

# Interspecific Hybridizations in *Citrullus* – Notes

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## Introduction

*Citrullus* is a relatively small genus that includes only seven known species. The phylogenetic relationships among these species have been well established (Chomicki and Renner, 2015). Based on its relationship to the cultivated watermelon (Renner, 2019) it might be assumed that *C. lanatus* var. *cordophanus* would hybridize readily with commercial watermelon cultivars and breeding lines. In a similar manner, it is generally known that *C. amarus* (citron) and *C. mucospermus* (egusi), the two species most closely related to *C. lanatus*, can be hybridized with *C. lanatus* and produce fertile progeny. In fact, a pollenizer (SP-6) with multiple disease resistance was developed through the crosses of these three species (Brusca and Zhang, 2012). This places both *C. amarus* and *C. mucospermus* firmly in the secondary gene pool (as defined by Harlan and de Wet, 1971).

*Citrullus colocynthis*, a species distantly related to *C. lanatus*, also hybridizes with *C. lanatus* and produces fertile progeny (Levi et al., 2002). In a cross of RCAT055816 x PI537300 (*C. amarus* x *C. colocynthis*), the F<sub>1</sub> was essentially sterile. However, X. Zhang and colleagues have developed unique lines with very small fruit size and very thin rind using *C. colocynthis* (PI537300) as a pollen donor. These findings suggest that *C. colocynthis* might also be considered to be a member of the secondary gene pool.

These earlier reports indicate that several of watermelon's wild relatives, both closely related and distantly related, can be hybridized with *C. lanatus*. However, little information, with few exceptions (de Winter, 1990; Jarret et al., 2017), has been presented on the ability to hybridize several of watermelon's other crop wild relatives (CWRs) with *C. lanatus*, or among themselves. These other desert-dwelling species offer potential opportunities for the introgression of desirable traits (Simmons et al., 2019) into the cultivated watermelon, either

directly or indirectly. These species include *C. ecirrhosus*, *C. rehmi* and *C. naudinianus*.

This brief note is offered to provide information obtained over several years of working with these less well investigated species. The work is hardly complete, and it is hoped that further studies will be undertaken.

## Materials and Methods

All plant materials were obtained from the S-009 genebank in Griffin, GA as described previously (Jarret et al., 2017) and grown in the greenhouse or field on the GA Experiment Station. Unless noted otherwise, the excised embryos of hybrid seeds were germinated in vitro, or entire seeds were germinated in Petri dishes on moist paper towel after cracking the seed coat with a small vise-grip (Jarret et al., 2017). The success of hybridizations was evaluated based on the phenotypic characteristics of the resultant progeny, or in the case of crosses with *C. naudinianus*, via molecular analysis.

**Molecular Analysis.** Genomic DNA isolation involved use of the plant DNA isolation kit (QIAGEN cat# 69104). PCR reactions consisted of 50 ng genomic DNA, 0.20 μM mixed forward and reverse primers, 1X Buffer (10 mM Tris-HCl pH 8.2, 50 mM KCl, Triton 0.1%, BSA 1 mg/ml), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs and 1 U *Taq* polymerase (Promega) in 10-μL reaction volumes. Amplification was performed in a GeneAmp PCR 9700 System thermal cycler (Applied Biosystems) programmed to 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 50-65°C for 30 s, 72°C for 1 min, then 72°C for 10 min. Amplified products were separated on a high-throughput DNA fragment analyzer (AdvanCE FS; Advanced Analytical Technologies, Ames, IA) and diluted in a 1:11 ratio depending on the concentrations of products; the dilution and the injection voltage were adjusted to prevent overloading the PCR product on the fragment analyzer. PCR product of 2 μl was pipetted directly into the wells of the sample plate containing

22 µl 1X TE dilution buffer. Alternatively, to prevent evaporation a drop of mineral oil was overlaid on each sample. The samples were size-separated by use of a 96-capillary automated system with capillaries 80 cm long. Polymer and other required reagents were from the DNF-900 dsDNA reagent kit (Advance Analytical Technologies). The DNF-900 dsDNA reagent kit can effectively separate amplicon ranges between 35 and 500 bp and resolve 1-bp differences between alleles. Following the capillary electrophoresis, the data were processed by use of PRO Size 2.0, software (Advance Analytical Technologies). The data were normalized to the 35-bp lower marker and 500-bp upper marker and calibrated to the 75- to 400-bp range.

## Results and Discussion

### Crosses with *C. lanatus* cv. Sugar Baby as Male Parent

***C. ecirrhosus* x cv. Sugar Baby.** *C. ecirrhosus* is native to the Namib Desert. This species is a perennial and F<sub>1</sub> hybrids with *C. ecirrhosus* generally exhibited a perennial growth habit. Unlike most other *Citrullus* species, *C. ecirrhosus* (and its hybrids with *C. lanatus*) could be readily propagated via vine cuttings (Simmons et al., 2019). This species produces a caudex (Fig. 1), an organ utilized for water storage (Rowley, 1978; Romero, 2022).

The cross *C. ecirrhosus* x cv. Sugar Baby was made with relative ease. However, limited attempts to cross *C. ecirrhosus* with 2 other cultivars of *C. lanatus* (i.e. cvs. Charleston Gray and Bush Jubilee) met with less success indicating a genotypic effect on crossability. The F<sub>1</sub> plants of *C. ecirrhosus* x cv. Sugar Baby were vigorous. In 2020, a single F<sub>1</sub> plant was propagated to produce 12 rooted cuttings that were placed in the field to produce fruit/seed on the GA Experiment Station. Plants were allowed to open pollinate. Fruit set on the earliest flowers was low but improved as the season progressed and the plants increased in size. The total yield of fruit from these plants was substantial (Fig. 2). Seed yields averaged 85 seed/fruit in fruit that averaged 8-10" in diameter. Fruit rinds were uniformly dark green and smooth. Fruit flesh was moderately firm and whitish-yellow. Fruit were often irregular in shape, but were generally near round. F<sub>2</sub> seed germination averaged 55%.

***C. rehmi* x cv. Sugar Baby.** The fruit of *C. rehmi* have a unique rind that is patterned and springy (not hard) bearing some resemblance to the rind of *Cucumis melo* but of a different color and texture. This species is an annual, also native to the Namib Desert (De Winter, 1990), and is sometimes referred to as the Namib melon. Its distribution is sympatric with that of *C. lanatus* and *C. ecirrhosus* (De Winter 1990).

Successful crosses of *C. rehmi* x cv. Sugar Baby were readily made in the greenhouse. The viability of F<sub>1</sub> seed was ~ 45%. In 2022, a small population (10 F<sub>1</sub> plants) was grown in the

field on the GA Experiment Station. F<sub>1</sub> plants were near normal in fertility as judged by late season fruit set. The fruit harvested from these F<sub>1</sub> plants were similar in size and general appearance (coloration) to the fruit harvested from the F<sub>1</sub> plants of the *C. lanatus* x *C. ecirrhosus* population described earlier (Fig. 3). However, the fruit were often smaller, irregular in shape and with a waxy coating and a thin rind. Fruit flesh was off-white to pale yellow and spongy. Ten randomly selected mature fruit yielded 55-105 F<sub>2</sub> seed each. Our accession of *Citrullus rehmi* hybridized readily with cv. Charleston Gray.

***C. colocynthis* x cv. Sugar Baby.** The ability to produce fertile F<sub>1</sub> progeny from the cross *C. lanatus* x *C. colocynthis* has been previously reported (Levi et al., 2017). Hence, it will not be discussed except to note that a series of 6 hybridizations with *C. colocynthis* as the male parent resulted in an average of 22 seed/fruit and the reciprocal an average of 9 seed/fruit.

***C. naudinianus* x cv. Sugar Baby.** *C. naudinianus* is the species most distantly related to the cultivated *C. lanatus* and is the *Citrullus* species most closely related to *C. colocynthis*. *C. naudinianus* is a perennial, dioecious, and until recently was classified as *Acanthosicyos naudinianus*. Based on the known systematic relationships of *Citrullus* spp. (Chomicki and Renner, 2015), and the extremely limited availability of *C. naudinianus* flowers (see *C. colocynthis* x *C. naudinianus*), we elected to utilize most *C. naudinianus* female flowers to perpetuate the line or to cross with that species most likely (based on taxonomic relationships) to produce viable seed, that species being *C. colocynthis*. Hence, no hybridizations with cv. Sugar Baby were attempted.

### Crosses with cv. Sugar Baby as Female Parent

**cv. Sugar Baby x *C. ecirrhosus*.** This cross was also made with relative ease. The F<sub>1</sub> plants of cv. Sugar Baby x *C. ecirrhosus* were vigorous and also readily propagated by vine cuttings. In 2021, 10 cuttings were clonally propagated from a randomly selected F<sub>1</sub> plant and grown on the GA Experiment Station as described earlier for the reciprocal cross. Fruit set, fruit size, fruit shape and coloration of the fruit harvested from these F<sub>1</sub> plants were similar to those harvested from the *C. ecirrhosus* x cv. Sugar Baby hybrid population. Seed yields averaged 95 seed/fruit (10 fruit sample) in fruit averaging 9-11" in diameter. In this cross, as in the reciprocal, plants exhibited a perennial growth habit.

**cv. Sugar Baby x *C. rehmi*.** This cross was made with relative ease. Ten F<sub>1</sub> fruit yielded an average of 95 seed/fruit. Germination of these has yet to be tested. *C. rehmi* crossed readily with *C. lanatus* cv. Charleston Gray.

### Hybridizations with *C. naudinianus*

***C. colocynthis* x *C. naudiniana*.** Plants of *C. naudiniana* are large with vines readily reaching 10 meters and longer. The size of this plant prevented our maintaining more than a single male and female plant in the greenhouse. While the male plant produced sufficient flowers for limited use as a pollen source, the female plant produced only 6 flowers over the course of several months. The paucity of female flowers effectively precluded efforts to hybridize this species as pollen recipient and so the few female flowers that were available were used to maintain the accession (PI 596694).

Over the course of several years, we noted that PI 596694 was susceptible to gummy stem blight (causal agent *Stagonosporopsis* spp.) but the plants did not succumb to the disease over that time period. Cankers were periodically observed along the older portions of the vines near the crowns of the plants. Fungicides served to control the pathogen. Plants derived from newly formed storage organs initially appeared to be free of the pathogen but have not been tested.

This cross (*C. colocynthis* x *C. naudiniana*) can be made with some difficulty. Ten hybridizations yielded 357 seeds of which 165 were fully developed (Fig. 4). Five randomly selected seeds from a single cross were successfully germinated *in vitro*. The F<sub>1</sub> plants exhibited a growth habit that was intermediate between the two parents and they produced a small tap root.

***C. rehmi* x *C. naudiniana*.** The cross of *C. rehmi* x *C. naudiniana* was accomplished resulting in F<sub>1</sub> plants that were moderately vigorous. Eight hybridizations produced 112 seed, 66% of which were fully developed. The vines of F<sub>1</sub> plants branched (Fig. 5 -middle) at nearly every node. Leaves were scabrid as is typical of *C. naudiniana*. This hybrid produced carrot-like storage roots (Fig. 5 upper) that were smaller and of a different shape than typical *C. naudiniana* storage organ. Plants of this hybrid produced multiple (two or sometimes three) male flower buds/node with all, except one bud, eventually aborting and the remaining bud sometimes developing to maturity. A single plant of this hybrid combination produced hermaphroditic-like flowers with a fully developed pistil, partially developed ovary and partially or near fully-developed anthers – Fig. 5 - lower).

***C. ecirrhosus* x *C. naudiniana*.** *C. ecirrhosus* was hybridized with *C. naudiniana* with moderate difficulty (based on the number of apparently viable seed produced). The reciprocal cross was also successful resulting in 64 fully developed seed. Structures believed to be rudimentary aerial roots were sometimes observed on the hybrid plants (Fig. 6).

#### **Miscellaneous Crosses**

***C. rehmi* x *C. ecirrhosus*.** This cross was readily accomplished with plentiful fully-developed viable F<sub>1</sub> seed (average ~95) present in mature fruit (Fig. 7 – upper).

***C. ecirrhosus* x *C. rehmi*.** The reciprocal of the previous cross yielded fewer than three seed (typically none) per mature fruit. Most fruit contained only empty seed coats (Fig. 7 - lower).

***C. rehmi* x *C. mucospermus*.** The cross *C. rehmi* x *C. mucospermus* and its reciprocal were made without difficulty. Fruit averaged 30 -70 seed each.

***C. rehmi* x *C. amarus*.** It is well known that *C. amarus* (citron melon) intercrosses with *C. lanatus*. However, the cross compatibility of *C. amarus* with other more distant species has not been reported. F<sub>1</sub> plants of *C. rehmi* x *C. amarus* (and the reciprocal cross) are vigorous with rather thin vines that are moderately branched. The F<sub>1</sub> fruit of greenhouse grown plants were roundish in shape and about the size of large *C. rehmi* with intermediate coloration (Fig. 8 - lower). The F<sub>1</sub> plants set fruit readily when selfed. A total of three hybridizations resulted in an average of ~95 seed/fruit. All were fully developed (Fig. 8 - upper).

***C. rehmi* x *C. colocynthis*.** This cross was readily accomplished with abundant fully-developed (60-150) viable F<sub>1</sub> seed present in a mature fruit (Fig. 9).

## **Conclusions**

Of the six known CWR of watermelon, five (*C. amarus*, *C. mucospermus*, *C. ecirrhosus*, *C. rehmi* and *C. colocynthis*) can be hybridized with *C. lanatus* to produce fertile F<sub>1</sub> progeny capable of generating an F<sub>2</sub> population. In the course of this work, genotypic effects on the success of individual hybridization partners were observed. Some crosses such as *C. rehmi* x *C. ecirrhosus* exhibited a strong uni-directional effect and were successful only when one species was used as the female parent. In general, the interspecific hybrid plants were vigorous and displayed, as expected, phenotypic characteristics that were intermediate to the two parents.

Although no evidence was found to indicate fertility in the single *C. naudiniana* interspecific hybrid plant available for observation (due to greenhouse space limitations), that obstacle might be overcome by inducing tetraploidy (Bae et al., 2020), a process known to sometimes restore the fertility of interspecific hybrids (Oates et al., 2012), or by producing a greater number of hybrid plants. Due to the limited number of male parent plants and genotypes available for evaluation in this work (1), any attempt to meaningfully predict the ease of hybridization of *C. naudiniana* with other *Citrullus* species based on the present study, or the resultant fertility of hybrid offspring so produced, would be premature.

Whether or not watermelon's CWRs, with their many adaptive and disease resistance traits, are ultimately more fully utilized to improve the crop via conventional or marker-assisted breeding strategies, remains to be seen. One or more of the existing CWR might be used as a bridging species to

access the genome of a more distantly related species. Crosses of *C. amarus* x *C. ecirrhosus* x *C. mucosospermus* have been used to develop lines for high femaleness and superior disease resistance for use as rootstock for commercial watermelon production (X. Zhang - personal communication, Fig. 10). Information obtained from the study of the CWR might also be expected to contribute to gene editing efforts (Feng et al., 2023). In order to realize the greater use of these CWRs, a significant investment in resources, and a realistic (possibly long-term) time frame, may be required.

Recently, large amounts of genetic data on watermelon and its CWR have become available as a result of multiple genomic, pangenomic and super-pangenomic studies (Guo et al., 2013; Jarret et al., 2021; Nie et al., 2023; Sun et al., 2023; Wu et al., 2023). These provide a guide for potentially circumventing some of the obstacles that typically limit the introgression of desirable traits from CWR to the cultivated crop. This outpouring of *Citrullus* spp. genomic data will facilitate future utilization of the CWR and curtail or ameliorate some of the constraints to their broader use.

As a final note, the genepool assignments mentioned earlier are somewhat tentative as estimates of crossability among *Citrullus* species can be highly genotype dependent.

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Figure 1. Caudex on 2-year-old *C. ecirrhosus*.



Figure 2. Some of the fruit harvested from 12 plants of a *C. ecirrhosus* x cv. Sugar Baby F<sub>1</sub> hybrid in 2020 (Griffin, GA).



Figure 3. Mature fruit harvested from selfed *C. rehmii* x cv. Sugar Baby F<sub>1</sub> plant grown in the field.



Figure 4. Mature fruit of *C. colocynthis* x *C. naudiniana* F<sub>1</sub> hybrid.



Figure 5. Upper: Carrot-like tap/storage root of a *C. rehmi* x *C. naudinianus* F<sub>1</sub> plant. Middle: Vine branching pattern of a *C. rehmi* x *C. naudinianus* F<sub>1</sub> plant. Lower: Hermaphroditic-like flower on a *C. rehmi* x *C. naudinianus* F<sub>1</sub> hybrid.



**Figure 6. Aerial roots on *C. ecirrhosus* x *C. naudinianus* F<sub>1</sub> hybrid.**





Figure 7. Mature F<sub>1</sub> fruit of *C. rehmanii* x *C. ecirrhosus* (upper) and *C. ecirrhosus* x *C. rehmanii* (lower).



Figure 8. Upper: Interior of a mature fruit of a *C. rehmii* x *C. amarus* F<sub>1</sub> hybrid fruit. Lower: Exterior of a *C. rehmii* (left) and a mature *C. rehmii* x *C. amarus* F<sub>1</sub> hybrid fruit.



Figure 9. Mature fruit of *C. colocynthis* (left), *C. rehmi* (right) and their F<sub>1</sub> hybrid (middle).



Figure 10. Lines with strong plant (to be used as rootstock), high femaleness derived from the crosses *C. amarus* x *C. ecirrhosus* x *C. mucosospermus*.