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Pollen Viability of F_1 Hybrids between Watermelon Cultivars and Disease-resistant, Infraspecific Crop Wild Relatives

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Abstract. **Crop wild relatives (CWRs) are important sources of variation for domesticated crops like watermelon (*Citrullus lanatus*) where cultivated varieties have a very narrow genetic base. The use of CWRs in plant breeding can be hampered by low fertility, chromosomal rearrangements, marker distortion, and linkage drag in the progeny. Pollen viability can be a quick and easy way to estimate male fertility, which can be a cause of marker distortion and an indicator of chromosomal rearrangements. Pollen viability was determined for F_1 hybrids between cultivars and resistant citron and egusi types and the data were used to determine whether the parental cultivars/lines used or the directionality of the cross play a role in pollen viability. F_1 hybrids between cultivars and the egusi type showed no reduction in pollen viability compared with parental lines, whereas pollen viability of hybrids with citron types varied between 61.8% and 91.7%. Significant main effects were observed for the cultivar and donor lines used, but the directionality of the cross did not affect pollen viability. F_1 hybrids with 'Crimson Sweet' as the cultivar parent had significantly higher pollen viability than those with 'Sugar Baby' or 'Charleston Gray'. Our results indicate that the directionality of the crosses between watermelon cultivars and infraspecific CWRs does not affect pollen viability but that the specific cultivars and donor lines used can have a significant effect. The high pollen viability of cultivar–egusi hybrids is supported by previous genetic data and strongly suggests that it should be easier to introgress traits from egusi types than citron types.**

In 2010 ≈89 million metric tons of watermelon (*Citrullus lanatus*) were produced worldwide, of which 1.8 million metric tons were produced in the United States (Food and Agriculture Organization of the United Nations, 2011). The elite watermelon cultivars used in production today are susceptible to a large range of pests and diseases and molecular data have shown that these elite cultivars have a very narrow genetic base (Guo et al., 2013; Lee et al., 1996; Levi et al., 2001a, 2001b, 2004a; Navot and Zamir, 1987). Although this phenomenon is common in many cultivated crops (Zamir, 2001), it is particularly severe in watermelon where the reported level of diversity is lower than observed for important agronomic crops such as maize, soybean, and rice (Guo et al., 2013). Data from the complete watermelon genome sequence (Guo et al., 2013) also confirm the previous theory (Harris et al., 2009; Levi et al., 2001b) that many disease resistance genes were lost during watermelon domestication.

To alleviate the problem of low genetic diversity, plant breeders often use CWRs (Maxted et al., 2006) as a source of variation in cultivated crops (Gill et al., 2011; Hajjar and Hodgkin, 2007; Zamir, 2001). The CWRs of watermelon include *C. lanatus* subspecies (infraspecific) as well as the other three diploid (2n = 2x = 22) *Citrullus* species, *C. ecirrhosus*, *C. colocynthis,* and *C. rehmii*, which are all cross-compatible to some degree (Robinson and Decker-Walters, 1997). Infraspecific classification within *C. lanatus* is not clear-cut and various interpretations of the description by Jeffrey (2001) and the designation used by the U.S. Department of Agriculture–Agricultural Research Service National Plant Germplasm System (Plant Genetic Resources Conservation Unit, Southern Regional Plant Introduction Station, Griffin, GA; http://www.ars-grin.gov/npgs/index.html) seem to be applied differently by different researchers (Guo et al., 2013; Jarret et al., 1997; Nimmakayala et al., 2009; Sandlin et al., 2012). In this study, we will refer to samples as cultivars (*C. lanatus* var. *lanatus*), citron types (*C. lanatus* var. *citroides*), or egusi types (*C. lanatus* subsp. *mucosospermus*).

Citron and egusi types have many desirable characteristics, especially disease resistance (Boyhan et al., 1992, 1994; Gillaspie and Wright, 1993; Gusmini et al., 2005; Martyn and Bruton, 1989; Martyn and Netzer, 1991; Sowell, 1975; Strange et al., 2002) and because they can be readily crossed with watermelon cultivars, they are obvious candidates for use in breeding programs. However, high levels of marker segregation distortion, low fruit set, and diminished pollen viability have been observed in mapping populations developed from crosses between cultivars and citron types (Hawkins et al., 2001; Levi et al., 2004b; Prothro, 2010; Ren et al., 2012; Sandlin et al., 2012). Male fertility can be one of the causes of segregation distortion (Taylor and Ingvarsson, 2003; Törjék et al., 2006) and pollen viability is often used as a convenient and quick way to estimate male fertility (Coulibaly et al., 2003). Interspecific F_1 pollen viability has also been an indicator of chromosomal rearrangements and abnormal meiotic behavior (Chandler et al., 1986; Lai et al., 2005; Pertuzé et al., 2002) and Quillet et al. (1995) found that quantitative trait loci associated with pollen viability were located in regions with segregation distortion. Reduced recombination and segregation distortion can hamper the use of CWRs in plant breeding as a result of linkage drag and deviations from expected inheritance ratios in progeny (Quillet et al., 1995; Verlaan et al., 2011; Zamir, 2001). The study of TYLCV resistance (Ty-1) in tomato very elegantly demonstrates the challenges presented by suppressed recombination during CWR trait introgression (Verlaan et al., 2011).

The difficulties watermelon breeders have experienced trying to introgress fusarium wilt race 2 resistance from the citron-type PI 296341-*FR* (Martyn and Netzer, 1991; Netzer and Martyn, 1989) into elite cultivars (Wechter et al., 2012) suggests that despite their easy crossability, introgression of traits might be hampered by linkage drag. Although the resistance has been incorporated into the pollenizer SP-4 (Syngenta Seeds Inc., 2009, 2010), 30 years after it was described, no edible elite cultivars have been developed using this resistance. A high level of segregation distortion was observed in the F_2 mapping population developed from a cross between PI 296341-*FR* (citron type) and 'New Hampshire Midget' (Hawkins et al., 2001).

F_1 hybrids between *C. lanatus* × *C. colocynthis* showed diminished pollen viability that depended on the directionality of the crosses (Boyhan, 1994; Khosoo, 1955; Sain and Joshi, 2003; Sain et al., 2002; Shimotsuma, 1960), suggesting that cytoplasmic factors were involved. The authors also reported that pollen viability was dependent on the particular parental lines used (Sain and Joshi, 2003; Sain et al., 2002).

Citron and egusi types are important sources for introgression of useful traits into watermelon cultivars and investigating pollen viability in infraspecific populations is a useful tool to study the potential obstacles to this approach. It would be useful to know which factors affect pollen viability, specifically those aspects that can be controlled by the breeder.

The aim of this study was therefore to compare pollen viability of F_1 hybrids between watermelon cultivars and potential wild *C. lanatus* CWRs to determine whether 1) the choice of cultivar and/or donor line;

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Table 1. *Citrullus lanatus* cultivars and crop wild relative donor lines used in this study.

Name	Description	Resistance	Supplier	Pollen collection
Charleston Gray	Cultivar	—	Burpee	Greenhouse and field
Crimson Sweet	Cultivar	—	SeedWay	Greenhouse and field
Sugar Baby	Cultivar	—	Reimer Seeds	Greenhouse and field
PI 189225	Donor (citron type)	Gummy Stem Blight (Sowell, 1975) Anthracnose (race 2) (Sowell et al., 1980)	USDA-ARS Germplasm collection[z]	Greenhouse and field
PI 244019	Donor (citron type)	Gummy Stem Blight, WMV-2 (Gillaspie and Wright, 1993), ZYMV (Strange et al., 2002), PRSV-W (Strange et al., 2002)	USDA-ARS Germplasm collection[z]	Greenhouse and field
PI 296341	Donor (citron type)	Fon 0, 1, and 2 (Martyn and Netzer, 1991; Netzer and Martyn, 1989)	USDA-ARS Germplasm collection[z]	Greenhouse and field
PI 595203[y]	Donor (egusi type)	WMV-2 (Gillaspie and Wright, 1993), ZYMV (Boyhan et al., 1992), PRSV-W (Strange et al., 2002)	G.E. Boyhan (University of Georgia, Athens, GA)	Field only

[z]U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS), National Plant Germplasm System, Plant Genetic Resources Conservation Unit, Southern Regional Plant Introduction Station, Griffin, GA.
[y]Also known as Egun (Ling et al., 2009; Murphy and Dane, 2009).
PRSV-W = *Papaya ringspot virus* type-W; WMV-2 = *Watermelon mosaic* 2; ZYMV = *Zucchini yellow mosaic virus*.

and 2) the directionality of the crosses influences pollen viability.

Materials and Methods

Plant material. Seed was obtained for three commercial watermelon cultivars (Charleston Gray, Crimson Sweet, and Sugar Baby) and four accessions (PI 189225, PI 244029, PI 296341, and PI 595203). These specific PIs were chosen because they are sources of resistance to important diseases (Table 1) and therefore likely to be used in breeding efforts. Throughout the study, we refer to these resistant accessions as donor lines, irrespective of their improvement status. Crosses were carried out in the greenhouse between all the cultivars and the donor lines in both directions. Flower emasculation was carried out where required and F_1 hybrid identity was confirmed using simple sequence repeat markers (data not shown).

Preliminary pollen germination and pollen staining comparison. Flowers from a single plant of each of the parental cultivars/lines and a subset of seven F_1 hybrids were used to compare pollen germination and two pollen staining methods. The method of Yi et al. (2003) as described by Wetzstein et al. (2011) was used to determine pollen germination percentage. Initially sucrose concentrations (w/v) between 5% and 20% were tested on parental materials and subsequent tests were carried out using 10% sucrose because it yielded a high percentage of uniform germination for all parental lines after 1 h incubation (data not shown). Three hundred pollen grains were counted per plant and all germination tests were carried out before 1030 HR on the day pollen was collected.

Pollen from a single flower of each plant was also fixed in Carnoy solution 2 (6 ethanol: 3 chloroform : 1 acetic acid) and dissected onto two microscope slides. One was stained using 1% acetocarmine and the other with the modified Alexander method (Peterson et al., 2010).

Pollen viability. Pollen was collected from all parents and hybrids in the greenhouse and the field in 2012, except for PI 595203 and crosses involving PI 595203, for which pollen was only collected in the field. In the greenhouse, pollen was collected from plants that were sown in seedling trays (cell size 3.1 cm × 3.1 cm × 2.3 cm; Landmark Plastic Corp., Akron, OH) filled with Fafard 3B mix (Conrad Fafard, Inc., Agawam, MA) and 0.37 g Osmocote (14N–4.2P–11.6K; Scotts Miracle-Gro, Marysville, OH) per cell in the University of Georgia South Milledge greenhouses in Athens, GA, as part of the regular watermelon breeding program. Artificial light was supplied for 14 h per day and the temperature ranged from 22 to 38 °C. Plants were fertilized once a week with water-soluble fertilizer (20N–8.7P–16.6K) to provide 200 ppm nitrogen. Pollen from newly opened flowers was dusted onto microscope slides and 1% acetocarmine (Sain and Joshi, 2003; Sain et al., 2002) was immediately added and a coverslip was sealed over the stained pollen.

In Summer 2012, seedlings from all parental cultivars/lines and hybrids were transplanted in the field 4 weeks after sowing (14 May 2012) in a randomized complete block design with 4-ft in-row spacing and 6-ft between-row spacing. Plants were grown according to University of Georgia Cooperative Extension Service recommendations. To prevent cross-contamination of pollen between flowers by bees, unopened buds were collected 1 d before they were estimated to open (petals turned yellow) and fixed in Carnoy solution 2 for 36 h. Buds were stored in 70% ethanol at 4 °C until acetocarmine slides were prepared by opening the petals and using a dissecting needle to disrupt the anthers and release the pollen onto a microscope slide.

In both the field and greenhouse, three flowers were collected from each of four plants for each parental cultivar/line and hybrid. At least 100 pollen grains were counted for each flower using a light microscope. Stained, uniform pollen were considered viable, while unstained, irregular pollen were considered non-viable (Fig. 1).

Statistical analysis. Data collected from the three flowers of the same plant were averaged before analysis. To determine whether there was any difference between the data collected in the greenhouse and field, initial analysis was carried out only on parental and hybrid data that were collected in both locations. Subsequently, the data from the two locations were pooled (n = 8) and the data from PI 595203 hybrids (field only; n = 4) were added for all analysis. Analysis of variance (ANOVA) was used to determine the significance of the cultivar, donor line, and directionality (of the cross) main effects and their interactions. Tukey's honestly significant difference test was used to separate the least square means of significant effects. All analyses were carried out using JMP Pro Version 10.0.1 (SAS Institute, Cary, NC).

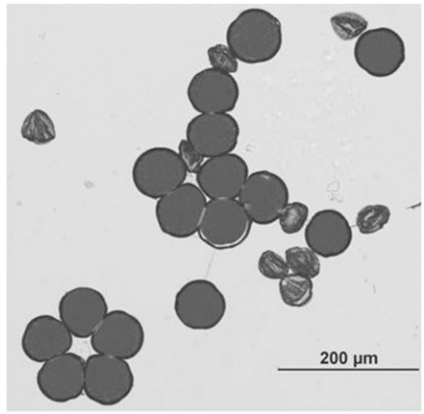

Fig. 1. Pollen from a cross between PI 244019 (citron type) and the cultivar Sugar Baby stained with 1% acetocarmine. Stained, uniform pollen were considered viable, whereas unstained, irregular pollen were considered non-viable.

Results and Discussion

A preliminary study was carried out to determine the validity of pollen staining as a way to estimate pollen viability. Significant correlations ($P < 0.01$) of 0.83 and 0.84 were

observed between pollen germination results and the acetocarmine and the modified Alexander staining method, respectively. Germination results were generally lower than results obtained with pollen staining (data not shown). The observed correlation between pollen staining and pollen germination is higher than correlations deemed sufficient to justify the use of pollen staining to estimate pollen viability in other crops (Rodriguez-Riano and Dafni, 2000). There was no significant difference between the results from the two staining methods and the correlation between the two methods was high (0.93).

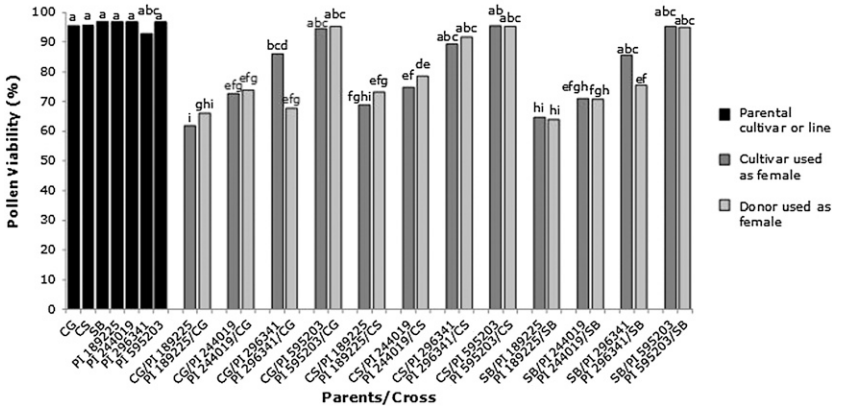

Fig. 2. Average pollen viability (%) of parental cultivars, donor lines, and their F_1 hybrids. Different letters indicate significant differences ($P < 0.05$) by Tukey's honestly significant difference test. CS = 'Crimson Sweet'; CG = 'Charleston Gray'; SB = 'Sugar Baby'.

Table 2. Cultivar (C), donor (D), and cross-directionality main and interaction effects for pollen viability of F_1 infraspecific *Citrullus lanatus* crosses.

Level	Pollen Viability (%)	Level	Pollen Viability (%)
Cultivar**[z]		Donor × directionality**	
CS	83.4 a[y]	PI 595203, D/C	95.2 a
SB	77.7 b	PI 595203, C/D	95.1 a
CG	77.3 b	PI 296341, C/D	87.1 b
		PI 296341, D/C	78.3 c
Donor**		PI 244019, D/C	74.3 d
PI 595203	95.2 a	PI 244019, C/D	72.8 d
PI 296341	82.7 b	PI 189225, D/C	67.7 e
PI 244019	73.6 c	PI 189225, C/D	65.2 e
PI 189225	66.4 d		
		Cultivar × donor × directionality**	
Cultivar × donor**		CS, PI 595203, C/D	95.5 a
CS, PI 595203	95.4 a	CS, PI 595203, D/C	95.3 a
SB, PI 595203	95.2 a	SB, PI 595203, C/D	95.3 a
CG, PI 595203	94.9 a	CG, PI 595203, D/C	95.2 a
CS, PI 296341	90.6 a	SB, PI 595203, D/C	95.1 a
SB, PI 296341	80.6 b	CG, PI 595203, C/D	94.5 a
CG, PI 296341	77.0 bc	CS, PI 296341,D/C	91.7 a
CS, PI 244019	76.6 bc	CS, PI 296341, C/D	89.5 a
CG, PI 244019	73.3 cd	CG, PI 296341, C/D	86.1 ab
CS, PI 189225	71.0 d	SB, PI 296341, C/D	85.7 ab
SB, PI 244019	70.9 d	CS, PI 244019, D/C	78.5 bc
SB, PI 189225	64.3 e	SB, PI 296341, D/C	75.5 cd
CG, PI 189225	63.9 e	CS, PI 244019, C/D	74.7 cd
		CG, PI 244019, D/C	73.9 cde
Directionality NS		CS, PI 189225, D/C	73.1 cde
C/D	77.9	CG, PI 244019, C/D	72.8 cde
D/C	76.6	SB, PI 244019, C/D	71.1 cdefg
		SB, PI 244019 D/C	70.7 cdefg
Cultivar × directionality*		CS, PI 189225, C/D	68.9 defgh
CS, D/C	84.6 a	CG, PI 296341, D/C	67.8 defgh
CS, C/D	82.1 ab	CG, PI 189225, D/C	66.1 efgh
SB, C/D	79.2 bc	SB, PI 189225, C/D	64.8 fgh
CG, C/D	78.8 bc	SB, PI 189225, D/C	63.9 gh
SB, D/C	76.3 c	CG, PI-A, C/D	61.8 h
CG, D/C	75.7 c		

[z]Significance of main and interaction effects as determined by analysis of variance, where NS = nonsignificant, $*P < 0.05$ and $**P < 0.0001$.

[y]Values followed by the same letters within a column within each effect are not significantly different ($P < 0.05$) by Tukey's honestly significant difference test.

CS = 'Crimson Sweet'; CG = 'Charleston Gray'; SB = 'Sugar Baby'.

The acetocarmine method was preferred for the current study to make results comparable with those obtained previously for *Citrullus* (Boyhan, 1994; Sain and Joshi, 2003; Sain et al., 2002).

For the six parental cultivars, donor citron lines, and the 18 F_1 hybrid progeny lines where data were collected in both the greenhouse and field, there was no statistically significant difference ($\alpha = 0.05$) between the results from the two locations (data not shown). The data were pooled and analyzed together for the remainder of the analysis. Similar results were reported by Sain and Joshi (2003) who found no significant difference between pollen collected in different seasons for interspecific F_1 *Citrullus* progeny.

The average pollen viability of the parental cultivars and lines ranged from 92.9% to 96.9% and did not differ significantly from one another (Fig. 2). Previous studies in *Citrullus* reported similar pollen viability within cultivars/lines (Sain et al., 2002; Trivedi and Roy, 1970). The pollen viability of the F_1 hybrids ranged from 61.8% ('Charleston Gray'/PI 189225) to 95.5% ('Crimson Sweet'/PI 595203). This range partially overlaps with the 18.5% to 84.3% observed in *C. lanatus* × *C colocynthis* hybrids (Sain and Joshi, 2003; Sain et al., 2002) but with higher lower and upper limits.

The pollen viability for the crosses involving PI 595203 ranged from 94.5% to 95.5% and there were no significant differences between any of the PI 595203 F_1 hybrids and, in fact, the pollen viability of the hybrids was not different from the parental cultivars or lines (Fig. 2). ANOVA of the F_1 progeny data identified significant main effects for the cultivar and donor parent used as well as significant interaction effects (Table 2). Hybrids with 'Crimson Sweet' as the cultivar parent had the highest pollen viability, whereas using 'Charleston Gray' or 'Sugar Baby' produced hybrids with lower pollen viability. Hybrids with PI 595203 as the donor parent had the highest pollen viability, whereas crosses involving PI 189225 had the lowest pollen viability (Table 2). The high pollen viability of PI 595203 hybrids supports previous molecular data that show that watermelon cultivars are more closely related to egusi types than citron types (Che et al., 2003; Dane and Lang, 2004; Dane and Liu, 2007; Guo et al., 2013; Jarret et al., 1997; Nimmakayala et al., 2009) and that cultivar × egusi type mapping populations have low segregation distortion (Sandlin et al., 2012). From a practical breeding standpoint this means that it should be easier to introgress traits from PI 595203 than the citron lines. Only one egusi line was tested in the current study and it remains to be seen whether this will also be true for other egusi lines. The close molecular relationship between egusi types and cultivars (Guo et al., 2013) strongly suggests broad applicability of this result. However, breeders often do not have a choice of which donor line to use, because often only a single source of a desirable trait is available. In such cases it would

be advantageous to use several different cultivars because the cultivar also has an effect on hybrid pollen viability, although the difference was not as dramatic (Table 2).

There was no significant main effect for the directionality of the cross (cultivar × donor or donor × cultivar) although there were significant interaction effects (Table 2). There was a significant difference between the two reciprocal crosses for two of the parental combinations ('Charleston Gray' and PI 296341 and 'Sugar Baby' and PI 293341). In both of these combinations involving PI 296341, higher pollen viability was observed when using the cultivar as the female parent.

In *C. lanatus* × *C. colocynthis* hybrids, higher pollen viability was observed when using *C. lanatus* as the female parent (Sain and Joshi, 2003; Sain et al., 2002), indicating that pollen viability was influenced by the cytoplasm in the interspecific crosses (Schwarzbach and Rieseberg, 2002). Breeders often prefer to use the cultivar as the female parent because the donor lines are often andromonoecious, which necessitates emasculation of the pistillate flower before crossing. In addition, wild accessions often start flowering much later than the cultivars and because plants usually produce male flowers first, male flowers from wild accessions will overlap with female cultivar flowers earlier than the other way around. Fruit on the cultivar also usually requires less time to reach maturity, which further shortens the generation time. Our results confirm that this is a good strategy and that there is unlikely to be a pollen viability advantage to using the wild accessions as the female parent.

Diminished pollen viability can be the result of genetic factors or an indication of chromosomal rearrangements (Kim and Rieseberg, 1999; Lai et al., 2005; Quillet et al., 1995; Schwarzbach and Rieseberg, 2002). In interspecific sunflower hybrids, loci controlling pollen viability were associated with regions with distorted markers and chromosome rearrangements (Kim and Rieseberg, 1999; Quillet et al., 1995). Although distorted markers are a common occurrence in cultivar × citron watermelon crosses (Hawkins et al., 2001; Levi et al., 2004b; Prothro, 2010; Sandlin et al., 2012), it remains to be seen whether pollen viability is associated with these regions in *C. lanatus*. We are currently mapping loci associated with pollen viability in a cultivar × citron F_2 population to further elucidate potential obstacles that may hamper the introgressing of traits from citron types into watermelon cultivars.

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