 8. *C. papaya* x *V. x heilbornii* 9. *C. papaya* 10. *C. papaya* x *V. stipulata* 11. *V. stipulata* 12. *C. papaya* x *V. stipulata*

Figure 30. Intergeneric isozyme gels

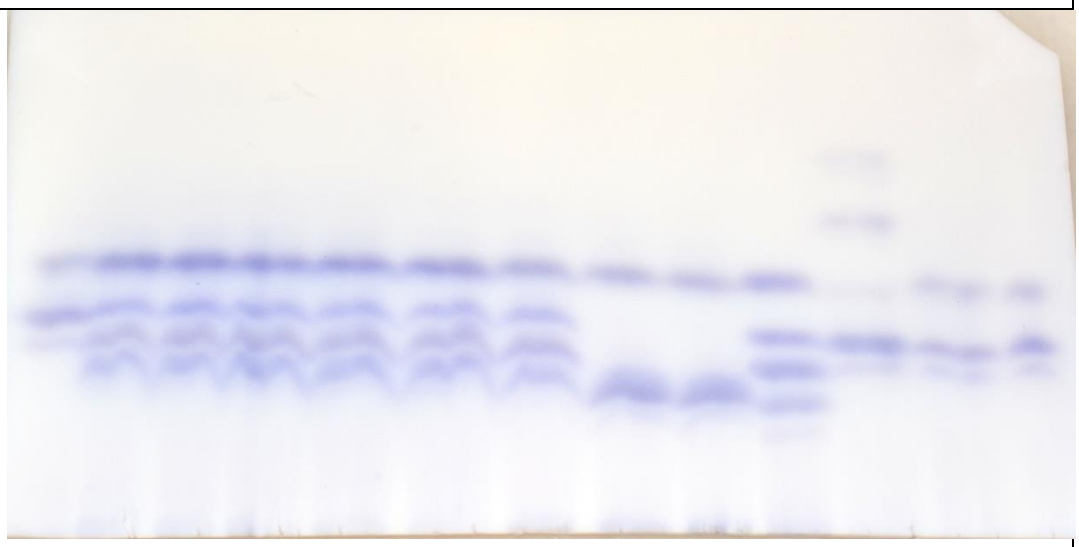
System	Gel
MDH	
PGM	
Lanes	1. *V. parviflora* 2. *V. quercifolia* x *V. parviflora* F_1 3. *V. quercifolia* x *V. parviflora* F_1 4. *V. quercifolia* x *V. parviflora* F_1 5. *V. quercifolia* x *V. parviflora* F_1 6. *V. quercifolia* x *V. parviflora* F_1 7. *V. quercifolia* x *V. parviflora* F_2 8. *V. quercifolia* 9. *V. quercifolia* 10. *V. parviflora* x [*V. quercifolia* x *V. parviflora* F_2] 11. *C. papaya* x *V. goudotiana* 12. *V. parviflora* 13. *V. goudotiana*

Figure 31. Interspecific isozyme gels

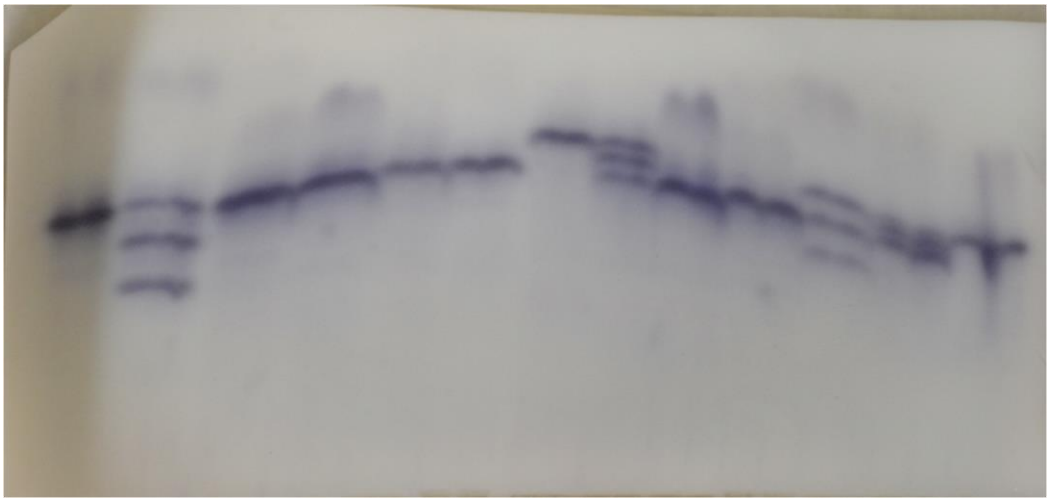
PGI	
Lanes	1. *V. monoica* x *V. parviflora* 2. *V. goudotiana* 3. *C. papaya* x *V. goudotiana* 4. *C. papaya* 5. *C. papaya* x *V. pubescens* 6. V. pub pubescens 7. *V. x heilbornii* 8. *C. papaya* x *V. x heilbornii* 9. *C. papaya* 10. C. p x *V. stipulata* 11. *V. stipulata* 12. *V. glandulosa* 13. *V. glandulosa* x *V. pulchra* 14. *V. pulchra*

Figure 32. PGI gel

4.4 Discussion

In general, all hybrids possessed reduced fertility. Several possessed poor vigor (*C. papaya* x *V. monoica* and *C. papaya* x *V. stipulata*), although several crosses were hardy and fast growing (C. papaya x V. goudotiana, C. papaya x V. pubescens, and C. papaya x V. x heilbornii). Of interest, in the case where crosses were made both directions using *Carica* and *V. goudotiana*, the direction of the cross impacted the vigor, in that the hybrids resulting from female *Vasconcellea* crossed with hermaphrodite papaya pollen showed diminished vigor. Nonetheless, the pollen of this cross, while still inviable, did appear to be further developed.

Two papaya crosses, *C. papaya* x *V. monoica* and *C. papaya* x *V. x heilbornii* are previously unreported. The sex expression of the *C. papaya* x *V. goudotiana* plant exhibited co-dominance of expression in the genetically male plant, resulting in the production of largely hermaphrodite flowers on a clearly male inflorescence.

Isozymes are useful and practical for confirming hybridity. All assayed hybrids were confirmed to be what they were thought to be, both by isozyme testing and by physical phenotypes. Although it is likely that crossing *V. glandulosa* x *V. pulchra* was successful, the fertility of the cross, morphology of the progeny, and isozyme results cast doubt on whether the parents are in fact separate species or just mislabeled accessions of the same species.

The *V. quercifolia* x *V. parviflora* F_2 possessed MDH bands confirming that it had parentage of both species, however, lacked indication of *V. quercifolia* parentage in the PGM system. This indicates a selective loss of *V. quercifolia* genetics.

Despite most hybrids displaying strong vegetative growth, the previously reported sterility of hybrids was present in all of crosses which flowered. Even in flowers where there was some fertility, fertility was still poor. A degree of fertility was recovered, however, in the F_2 plant, which was one of many attempts to produce an F_2.

Of some interest, the ability to form hybrids did not correlate with phylogenetic positioning. *V. parviflora* was able to cross with *V. goudotiana* and *V. pubescens*, but not *V. stipulata*, despite being described as most closely related to *V. stipulata* (Carvalho and Renner, 2012). Additionally, both *V. parviflora* and *V. stipulata* were able

to produce hybrids with *V. monoica*. *V. quercifolia* is described as being most closely related to *V. glandulosa*, however, that cross was unsuccessful.

CHAPTER 5: INDUCTION OF PLOIDY CHANGES

5.1 Introduction

Changes in ploidy have had significant impacts on the evolution and natural history of organisms, as well as in crop domestication and breeding. Some crops, such as cotton (Wendel and Cronn, 2003), rapeseed (Chalhoub et al., 2014), and coffee (Clarindo and Carvalho, 2008), are the result of natural polyploid events occurring in their ancient or recent evolutionary history, while others, such as seedless triploid watermelons (Andrus, 1971) and Gravenstein apples, are man-made. With this in mind ploidy manipulation can be seen as a tool for crop improvement.

Polyploids can be categorized on the basis of the origin of their chromosome sets. An allopolyploid is a plant with additional sets of chromosomes where the additional sets originate from a separate species. This is in contrast to an autopolyploid, where all chromosomes originate from the same source. A tetraploid papaya would be an autotetraploid, as all chromosomes are *Carica papaya*. A tetraploid *Carica* x *Vasconcellea* hybrid would be an allotetraploid, as for every set of four homeologous chromosomes, one pair of chromosomes are *Carica* chromosomes, and the other pair are *Vasconcellea* chromosomes. Such a hybrid would also be referred to as an amphidiploid, as the chromosomes originating in each species would preferentially pair with each other, behaving in a diploid-like fashion within the nucleus. Production of allotetraploid *Carica* x *Vasconcellea* hybrids by doubling diploid hybrids was the goal of this work.

Oryzalin was the agent chosen to be utilized in induced polyploidy. Oryzalin is a mitotic inhibitor which has the ability to disrupt the cell division process by arresting the development of spindle fibers. Under normal circumstances, spindle fibers pull apart chromosomes during anaphase of mitosis, and the original cell divides into two daughter cells. However, if the spindle apparatus is disrupted by oryzalin, the doubled chromosomes do not migrate to the poles and cell division fails to occur**,** resulting in a single cell with doubled chromosomes. Colchicine is an alternative agent, however, it is generally considered to be a more dangerous mutagen than oryzalin.

To practically utilize the oryzalin, meristem treatments were used. There are three tissue layers present in the meristem: the L1, the L2, and the L3 layers. Each of

these is the origin of different types of tissues found in the plant. The goal of a meristematic treatment is to convert all three layers in the meristem, or at the very least, to convert the L2 layer, which is the layer from which reproductive tissue originates.

An alternative methodology would be to first generate tetraploid parents and cross-breed those. This would allow for greater genetic diversity in the offspring, as there would still be two possible chromosomes which could be inherited from each parent. For example, pre-doubling chromosomes to get $A_1A_1A_2A_2$ in species A and $B_1B_1B_2B_2$ in species B has the potential to yield a hybrid with chromosomes of $A_1A_2B_1B_2$ (or eight other potential combinations). In contrast, making hybrids of A_1A_2 x B_1B_2 = A_1B_2 (or three other potential diploid (2x) combinations) and doubling afterwards can only yield plants with the identical homologous chromosomes of $A_1A_1B_2B_2$ (or three other potential tetraploid (4x) combinations).

5.2 Materials and Methods

5.2.1 Chromosome doubling procedure

A 30 ml solution of deionized water and Murashige & Skoog basal salts was prepared. The purpose of the MS salts was to provide divalent cations to facilitate proper gelling of the gelrite. Gelrite (2.8 g/L) and full strength MS salts (4.43 g/L) were combined in deionized water and brought to boiling in a microwave. Typically, only 30 mL was made at a time, as the gel block treatments use very little volume. After boiling, oryzalin was added, followed by 1% (vol. : vol.) dimethyl sulfoxide (DMSO). Concentrations of oryzalin were either 0.5% or 1% active ingredient. Care should be taken to avoid combining undiluted oryzalin and DMSO, as this was found to result in an exothermic reaction. The source of the oryzalin used was a commercially available herbicide formulation (Quali-Pro Oryzalin 4 Pro) containing 41% active ingredient. This made the exact ingredients, to produce 30 ml of the 1% oryzalin treatment, equal to 732 µl of 41% oryzalin, 300 µl DMSO, 90 mg gelrite, and 130 mg MS salts.

After incorporating the oryzalin and DMSO, the hot gelrite mixture was allowed to cool prior to application to avoid scalding damage to the meristems. Meristems were treated when the mixture would solidify in the narrow opening end of the Pasteur pipette used for application, indicating that the temperature of the mixture was just above the

solidification point during application. The glass pipette was inserted as close to the meristem as possible, and the gel was applied onto the meristematic region (Fig. 33-34).

Treated plants were enclosed in either clear plastic bags or clear plastic boxes with moistened paper towels to retain humidity and prevent the gel from desiccating. In cases where plants would not fit in bags or boxes, moist paper towels were placed near the meristem and plastic wrap was used to cover the treatment point.

After treatment, gel blocks were removed with cotton swabs in cases where the gel was still very moist, or with fine tweezers for dryer blocks, followed by spraying of the meristems with a thin stream of water. In some treatments, plants were allowed to keep the same gel block throughout the treatment, which lasted 2-5 days; in other cases, the gel block was removed and reapplied daily.

In addition, several plants were treated in the field to see if meristems of well-established plants could be converted to tetraploid. Plants were treated as above, with moistened cotton swabs affixed to the stems near the treatment point, and wrapped with aluminum foil to prevent overheating. Treatment lasted for 5 days, although the gel likely desiccated after one day.

Figure 33. Oryzalin treatments on papaya seedlings

Figure 34. Oryzalin treatment on an individual hybrid plant

5.2.2 Flow cytometry:

Flow cytometry was used to confirm polyploidy. Samples of plant nuclei were prepared using CyStain PI Absolute P DNA Staining Kit (Partec, Munster, Germany). Two leaf disks (~7 mm dia.) were removed per plant with a hole punch and placed in a 6 cm plastic petri dish, to which 0.5 ml of the Partec kit's extraction buffer was added. Disks were taken from different leaves to better determine if oryzalin treatment had produced a sectoral mixoploid. Using a razor blade, leaf disks were chopped for 60 seconds, and the liquid strained through a 50 µm Celltrics filter (Partec, Munster, Germany) into a tube. Partec staining buffer (2 ml), RNAse (6 µl), and propidium iodide (12 µl) were added to the tubes. The tubes were vortexed briefly. As the propidium iodide is light sensitive, tubes were wrapped in aluminium foil to minimize light exposure, and the tubes were kept on ice in a covered container. A Beckman-Coulter EPICS XL flow cytometer (www.soest.hawaii.edu/sfcf) using 15 mW argon ion laser (488 nm excitation wavelength) running Beckman-Coulter's Expo32 MultiComp software was used to analyze samples. The fluorescence, forward, and side scatter signals were

collected using a 610 BP filter. Histograms were produced in FlowJo software (Treestar Inc., www.flowjo.com).

5.3 Results

In the oryzalin treatments of papaya, four tetraploids were produced (Table 9, Fig. 35). Tetraploid papaya had no observable differences in leaf shape, however, the leaves did feel thicker and more leathery to the touch. Additionally, vigor was markedly diminished in the tetraploids. Mixoploid vigor was equivalent to the diploid plants, although these were not tested a second time to ensure that the diploid tissue was not outgrowing the tetraploid tissue. Leaf morphology did not appear to be noticeably different in the tetraploids. In the treatment of the interspecific or intergeneric hybrids, several mixoploid and tetraploid plants were produced (Fig. 36-38), although in some plants which were later re-tested, the proportion of tetraploid tissue had diminished, or it was no longer detectable (Fig. 36-37).

Table 9. Doubling treatments and results

Treatment	Species	Plants treated	Mixoploids (2x + 4x)	Tetraploids (4x)
2 days, 0.5%	*C. papaya*	49	8	4
3 days, 1%	C.papaya x *V. goudotiana*, *C. papaya* x *V. x heilbornii*, *C. papaya* x *V. stipulata*, *V. monoica* x *V. parviflora*	7	0	0
4 days, 1%	*C. papaya* x *V. pubescens*, C.papaya x *V. goudotiana*, *C. papaya* x *V. x heilbornii*, *V. monoica* x *V. parviflora*, *V. parviflora* x *V. monoica*, *V. monoica* x *V. stipulata*	25	8	2
4 days, replaced daily. 1%	*C. papaya* x *V. pubescens*, C.papaya x *V. goudotiana*, *C. papaya* x *V. x heilbornii*, *V. monoica* x *V. parviflora*	11	5	2
5 days, replaced daily, 1%	*C. papaya* x *V. goudotiana*	2	1	0
5 days, outdoors	*V. quercifolia* x *V. parviflora*, *C. papaya* x *V. goudotiana*	13	1	0

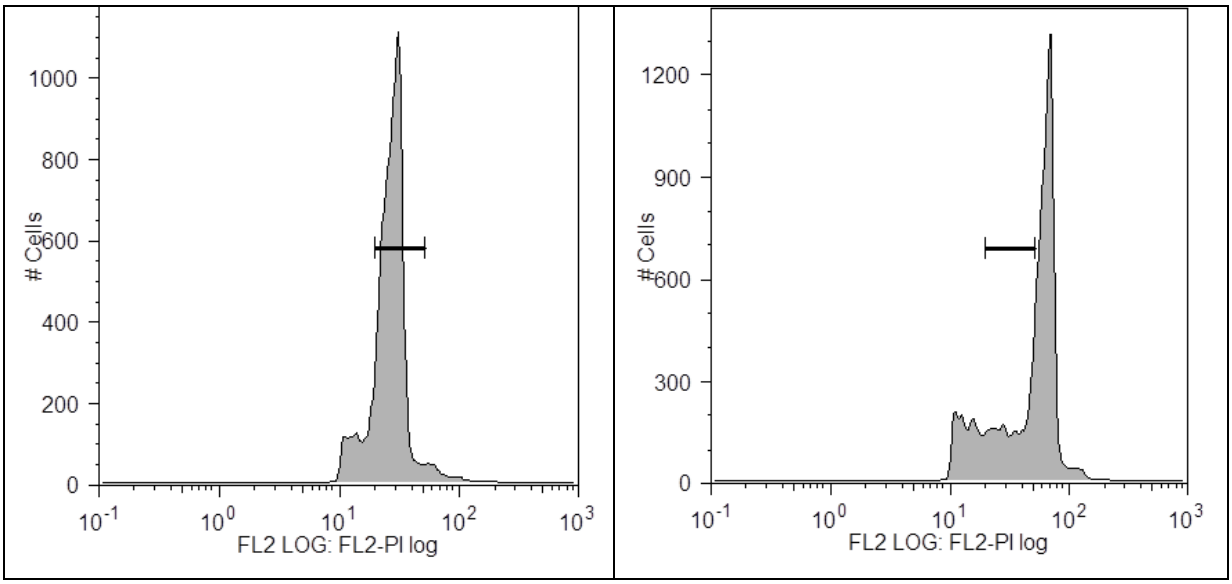


Figure 35. Papaya diploid control (left) and tetraploid (right), showing diploid gate

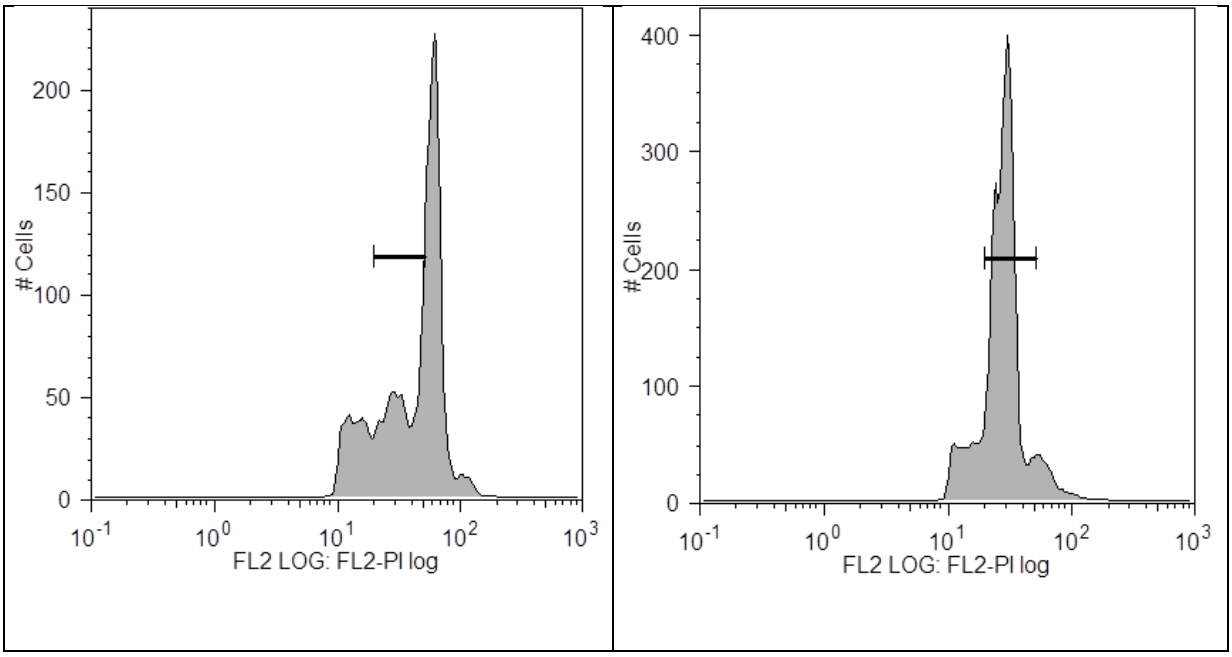


Figure 36. *V. quercifolia* x *V. parviflora* mixoploid sector (left), and same plant two months later (right)

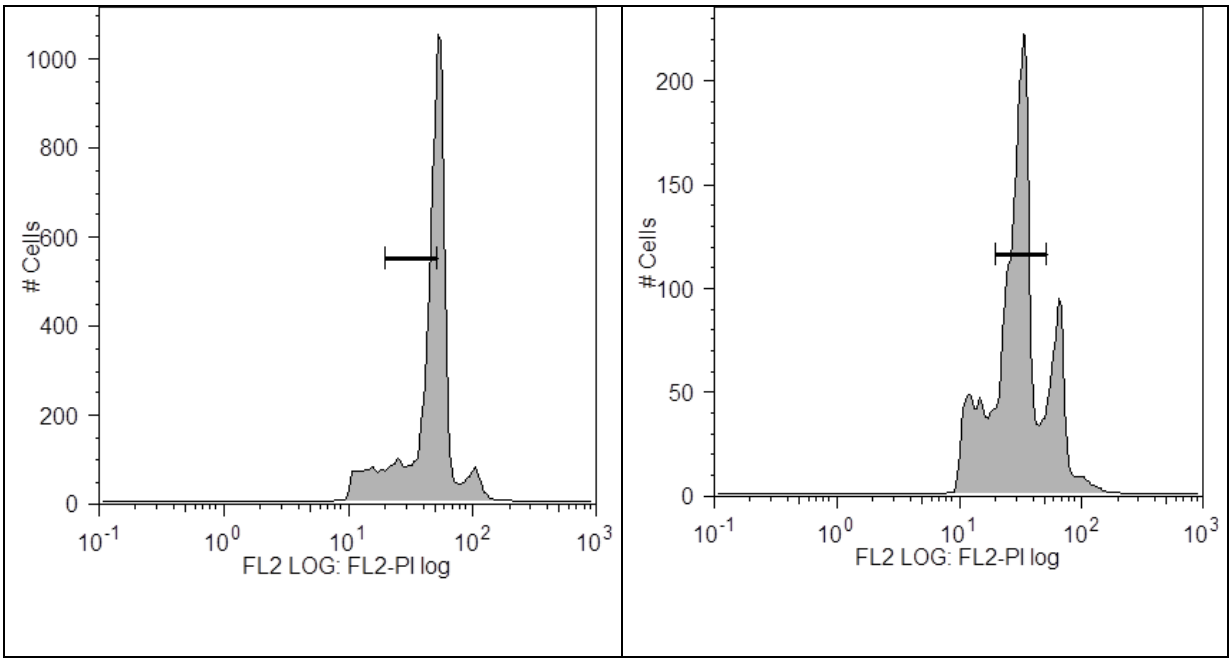


Figure 37. *C. papaya* x *V. pubescens*, 4x (left) and same plant 2 months later (right)

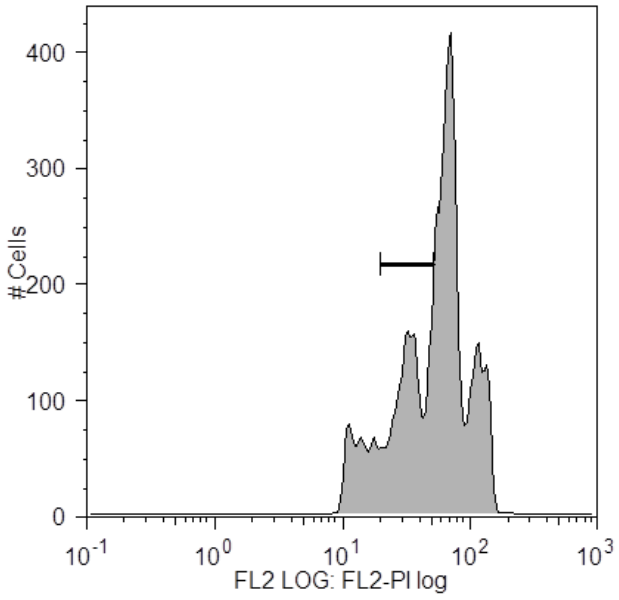


Figure 38. *C. papaya* x *V. goudotiana* mixoploid showing multiple peaks

In the interspecific plants, one *V. quercifolia* x *V. parviflora* hybrid was found to have sectoral mixoploidy. Flowers from this section appeared to be larger toward the base (Fig. 39), and were able to produce pollen which was double that of the smaller flowers from the diploid section (Table 10, Fig. 40).

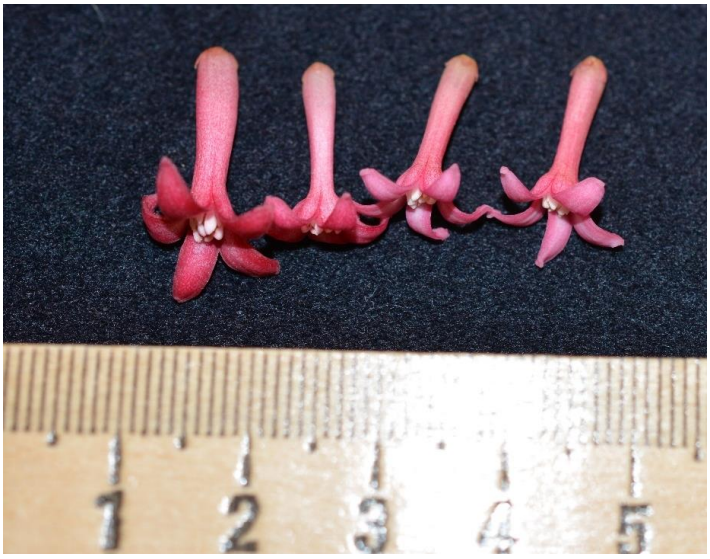


Figure 39. *V. quercifolia* x *V. parviflora* flower from the mixoploid sector (left) and three from the opposite side (right)

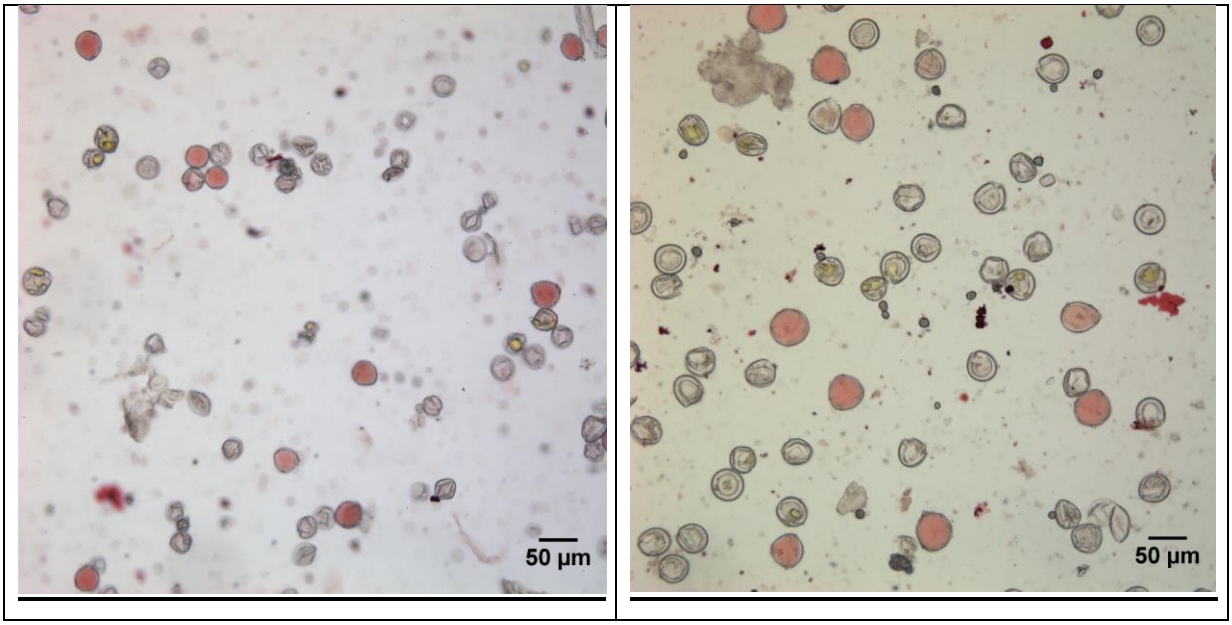


Figure 40. *V. quercifolia* x *V. parviflora* diploid (left) and tetraploid (right) pollen, 400x magnification

Table 10. Pollen grain stainability and cross-sectional area from diploid and potentially tetraploid flowers

Species	Average cross sectional area	Grains measured	Stainable pollen	Non-stainable pollen	Percent stainable
V. quercifolia x *V. parviflora*	830 μm^2	75	156	1353	10.40%
V. quercifolia x *V. parviflora* mixoploid	1611 μm^2	86	54	319	14.48%
V. quercifolia	901 μm^2	18	888	25	97.26%
V. parviflora	1222 μm^2	18	1109	147	88.30%

5.4 Discussion:

In no plants was any mortality of the meristem observed with the exception of one: a *V. monoica* x *V. parviflora* hybrid, which was in the group that was treated for four days with 1% oryzalin daily. In all others, although there was clear distortion of leaves near the growth point, there was no obvious instances of necrosis of the meristem. There was however instances of young leaves near the meristem abscising if the oryzalin could not be removed, and an instance of a dry streak of oryzalin leaving a scar on the base of a treated plant after being left unremoved for a two weeks.

One plant, a *C. papaya* x *V. pubescens* hybrid, was confirmed by flow cytometry to be tetraploid. This plant continued to display good vigor above the treatment point. Two side shoots originating below the treatment point had greater vigor. However, after initial testing, despite the clear tetraploid results, later testing indicated that the plant was mixoploid, with a ratio of 2 diploid:1 tetraploid counts (Fig. 37). A second plant, a *C. papaya* x *V. goudotiana*, was indicated by early tests to similarly be tetraploid (Fig. 38). Several others showed tetraploid and higher peaks, indicating a potential presence of octoploid tissue. These may also have ability to produce tetraploid flowers if the L2 layer was among the tissue converted. Additionally, there was a large degree of conversion in a *V. quercifolia* plant, which could be used to attempt hybridization at the tetraploid level.

In another instance, a section of a *V. quercifolia* x *V. parviflora* hybrid produced diploid and tetraploid tissue in a 1:1 ratio on one section of the plant. This was determined by doing separate flow cytometry analysis on leaves around the plant, and the ratios correlated with position (ie the positioning of the ratios was consistent with one side of the meristem being converted, not random). However, later sampling of this plant showed a loss of tetraploid tissue (Fig. 36). In this instance, it appears that mixoploid tissue in the plants is outgrown by diploid tissue. This converted sector was however able to flower, and the pollen from these flowers had twice the cross-sectional area of the pollen from diploid sectors (Table 10, Fig. 40). This is consistent with the pollen are of tetraploids of other species (Hecker, 1988, Randolph, 1935), and indicates that the L2 layer of this plant was successfully converted to the tetraploid level in that sector. However, in terms of stainable pollen percentage, this pollen was no more

viable than the diploid material. This suggests that, in the case of interspecific hybrids within Vasconcellea, doubling may be insufficient for fertility restoration. It should be noted however that actual fertility can only be determined by crossing attempts, which have not yet been attempted using these materials.

5.5 Conclusion:

Carica was able to successfully hybridize with 5 of the 9 species used. Embryo rescue was required in all instances. These hybrids were generally vigorous, although lacking in fertility. Sex expression was abnormal in the intergeneric flowering plants, with female characteristics being present in the genetically male plant. This is similar to previous reports of *C. papaya* x *V. pubescens* plants appearing as two types of ultimately female plants, but differs from reports indicating that *C. papaya* x *V. parviflora* hybrids are capable of producing male and female flowers.

Oryzalin doubling methods had a clear effect on the ploidy of tissue, as determined by flow cytometry. The main difficulty was in creating a complete conversion of meristem tissue to tetraploid. For future work in the production of tetraploids, it may take additional efforts to ensure the generation of completely converted meristems. Possible avenues for this include removing developing leaves near the meristem to allow for greater contact of the gel to the meristem and ensure greater penetration of the oryzalin. Additionally, as there were instances of sectoral conversion, determining sections of converted plants and encouraging lateral bud growth from those sections may yield results.

A sibling cross between *V. quercifolia* x *V. parviflora* F_1 displayed an increase of fertility. This F_2 hybrid appeared to be more like the pollen parent (*V. parviflora*) than the maternal *V. quercifolia*. A possible explanation for this could be that two unreduced gametes produced a spontaneous autotetraploid, however, flow cytometry indicated the plant to be diploid. This suggests the possibility that the F_2 plant is a rare segregant in which a favorable combination of parental genetics has produced a relatively stable genomic combination, capable of producing backcross offspring to *V. parviflora*. The greater presence of *V. parviflora* isozyme alleles suggest disruptive selection tending toward that parental species.

One oryzalin-treated interspecific *V. quercifolia* x *V. parviflora* F_1 male produced pollen from a sector with tetraploid leaf tissue. The pollen grains displayed larger size consistent with tetraploidy, but showed no increase in stainable percentage. However, fertility can only be determined in terms of the number and nature of progeny obtained from backcrossing to the parent species or to other allotetraploids. Flowering of male intergeneric hybrids with tetraploid tissue will permit the determination of whether similarly enlarged pollen grains occur in the wider crosses between *Carica* and *Vasconcellea*, and whether these are fertile in crosses to the parent species or to other intergeneric allotetraploids.

References:

Ammar K., Mergoum M., Rajaram S. 2004. The History and Evolution of Triticale. In: Mergoum M, Gómez-Macpherson H, editors. Triticale improvement and production. Rome (Italy): The Food and Agriculture Organization of the United Nations. p. 1-10.

Andrus, C. F. 1971. Production of Seedless Watermelons. Technical Bulletin No.1425 USDA-ARS

Aradhya, M.K., L.K. Yee, F.T Zee, and R. Manshardt. 1998. Genetic variability in Macadamia. Genetic Resources and Crop Evolution 45;19-32.

Badillo, VM. 1993 Monografia de la Familia Caricaceae. Universidad Central de Venezuela, Maracay, Venezuala

Carvalho, FA, Renner, S. 2012. A dated phylogeny of the papaya family (Caricaceae) reveals the crop's closest relatives and the family's biogeographic history. Mol Phylogenet Evol. 65(1);46-53

Chalhoub et al. 2014. Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science. 345(6199);950-3.

Clarindo, W.R. & Carvalho, C.R. 2008. First Coffea arabica karyogram showing that this species is a true allotetraploid. Plant Syst Evol 274;237–241

Deputy, J.C, Ming, R. Ma, H., Liu Z., Fitch M. M. M., Wang M., Manshardt R., Stiles J. I. 2002. Molecular markers for sex determination in papaya (Carica papaya L.) Theor Appl Genet 106;107–111.

Dieleman, F.L. and Eenink, A.H. 1980. Breeding lettuce (*Lactuca sativa*) for resistance to the aphid Nasonovia ribisnigri in Minks, A.K. & Gruyss, P. (Eds) Integrated Control of Insect Pests in The Netherlands. Pudoc. Wageningen. pp 183-185

Drew, RA., Magdalita, M., and O'Brien, CM. 1998. Development of *Carica* Interspecific Hybrids. Acta Hortic. 461;285-292

Drew RA. 2011. The Use of Non-Transgenic Technologies for the Development of Papaya Ringspot Virus Resistance in *Carica papaya*. Acta Hortic. 1022;23-29

FAOSTAT 2016 http://faostat.fao.org/beta/en/#data/QC

Fitch MMM. 1993. High frequency somatic embryogenesis and plant regeneration from papaya hypocotyl callus. Plant Cell, Tissue and Organ Culture 32(2);205–212

Gardner R. G. and Panthee D.R. 2010. 'Plum Regal' Fresh-market Plum Tomato Hybrid and Its Parents, NC 25P and NC 30P. Hortscience 45(5);824–825.

Gonsalves, D., Gonsalves, C., Ferreira, S., Pitz, K., Fitch, M., Manshardt, R., and Slightom, J. 2004. Transgenic Virus Resistant Papaya: From Hope to Reality for Controlling Papaya Ringspot Virus in Hawaii. APSnet Features. http://www.apsnet.org/publications/apsnetfeatures/Pages/PapayaRingspot.aspx

Harlan J. R., and de Wet J. M. J, 1971. Toward a Rational Classification of Cultivated Plants. Taxon 20(4);509-517

Hecker, R. J. 1988. Pollen Characteristics of Diploid and Tetraploid Sugarbeet. Journal of Sugar Beet Research 25(1);55-62

Horovitz S., Jiménez H. 1967. Cruzamientos interespecíficos e intergenericos en Caricaceas y sus implicaciones fitotecnicas. Agronomía Tropical 17;323–343.

Kim, M.S. 2002. Genetic diversity of *Carica papaya* as revealed by AFLP markers. Genome. 45(3);503–512

Manshardt R.M., Wenslaff T.F. 1989. Interspecific hybridization of papaya with other *Carica* species. JAm Soc Hortic Sci 114;689-694

Mekako H.U., Nakasone H.Y. 1975. Interspecific hybridization among 6 *Carica* species. JAm Soc Hortic Sci 100;237–242

Moore, P.H 2013. Phenotypic and Genetic Diversity of Papaya. Genetics and Genomics of Papaya pp 35-45 eds. Ming R., Moore P. H.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15;473—97

NASS (2011) Statistics of Hawaii Agriculture 2011 https://www.nass.usda.gov/Statistics_by_State/Hawaii/Publications/Annual_Statistical_Bulletin/2011/2011HawaiiAgStats.pdf

The National Academies Press, 1989. Highland Papayas In: Lost Crops of the Incas: Little-Known Plants of the Andes with Promise for Worldwide Cultivation. Washington, DC (USA): The National Academies Press. p. 252-261.

O'Brien, C. 2009. Marker-Assisted Breeding for Papaya Ringspot Virus in Carica Papaya L. (Master's thesis) Retrieved from: https://www120.secure.griffith.edu.au/rch/file/00b624df-8197-1b12-e9ce-fc7a93ed17fa/1/O%27BrienC_2010_02Thesis.pdf

O'Brien C. M. and Drew R. A. 2009 Potential for using Vasconcellea parviflora as a bridging species in intergeneric hybridisation between *V. pubescens* and *Carica papaya Australian Journal of Botany* 57(7);592-601

Olsen, RT., Ranney, TG., Viloria, Z. 2006. Reproductive Behavior of Induced Allotetraploid × *Chitalpa* and In Vitro Embryo Culture of Polyploid Progeny. J Am Soc Hortic Sci 131;716–724

Pan M.J & van Staden J. 1998. The use of charcoal in in vitro culture – a review. Plant Growth Regulation 26;155–163

Porch et al. 2013. Use of Wild Relatives and Closely Related Species to Adapt Common Bean to Climate Change. Agronomy 3;433-461

Randolph, L. F. 1935. Cytogenetics of Tetraploid Maize. Journal of Agricultural Research 50(7);591-605

Reddy M.T. 2015 Crossability Behaviour and Fertility Restoration Through Colchiploidy in Interspecific Hybrids of Abelmoschus esculentus × Abelmoschus manihot subsp. Tetraphyllus. International Journal of Plant Science and Ecology 1(4);172-181

Sauls J. "Papaya." Fruit & Nut Resources. http://aggie-horticulture.tamu.edu/fruit-nut/fact-sheets/papaya

Tennant PF. 1994. Differential Protection Against Papaya Ringspot Virus Isolates in Coat Protein Gene Transgenic Papaya and Classically Cross-Protected Papaya. Phytopathology 84:1359-1366

Tonguç M., Griffiths P.D. 2004. Transfer of powdery mildew resistance from Brassica carinata to Brassica oleracea through embryo rescue. Plant Breeding 123, 587—589

Warnert J. 2004. Conventionally bred papaya still possible, even in California. California Agriculture 58(2):74-74.

Wendel, J. F. and Cronn, R. 2003. Polyploidy and the Evolutionary History of Cotton. Advances in Agronomy, 78;139-186

Whitaker T. and Robinson R. 1980. Squash Breeding. Breeding Vegetable Crops. Bassett M. J. (Ed) The Avi Publishing Company, Connecticut. pp 218-224