Resistance Response of *Citrullus* Genotypes to *Stagonosporopsis* spp. Isolates Causing Gummy Stem Blight

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Introduction

Gummy Stem Blight (GSB) is a major fungal disease affecting watermelon (*Citrullus lanatus*) and other cucurbits (Sherbakoff 1917; Chiu and Walker 1949; Sherf and MacNab 1986). The disease is also commonly known as black rot, when infection occurs on fruit (Chiu and Walker 1949; Maynard and Hopkins 1999). It is a serious problem for cucurbit growers, especially in tropical, subtropical and some temperate areas, where the warm and humid conditions are conducive for disease development (Robinson and Decker-Walters 1997). In the southeastern United States (US), GSB was identified as the second most important research priority in watermelon after fusarium wilt (Kousik et al. 2016).

GSB was previously thought to be caused by a single pathogen: *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*). However, it was recently determined that GSB is caused by three species of the genus *Stagonosporopsis: S. cucurbitacearum, S. citrulli* and *S. caricae* (Stewart et al. 2015). Morphologically, the species appear similar, but they differ genetically. Among the three *Stagonosporopsis* species*, S. citrulli* was found to be the most widely distributed worldwide (Brewer et al. 2015; Stewart et al. 2015). The study by Stewart et al. (2015) established that most of the isolates obtained from different hosts in North and South America, Europe, north Africa, and Asia were *S. citrulli*. *S. caricae* isolates, some of which were obtained from *Carica papaya*, were found in samples from North and South America, Asia, and southeast Asia, while *S. cucurbitacearum* were specifically from temperate regions in North America, Europe, Asia, and New Zealand. Within the US, *S. citrulli* was the most abundant, especially in the southeast US, while *S. cucurbitacearum* isolates were more common in northeast US (Stewart et al. 2015).

Several studies have shown that the different pathogen species exhibit variation in fungicide sensitivity (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019). For example, tebuconazole resistance was reported in *S. caricae* isolates, whereas *S. citrulli* and *S. cucurbitacearum* isolates were shown to be sensitive to this fungicide (Li et al. 2016). A subsequent study reported sensitivity to boscalid and fluopyram among all *S. caricae* isolates but varied sensitivity among *S. citrulli* isolates to boscalid (Li et al. 2019). Resistance to thiophanate-methyl has also been detected among *S. citrulli* isolates from East China while most isolates from Florida remained sensitive to this fungicide (Newark et al. 2019). This differential sensitivity poses a major challenge in management of GSB, especially because current management efforts rely heavily on fungicide applications, since no commercial watermelon cultivars currently possess genetic resistance to the GSB in the field.

Cultivated watermelon has a very narrow genetic base as a result of domestication that led to loss of some traits while selecting for desirable fruit quality (Levi et al. 2017; Guo et al. 2019). Other *Citrullus* species have been used as a major source of disease resistance traits for various diseases in watermelon (Boyhan et al. 1994; Guner 2005; Thies and Levi 2007; Tetteh et al. 2010; Wechter et al. 2012; Levi et al. 2017). *Citrullus* germplasm resistant to GSB have been identified as early as 1962 (Sowell and Pointer 1962) and efforts to introgress this resistance into commercial cultivars has been attempted, though unsuccessful (Norton 1979; Norton et al. 1986; Norton et al. 1993; Sumner and Hall 1993; Norton et al. 1995; Song et al. 2002). Plant Introduction (PI) 189225 was initially identified as the most resistant accession evaluated from the USDA-ARS watermelon germplasm collection (Sowell and Pointer 1962). PI 271778, was later identified as an additional source of resistance (Sowell 1975; Norton 1979). Both PI 189225 and PI 271778 are wild accessions of *C. amarus,* a close relative of watermelon (Chomicki and Renner 2015; Renner et al. 2017). Crosses between elite cultivars and resistant PIs were made to produce two lines: 'AU-Jubilant' ('Jubilee' x PI 271778) and 'AU-Producer' ('Crimson Sweet' x PI 189225) (Norton et al., 1986). These cultivars have however not shown resistance to GSB in the field (Song et al. 2002).

Gusmini et al. (2005) identified a further ten PIs that displayed significant levels of resistance to GSB under both field and greenhouse conditions. These accessions consisted of genotypes from both *C. amarus* and *C. lanatus* species and included PI 164248, PI 244019, PI 254744, PI 271771, PI 279461, PI 296332, PI 482276, PI 482379, PI 490383 and PI 526233 (Gusmini et al. 2005). Despite all the resistant sources described, breeding efforts for GSB resistant watermelon cultivars have been unsuccessful. With the discovery that GSB can be caused by three different *Stagonosposopsis* species, the question arises whether differential host resistance to the species might be partially responsible for the lack of success in resistance breeding efforts. To date, no studies have examined the effect of different *Stagonosporopsis* species on putative resistant *Citrullus* genotypes. It is vital to establish whether the three species have similar host responses. Understanding the level and breadth of resistance found in *Citrullus* genotypes will be essential in determining the appropriate sources of resistance to use in breeding efforts. The objective of this study was therefore to evaluate the level of resistance of 12 different *Citrullus* genotypes to six isolates from three different *Stagonosporopsis* species.

Materials and Methods

Seeds of 12 different *Citrullu*s genotypes that included both wild and elite genotypes were sown in the greenhouse in 48-cell seedling trays, approximately 2 weeks prior to the screen. The genotypes consisted of cultivars and PIs belonging to *C. amarus, C. mucosospermus* and *C. lanatus* species. They included: 'AU-Producer' (AUP), 'Crimson Sweet' (CS), 'Mickylee' (MICK), 'Sugar Baby' (SB), PI 189225, PI 244019, PI 279461, PI 482276, PI 482379, PI 549160, PI 560023 and PI 593359 (Table 1). The panel of genotypes used in this study were specifically chosen to represent a broad genetic background for watermelon. Moreover, five of the PI used in this study (PI 189225, PI 244019, PI 279461, PI 482276, PI 482379) were chosen because they had been previously described as resistant to GSB (Sowell and Pointer 1962; Gusmini et al. 2005)

The *C. lanatus* genotypes used in the study were from North America (CS, AUP, SB and MICK), Asia (Japan: PI 279461; China: PI 593359) and Africa (PI 549160). Many modern watermelon cultivars are related to CS, which is a parent of AUP (Norton et al. 1986; Wehner and Barrett 2002). SB, which is genetically distant from other North American cultivars, is an ancestral parent of MICK (Wehner and Barrett 2002). PI 549160 is a wild *C. lanatus* from northeast Africa, which is a center of domestication for watermelon (Renner et al. 2017). PI 560023 was the only *C. mucosospermus* (egusi) species used in this study. Egusi watermelon are utilized in West Africa for their edible seeds. The *C. amarus* species included PI 244019, PI 482379, PI 482276 and PI 189225 which are from South Africa, Zimbabwe, Zaire and Zimbabwe, respectively.

Six *Stagonosporopsis* isolates, provided by Marin Brewer (University of Georgia, Department of Pathology), were grown (16h/8h light/dark cycle) on potato dextrose agar (PDA) (Becton, Dickinson and Company, NJ, USA) for 2 weeks. Approximately 1 cm^2 agar plugs were then sub cultured on quarter-strength PDA (qPDA) where they were grown for another 2 weeks. The isolates included *S. citrulli:* 12178A and AcSq5, *S. cucurbitacearum:* RT2 and GSB26 and *S. caricae:* GA8007H and RG3 (Stewart et al., 2015; M. Brewer, *personal communication*) (Table 2).

Three independent screens were performed in a growth chamber. During each screen, seven trays (6 isolates and 1 control) were sown, with four seeds of each genotype (12 genotypes total) per tray. On the day of inoculation, qPDA cultures were flooded with 10 ml of 0.1% tween20 and gently scraped with a sterile spatula to release spores. The inoculum was filtered through 2 layers of sterile cheese cloth and spore concentration was determined using a hemacytometer (Hausser Scientific, PA, USA). Spore concentrations were then adjusted to 5 x 10^5 spores/ml using 0.1% tween20 solution.

At the 2nd true leaf stage, seedling trays were placed in plastic tubs and each tray was sprayed with freshly made inoculum from one isolate using an airbrush sprayer (Master Airbrush Model E91) for 60 seconds. The control tray was sprayed with a mock inoculation consisting of 0.1% tween20 solution. The tubs were then sealed in a transparent, plastic bag to promote high relative humidity of approximately 95% which was measured using a data logger (Lascar Electronics UK). The tubs were placed in a growth chamber set to 26 °C day and 23 °C night with a 12h/12h light/dark cycle. On the 3rd day post-inoculation (dpi), the trays were removed from the tubs and disease severity data was collected 7dpi. Disease symptoms were evaluated on a scale of 0 to 9 as described by (Lou et al. 2013), where 0 = no disease; 1 to 2 = mild trace of infection with less than 10% of leaves covered with lesions; 3 to 4 = 10 to 20% of leaves covered with lesions, 5 to 6 = 21 to 50 % of the leaves covered with small lesions; 7 to 8 = wilting plant and more than 50 % of the leaves covered with lesions; and 9 = dead plant.

Statistical analyses were conducted using a fitted mixed linear model in R, whereby genotype, isolate and their interaction were the fixed effects while screen was treated as a random effect. Post hoc comparisons among groups after fitting the model were done using emmeans to obtain treatment values and significance levels after taking into account other terms in the model. Hierarchical cluster analysis

was performed for both the isolates and the genotypes using JMP® Pro 14.1.

Results and Discussion

No lesions were observed on mock inoculated plants in any of the screens. One of the isolates, *S. citrulli* AcSq5 had slightly lower spore concentration (4.34 x 10^5 spores/ml) in the first screen. In the subsequent screens, spores were not observed and therefore data from only one replication was included in the analysis for this isolate. In the treated trays, similar trends were observed in the three screens with *S. citrulli* 12178A exhibiting higher aggressiveness than the other isolates, with most of the seedlings dead by 7dpi (data not shown). Results of the ANOVA indicated a significant difference between the watermelon genotypes used ($P < 0.001$) as well as the isolates ($P < 0.001$), but no significant genotype × isolate interaction (Table 3).

S. citrulli 12178A and *S. caricae* RG3 were significantly the most aggressive of the isolates, followed by *S. cucurbitacearum* RT2 (Fig. 1). The least aggressive isolate was *S. cucurbitacearum* GSB26, however it was not significantly different from *S. caricae* GA8007H and *S. citrulli* AcSq5. Based on the hierarchical cluster analysis the isolates formed two major clusters, with *S. citrulli* 12178A and *S. caricae* RG3 diverged from the four other isolates (Fig. 1). These results indicate that the level of aggressiveness was not species-dependent and that certain isolates within a species could be more aggressive than others.

The watermelon genotypes exhibited a wide distribution of resistance levels to the different isolates of *Stagonosporopsis* (Fig. 1) as would be expected from our choice of genotypes. The genotypes separated into two major clades in the hierarchical cluster analysis, with the *C. amarus* genotypes forming one clade and all the *C. lanatus* and the *C. mucosospermus* (PI 560023) genotypes in the other clade. Among the genotypes, PI 189225 and PI 482276 were generally more resistant than the other genotypes and they clustered together. These two lines had been previously described as resistant to GSB (Norton et al. 1993; Gusmini et al. 2005) and this study confirms their broad resistance to GSB isolates. The other two *C. amarus* lines, PI 482379 and PI 244019 also displayed intermediate resistance to most of the isolates, however the latter was more susceptible to *S. cucurbitacearum* RT2.

AUP had the highest disease severity score overall (7.51) followed by SB (7.26), CS (7.09) and PI 279461 (6.63) (Fig. 1). AUP and CS clustered together in the hierarchical clade. It is worth noting that AUP, which was formerly described as resistant to GSB (Norton et al. 1986) but demonstrated to be susceptible in the field (Song et al. 2002), only showed resistance to *S. cucurbitacearum* GSB26, the least severe of the isolates tested (Fig. 1). PI 279461 was among the most resistant lines described by Gusmini et al. (2005) but displayed high disease severity in the present study. Similar to AUP, it seemed slightly more resistant to the least aggressive *S. cucurbitacearum* GSB26. It is tempting to speculate that an isolate similar to *S. cucurbitacearum* GSB26 was used in these studies for phenotyping, but the current study does not allow us to determine that with any certainty. AUP however displayed very high susceptibility to all other isolates, confirming the susceptibility of this cultivar to GSB. The elite cultivars were generally susceptible to the various isolates (Fig. 1). PI 189225 (2.89) and PI 482276 (2.83) were more resistant than the other genotypes across isolates. The results observed on these genotypes confirm the resistance of these two *C. amarus* genotypes against GSB as previously described by Norton et al., (1993) and Gusmini et al., (2005).

This study confirms that some *Stagonosporopsis* isolates are more aggressive than others, but with the isolates tested in this study, there is no pattern of aggressiveness within species. The two most aggressive isolates (12178A and RG3), which were *S. citrulli* and *S. caricae*, respectively, were originally obtained from *C. lanatus* hosts, therefore it could be argued that there could be some host specificity. However, RT2, which also displayed high aggressiveness, was obtained from *Cucurbita moschata*, while GA8007H which displayed lower aggressiveness was isolated from watermelon (Stewart et al., 2015). One limitation of this study was that one isolate (AcSq5) only had one replication due to low sporulation.

Our results could explain the inconsistency that has been observed with GSB phenotyping in different research programs and why efforts to introgress GSB resistance into commercial cultivars have been complex and unsuccessful. It is possible that different *Stagonosporopsis* isolates with varying levels of aggressiveness are used for phenotyping, especially considering the pathogen in the screens is only referred to as *Didymella bryoniae*. It is also highly likely that a mixture of isolates exists in the field (Brewer et al., 2015). This further complicates the breeding process for GSB resistance. From the results of this study, it should be noted that phenotyping using a less aggressive isolate may confer resistance to the specific isolate, but when the genotype is challenged with a more aggressive isolate present in the field, it may not survive. Results from Gusmini et al. (2017) also displayed large environmental effects associated with GSB, which would impact the severity of symptoms observed in the field.

It is still unknown whether the same resistant loci in *Citrullus* genotypes confer broad resistance against different *Stagonosporopsis* isolates. Utilization of highly aggressive

Stagonosporopsis isolates during GSB resistance breeding provides a greater likelihood of obtaining field-level resistance to natural GSB epidemics. Knowledge of the effect of different *Stagonosporopsis* isolates on *Citrullus* genotypes may inform breeders on the appropriate resistance sources and pathogen isolates to utilize for breeding. These results can inform watermelon breeders in developing strategies for phenotyping and resistance loci deployment when breeding for GSB resistance.

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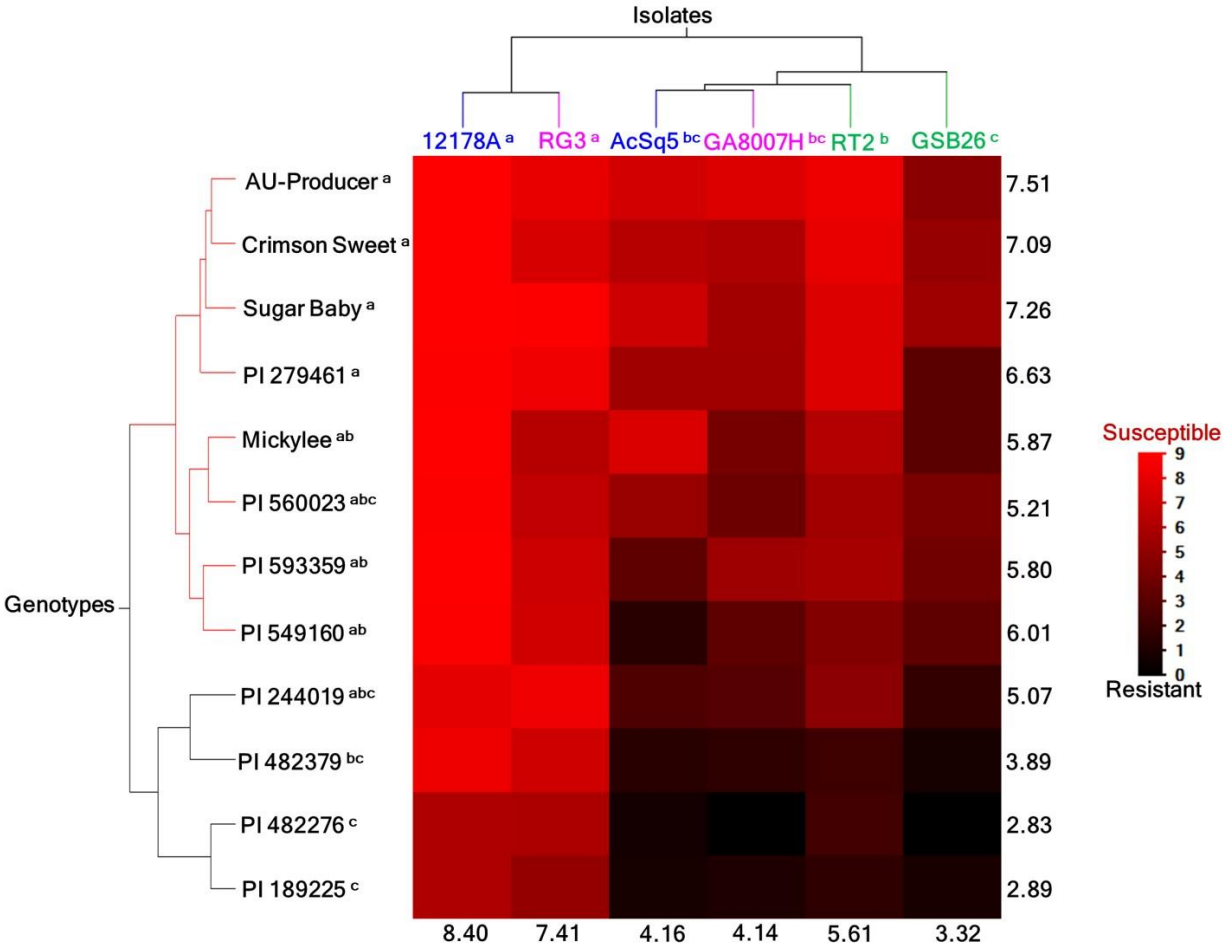

Fig. 1 Heat map displaying the disease severity of different *Citrullus* genotypes (x-axis) against various *Stagonosporopsis* spp. isolates (y-axis). The isolates are *S. citrulli* (blue), *S. caricae* (pink) and *S. cucurbitacearum* (green). On the right and bottom are the mean severity scores for each genotype and isolate, respectively. Levels not connected by the same letter (superscript) are significantly different.

Table 1. Seed sources for *Citrullus* genotypes used in this study.

Genotype	Seed source	Species
'AU-Producer' (AUP)	Hollar Seeds	*C. lanatus*
'Crimson Sweet' (CS)	Seedway Seeds	*C. lanatus*
'Sugar Baby' (SB)	Reimer Seeds	*C. lanatus*
'Mickylee' (MICK)	Hollar Seeds	*C. lanatus*
PI 279461	USDA-ARS, Griffin, GA	*C. lanatus*
PI 593359	USDA-ARS, Griffin, GA	*C. lanatus*
PI 549160	USDA-ARS, Griffin, GA	*C. lanatus*
PI 560023	USDA-ARS, Griffin, GA	*C. mucosospermus*
PI 244019	USDA-ARS, Griffin, GA	*C. amarus*
PI 482379	USDA-ARS, Griffin, GA	*C. amarus*
PI 482276	USDA-ARS, Griffin, GA	*C. amarus*
PI 189225	USDA-ARS, Griffin, GA	*C. amarus*

Table 2. Sources of isolates used in this study (Stewart et al., 2015, M. Brewer, *personal communication*).

Isolate name	Original host species	State of origin	*Stagonosporopsis* spp.
12178A	*Citrullus lanatus* (watermelon)	Georgia	*S. citrulli*
AcSq5	*Cucurbita pepo* (acorn squash)	North Carolina	*S. citrulli*
RG3	*Citrullus lanatus* (watermelon)	California	*S. caricae*
GA8007H	*Citrullus lanatus* (watermelon)	Georgia	*S. caricae*
RT2	*Cucurbita moschata* (butternut squash)	Michigan	*S. cucurbitacearum*
GSB26	*Cucumis melo* (muskmelon)	New York	*S. cucurbitacearum*

Table 3. Analysis of variance for mean disease severity scores of 12 watermelon genotypes inoculated with six *Stagonosporopsis* species isolates.

Source of variation	Sum Sq	Mean Sq	DF	F value	Pr (>F)
Genotype***	422.99	38.45	11	7.14	3.644×10^{-9}
Isolate***	671.30	134.26	5	24.92	$< 2.2\times10^{-16}$
Genotype × Isolate^{NS}	142.04	2.58	55	0.48	9.986×10^{-1}