Inheritance of Resistance to Gummy Stem Blight in Watermelon

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Abstract. **Gummy stem blight (GSB), caused by three related species of *Stagonosporopsis* [*Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*), *Stagonosporopsis citrulli*, and *Stagonosporopsis caricae*], is a major disease of watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] in most production areas of the United States. We studied the inheritance of resistance to GSB using three PI accessions of watermelon. Four families of six progenies (P_r, P_s, F_1, F_2, BC_1P_r, and BC_1P_s) were developed from four crosses of resistant PI accessions by susceptible cultivars. Each family was tested in 2002 and 2003 in North Carolina under field and greenhouse conditions for resistance to GSB. Artificial inoculation was used to induce uniform and strong epidemics. The effect of the Mendelian gene for resistance, *db*, was tested. Partial failure of the data to fit the single-gene inheritance suggested that resistance to GSB of PI 482283 and PI 526233 may be under the control of a more complex genetic system.**

Gummy stem blight is a major disease of watermelon [*C. lanatus* (Thunb.) Matsum. & Nakai]. It is caused by three genetically distinct *Stagonosporopsis* species, *S. cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae* (Stewart et al., 2015). The three species are pathogenic to cucurbits, but *S. caricae* also causes leaf spot and stem and fruit rot in papaya (*Carica papaya*) (Stewart et al., 2015). This disease was first observed in 1891 by Fautrey and Roumeguere in France on cucumber (*Cucumis sativus* L.) and in Delaware in watermelon (Chiu and Walker, 1949; Sherf and MacNab, 1986). In 1917, GSB was reported in the Southern United States, affecting watermelon fruit in Florida (Sherbakoff, 1917). Gummy stem blight remains an important limiting factor for watermelon production in Florida (Keinath, 1995; Power, 1992). Gummy stem blight on watermelon plants is evident as crown blight, stem cankers, and extensive defoliation, with symptoms observed on the cotyledons, hypocotyls, leaves, and fruit (Maynard and Hopkins, 1999). *Stagonosporopsis cucurbitacearum* is seedborne (Lee et al., 1984), airborne (van Steekelenburg, 1983), and soilborne (Bruton, 1998; Keinath, 1996).

Adequate control of GSB through fungicide applications (Keinath, 1995, 2000, 2016) and appropriate cultural practices (dos Santos et al., 2016; Rankin, 1954; Keinath,

1996) is difficult, particularly during rainfall when relative humidity remains high for extended times (Café-Filho et al., 2010). In addition, there is concern among pathologists and breeders for the development of resistance by *S. cucurbitacearum* to fungicides (Avenot et al., 2012; Kato et al., 1984; Keinath and Zitter, 1998; Li et al., 2016; Malathrakis and Vakalounakis, 1983; Miller et al., 1997; Thomas et al., 2012; van Steekelenburg, 1987). Resistance to GSB has received attention since the 1970s as a possible alternative to chemical control (Lou et al., 2013; Norton et al., 1986, 1993, 1995).

Differences in GSB resistance among commercial cultivars of watermelon (*C. lanatus*) were reported, with 'Congo' the least susceptible, 'Fairfax' intermediate, and 'Charleston Gray' the most susceptible (Schenck, 1962). Resistance assays by controlled inoculation of watermelon plants using spore suspensions of *S. cucurbitacearum* identified PI 189225 and PI 271778 as the most resistant accessions available in the USDA-ARS watermelon germplasm collection (Sowell, 1975; Sowell and Pointer, 1962). In crosses with susceptible 'Charleston Gray', a single recessive gene *db* was determined to confer resistance in PI 189225 (Norton, 1979). Resistant watermelon cultivars were developed from two crosses ('Jubilee' × PI 271778 and 'Crimson Sweet' × PI 189225) by selecting disease-resistant seedlings from backcrossed families that had a high yield of excellent quality fruit (Norton et al., 1986). 'AU-Jubilant', 'AU-Producer' (Norton et al., 1986), 'AU-Golden Producer' (Norton et al., 1993), and 'AU-Sweet Scarlet' (Norton et al., 1995) were released, with moderate resistance to GSB. However, they were found less resistant to GSB than the resistant parents PI 189225 and PI 271778. To date, no cultivars of

watermelon have been released that have a high level of resistance to natural epidemics of GSB.

The expanding watermelon industry in the southeastern United States and the increasing losses due to GSB outbreaks in the last decade led to a new set of studies for the use of genetic resistance to control GSB in watermelon (Gusmini et al., 2005; Li and Brewer, 2016). The watermelon breeding program at North Carolina State University developed an efficient screening method for testing watermelon germplasm (Gusmini and Wehner, 2002; Song et al., 2004), including systems for mass production of inoculum of *S. cucurbitacearum* for large field screening experiments (Gusmini et al., 2003), and a disease assessment scale for rating foliar and stem lesions (Gusmini et al., 2002). Available PI accessions (totaling 1274) from the USDA-ARS watermelon germplasm collection, along with 51 adapted cultivars, were tested to identify new genetic sources of resistance to GSB (Gusmini et al., 2005). A total of 59 new accessions were identified that had resistance to GSB as good as or better than PI 189225 and PI 271778 at the field and greenhouse tests. Two of the best were PI 482283 and PI 526233.

The objective of this study was to determine the inheritance of resistance to GSB in watermelon accessions PI 482283 and PI 526233, along with the previously identified accession PI 189225. Because of the unsuccessful breeding history for this trait, we hypothesize that resistance to GSB is due to a more complex mode of inheritance, which will be tested by validating the monogenic inheritance of *db* gene in PI 482283 and PI 526233.

Material and Methods

Plant material. We used four families developed from the four crosses PI 189225 × 'NH Midget', PI 482283 × 'NH Midget', PI 482283 × 'Calhoun Gray', and PI 526233 × 'Allsweet'. 'NH Midget', 'Calhoun Gray', 'Allsweet', and PI 526233 were *C. lanatus* subsp. *vulgaris* (Chomicki and Renner, 2015). PI 189225 and PI 482283 were *Citrullus amarus* (Chomicki and Renner, 2015). PI 189225, PI 482283, and PI 526233 were used as resistant parents, and 'NH Midget', 'Calhoun Gray', and 'Allsweet' were used as susceptible parents (Gusmini et al., 2005). The cultivars were obtained from commercial seed stocks, and the PI accessions were obtained from the Southern Regional Plant Introduction Station at Griffin, GA. For each family, we developed six progenies (P_r, P_s, F_1, F_2, BC_1P_r, and BC_1P_s) using greenhouses at North Carolina State University in Raleigh, NC.

Plating and management. In the greenhouse, temperatures averaged 23 to 43 °C (0800–2000 HR) and 12 to 24 °C (2000–0800 HR) when the assays were performed. We seeded directly in plastic pots (100 × 100 mm size, 600 mL volume) filled with a soilless mix (Canadian sphagnum peatmoss, perlite,

vermiculite, processed pine bark). We planted two seeds per pot and thinned to one to ensure a uniform experiment. In the field, seeds were sown on raised, shaped beds on 3.1 m centers in single hills, 1.2 m apart. Border rows of the susceptible 'Charleston Gray' and 'Calhoun Gray' were planted around each test.

We conducted our tests in greenhouses at North Carolina State University in Raleigh, NC, and in the field at the Horticultural Crops Research Station at Clinton, NC. The two families PI 526233 × 'Allsweet' and PI 482283 × 'Calhoun Gray' were tested in 2002, whereas the other two were tested in 2003.

Inoculum preparation. Originally, the isolate of *S. cucurbitacearum* was obtained from diseased cucumber tissues harvested from naturally infected plants in Charleston, SC, in 1998. In the fall of 2001, we reisolated *S. cucurbitacearum* from watermelon plants that were artificially inoculated with the isolate from South Carolina and developed a new stock of inoculum from single spores. Pycnidia were identified with a dissecting microscope (20×) and transferred to petri plates containing potato dextrose agar (PDA) (25 mL/petri plate). Isolates were selected from the first subculture on PDA based on macroscopic observations: colonies dark in color and showing concentric circles of growth were kept and transferred to fresh PDA. Cultures that did not appear contaminated by other fungi or bacteria were transferred to a medium containing 25% PDA to stimulate abundant sporulation. Finally, we observed pycnidia/pseudothecia and spores to verify that their shape and size matched those of *S. cucurbitacearum* as published (Zitter et al., 1996). For long-term storage (Dhingra and Sinclair, 1995), we transferred the fungus onto sterile filter paper (Whatman #2, 70 mm diameter), subcultured the fungus for 2–4 weeks, dehydrated the filter paper disk and the mycelium for 12–16 h at room temperatures (24 ± 3 °C) under a sterile laminar flow hood, cut the filter paper into squares (5 × 5 mm), and stored them in sterile test tubes in a refrigerator (3 ± 1 °C) in the dark.

Cultures of *S. cucurbitacearum* were grown in Nalgene autoclavable pans (420 × 340 × 120 mm) containing 1000 mL of 50% PDA (Gusmini et al., 2003) before inoculation. We incubated the Nalgene pans for 2–4 weeks at 24 ± 2 °C under alternating periods of 12 h of fluorescent light (40–90 μmol·m^{-2}·s^{-1} photosynthetic photon flux density) and 12 h of darkness until pycnidia formed. For all inoculations, we prepared a spore suspension by flooding the culture plates with 10 mL of sterile, distilled water, and gently scraping the surface of the agar with an L-shaped sterile glass-rod to remove the spores from the mycelia. We filtered the liquid from each pan through four layers of sterile cheesecloth to remove dislodged agar and some mycelia. We measured spore concentration with a hemocytometer and adjusted to a concentration of 5 × 10^5 spores/mL by adding deionized water. Tween 20 (0.06 g·L^{-1}) was added to the inoculum to keep the spores well dispersed in the inoculum solution (Song et al., 2004).

Inoculation. In the greenhouse, we inoculated plants at the second-true-leaf stage (≈2 weeks after planting), after damaging the trichomes on the leaf surface by brushing the plants with a wooden stake 200 mm long and 20 mm wide. The inoculum was delivered using a hand-pumped spray bottle. Immediately after inoculation, we moved the plants into a humidity chamber made of clear polyethylene on the sides and top. The top was kept open during the summer and closed during the winter to keep the internal temperature close to 24 °C, the optimum for *S. cucurbitacearum*. We used humidifiers in the chamber running continuously for the treatment time (1 d before inoculation through 3 d after inoculation) to keep the relative humidity close to 100% day and night. Plants were watered daily using overhead sprinklers when humidifiers were not being used.

In the field, we inoculated plants when they reached the fourth-true-leaf stage (≈4 weeks after planting), after overhead irrigation of about 12 mm of water during the two previous days to promote guttation on the day of inoculation, and damaging the trichomes on the leaf surface at each time before the inoculum was delivered by brushing the plants with a wooden stake 200 mm long and 20 mm wide mounted on an aluminum handle 600 mm long (Lou et al., 2013; Song et al., 2004). Plants were inoculated at least two times at 2-wk intervals by spraying the inoculum onto all upper leaf surfaces. We delivered the inoculum as a fine mist using a backpack-sprayer operated at a pressure of 200–275 kPa (30–40 psi). In the late afternoon of the day of inoculation, we irrigated with ≈12 mm of water to promote disease development with high relative humidity at night.

Data collection. Plants were rated ≈5 weeks after planting (≈3 weeks after inoculation) in the greenhouse, and when symptoms appeared on the leaves and stems of the susceptible checks in the field (≈7 weeks after planting = ≈1 week after the second inoculation). We adopted an ordinal disease assessment scale (Gusmini et al., 2002), with 0 = no disease; 1 = yellowing on leaves (a trace of disease only); 2–4 = symptoms on leaves only; 5 = some leaves dead, no symptoms on stem; 6–8 = symptoms on leaves and stems; 9 = plant dead. Plants with a disease rating of 6 or greater had lesions on the stem, thus being prone to death from subsequent development of the disease. Plants with a disease rating of 5 or less had lesions only on the leaves. Leaf ratings are important because plant yield and survival are affected by leaf area, which is reduced by severe disease outbreaks. Stem ratings are important because large, localized lesions can kill the plant, especially if located near the crown (base) of the plant.

Statistical analysis. We tested the validity of the monogenic inheritance of the *db* gene, described by Norton (1979), after classifying

each plant as susceptible or resistant based on their rank relative to the mean value of the disease assessment scale adopted (4.5). Plants with a disease rating >4.5 were considered susceptible and plants with a disease rating <4.5 were considered resistant (Gusmini et al., 2002, 2005). We performed segregation analysis and the chi-square goodness-of-fit tests ($P < 0.05$) for each F_2 and BC_1P_r progeny with the SAS-STAT statistical package (SAS Institute, Cary, NC) and the SASGENE 1.2 program (Liu et al., 1997).

Yate's correction was used for those chi-square tests where counts were below five for any class in the ratio. However, there was no difference in the conclusion, so we presented the data without the correction to avoid the tendency of Yate's to overcorrect (Conover, 1974; Haviland, 1990).

Results

In our study, several observed segregation ratios for resistance to GSB in watermelon significantly deviated from the expected monogenic inheritance ratios, in both field and greenhouse tests (Table 1). The field and greenhouse tests appeared to be equally useful and mostly consistent in their classification of the resistant and susceptible parent lines. However, there was a combination of environmental variation for disease development and genotype × environment interaction for GSB resistance and perhaps escapes (from inoculation).

In PI 189225 × 'NH Midget', F_2 plants in the field test segregated 74:36 (susceptible: resistant) (Table 1). In this case, the chi-square test would be favorable to the single gene hypothesis except for the small P value. Furthermore, the F_2 greenhouse test did not indicate the validity of the single gene hypothesis suggested by the F_2 segregation ratio in the field. Similarly, the segregation ratios of BC_1P_r plants in the field and greenhouse significantly deviated from the expected ratios (Table 1). Thus, we rejected the hypothesis of a single gene controlling resistance to GSB, as previously reported by Norton in PI 189225.

In PI 482283 × 'NH Midget', both field and greenhouse F_2 segregation ratio chi-square tests reject the null hypothesis of a single gene controlling the expression of resistance to GSB (Table 1). Similarly, greenhouse BC_1P_r segregation ratio chi-square test rejects the null hypothesis of resistance being controlled by a single gene, contributed by PI 482283 (Table 1).

In PI 482283 × 'Calhoun Gray', field F_2 and BC_1P_r segregation ratio chi-square tests failed to reject the null hypothesis of resistance being controlled by a single gene (Table 1). However, the F_1 progeny segregated close to 1:1, thus casting doubts on the validity of the single gene hypothesis. Furthermore, also in this cross, the greenhouse tests did not validate the segregation ratios recorded in the field. The F_2 and BC_1P_r segregation ratio

Table 1. Single locus chi-square goodness-of-fit-test ($P < 0.05$) for resistance to gummy stem blight (GSB) in watermelon.[z]

Progeny	Susceptible[y]	Resistant[x]	Expected[w]	χ^2 value	df	P value
PI 189225 × 'NH Midget'						
Field test						
P_r^v	3	15				
P_s^u	12	0				
F_1	25	0				
F_2	74	36	3:1	3.50	1	0.06
BC_1P_r	32	14	1:1	7.04	1	0.01
BC_1P_s	44	12				
Greenhouse test						
P_r^v	0	40				
P_s^u	40	0				
F_1	60	0				
F_2	242	118	3:1	11.61	1	<0.001
BC_1P_r	98	42	1:1	22.40	1	<0.001
BC_1P_s	136	4				
PI 482283 × 'NH Midget'						
Field test						
P_r^v	0	16				
P_s^u	15	0				
F_1	18	0				
F_2	64	60	3:1	36.17	1	<0.001
BC_1P_r	25	19	1:1	0.82	1	0.36
BC_1P_s	41	17				
Greenhouse test						
P_r^v	0	40				
P_s^u	40	0				
F_1	46	14				
F_2	248	152	3:1	36.05	1	<0.001
BC_1P_r	38	102	1:1	29.26	1	<0.001
BC_1P_s	140	0				
PI 482283 × 'Calhoun Gray'						
Field test						
P_r^v	0	3				
P_s^u	3	0				
F_1	3	4				
F_2	50	11	3:1	1.58	1	0.20
BC_1P_r	8	6	1:1	0.29	1	0.59
BC_1P_s	11	0				
Greenhouse test						
P_r^v	1	2				
P_s^u	3	0				
F_1	6	0				
F_2	45	30	3:1	9.00	1	<0.001
BC_1P_r	18	3	1:1	10.71	1	<0.001
BC_1P_s	12	0				
PI 526233 × 'Allsweet'						
Field test						
P_r^v	1	3				
P_s^u	1	0				
F_1	2	0				
F_2	72	3	3:1	17.64	1	<0.001
BC_1P_r	19	0	1:1	19.00	1	<0.001
BC_1P_s	11	0				
Greenhouse test						
P_r^v	0	3				
P_s^u	3	0				
F_1	5	1				
F_2	31	44	3:1	45.34	1	<0.001
BC_1P_r	15	6	1:1	3.86	1	0.05
BC_1P_s	12	0				

[z]Data are ratings from four families of resistant PI accessions by susceptible cultivars of *C. lanatus* subsp. *vulgaris*. Disease assessment scale adopted for evaluating watermelon for resistance to GSB: 0 = no disease; 1 = yellowing on leaves (suspect of disease only); 2–4 = symptoms on leaves only; 5 = some leaves dead, no symptoms on stem; 6–8 = symptoms on leaves and stems; 9 = plant dead.
[y]Susceptible plants had a disease rating >4.5.
[x]Resistant plants had a disease rating <4.5.
[w]Expected was the hypothesized segregation ratio for single-gene inheritance for each segregating progeny.
[v]P_r was the hypothetic carrier of the recessive gene (*dbdb*).
[u]P_s was the hypothetic carrier of the dominant gene (*DbDb*).

chi-square tests reject the null hypothesis of resistance being controlled by a single gene (Table 1).

In PI 526233 × 'Allsweet', both field and greenhouse F_2 segregation ratio chi-square tests reject the null hypothesis of a single gene controlling the expression of resistance to GSB (Table 1). Also in this cross, field BC_1 P_r segregation ratio chi-square test reject the

null hypothesis of resistance being controlled by a single gene, in this case, contributed by PI 526233. Although greenhouse BC_1P_r segregation ratio chi-square test failed to reject the null hypothesis of resistance being controlled by a single gene, the small P value did not support validity (Table 1).

In Fig. 1, the distribution of our F_2 data displayed continuous variation and deviated from the expected bell-shaped (normal) distribution. This distribution pattern suggests the presence of one or more resistant genes segregating in the progenies.

Discussion

Gummy stem blight has shown significant variability for resistance in cucurbits (Boyhan et al., 1994; Gusmini et al., 2005; Wehner and Shetty, 2000). For example, plant-to-plant variability in four replications of two plants each was 4–8 for 'Charleston Gray' compared with a 2–4 rating for PI 482276 on a 0 to 9 scale (Gusmini et al., 2005). Thus, the resistant PI accession was not more heterogeneous than the pure-line cultivar. In addition, the test for GSB resistance has variation from plant to plant and rep to rep of 2–4 rating units. Observed variability of GSB outbreaks may be the results of genetic or environmental effects (Wehner and Amand, 1993). These effects can modify pathogen aggressiveness, causing differences over years, and between field and greenhouse tests (Gusmini and Wehner, 2002; Stewart et al., 2015). Recently, genetically distinct species have been reported as causal agents of GSB, suggesting that variability of resistance to GSB across years and environments (e.g., greenhouse and field) may be due to interactions of environment with fungal species (Brewer et al., 2015; Keinath, 2014; Stewart et al., 2015).

Resistance to GSB in watermelon has been previously described as dependent solely on the inheritance of the recessive gene db from PI 189225 (Norton, 1979). Watermelon cultivars have been improved by introgression of the db gene, but the cultivars were less resistant than the resistant parent accessions in the field. In addition, PI 189225 was identified as a source of resistance to anthracnose (*Colletotricum obiculare*) race 2 (Sowell at al., 1980). Both *C. obiculare* and *S. cucurbitacearum* were reported as prevalent pathogens, occupying different niches in the plant canopy during field evaluations (Peterson and Campbell, 2002). Interestingly, similarities in the symptomatology of these pathogens could lead to misreading visual evaluations during field outbreaks (Boyhan et al., 1994; Gusmini et al., 2005). In addition, the lack of progress for resistance to GSB in the development of new cultivars could be explained partially by the confounded epidemics of several pathogens during field evaluations (Peterson and Campbell, 2002; Rankin, 1954).

A wide array of testing methods for resistance to GSB have been deployed by breeders during the last three decades to follow the inheritance of the db gene in their populations, and the one adopted in this study was demonstrated to be the most effective of the methods tried (Gusmini and Wehner,
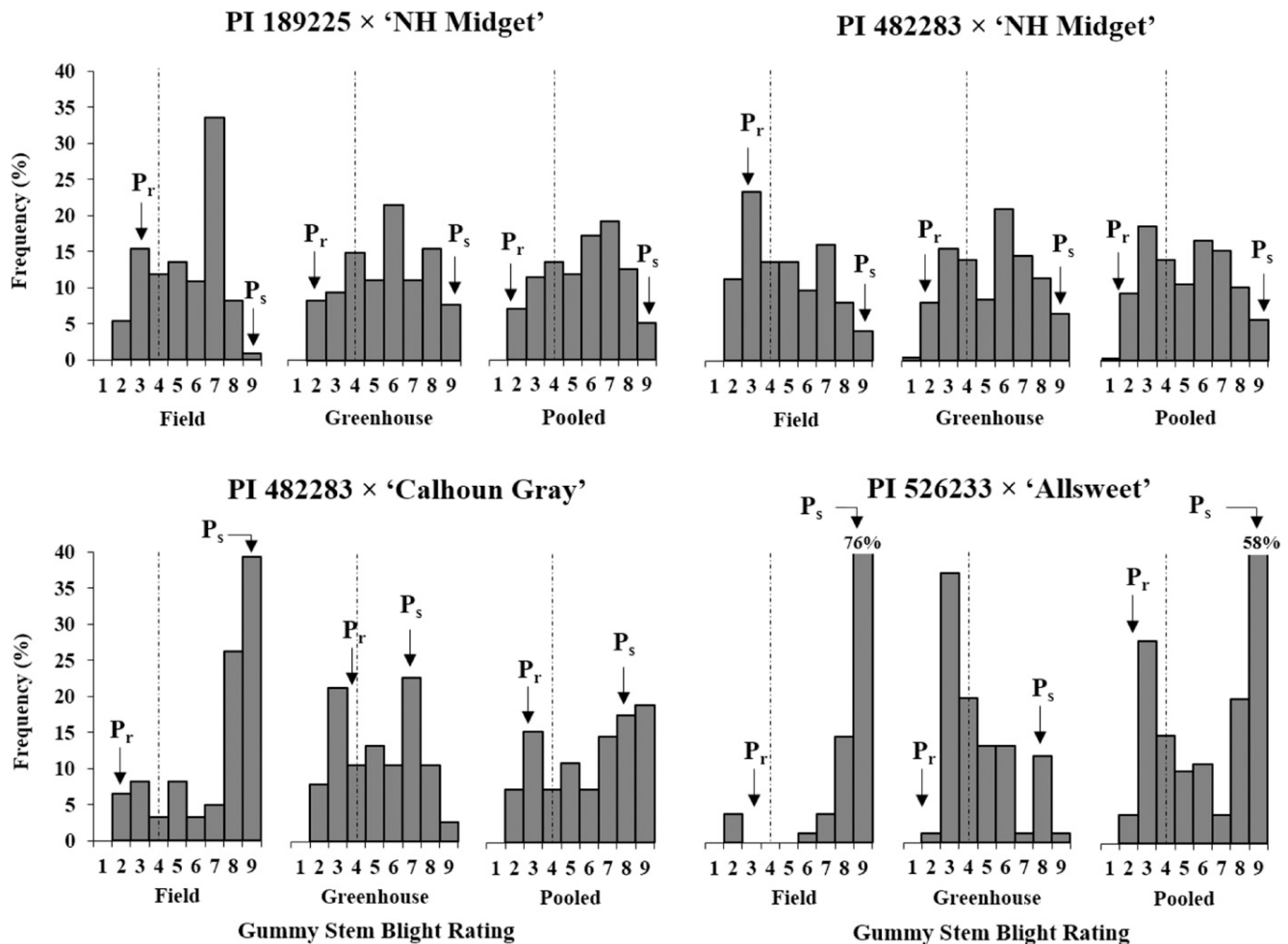

Fig. 1. Frequency distribution plots of F_2 data for the four watermelon families (resistant PI accessions by susceptible cultivars of *C. lanatus* subsp. *vulgaris*) screened for resistance to gummy stem blight (GSB) in greenhouse and field tests in North Carolina (2002–03). Disease assessment scale adopted for the screening: 0 = no disease; 1 = yellowing on leaves (suspect of disease only); 2–4 = symptoms on leaves only; 5 = some leaves dead, no symptoms on stem; 6–8 = symptoms on leaves and stems; 9 = plant dead. P_r and P_S indicate GSB ratings of the resistant and the susceptible parents, respectively. The dotted lines indicate the mean value of the disease assessment scale (4.5).

2002; Song et al., 2002, 2004). In those studies, the most resistant PI accession had a rating of 2.3 and the most susceptible had a rating of 6.3, with a least significant difference (0.05) of 0.4. The robustness of the method used in this study (0 = no disease to 9 = plant dead) relies on repeatability and replications within years and sites (Gusmini et al., 2005). However, this method was not able to prevent large differences between field and greenhouse tests. Also, there was variation among single plants of pure-line cultivars. For example, 'Charleston Gray' varied from a rating of 4 to a rating of 8 among seven plants evaluated (Gusmini et al., 2005).

Clear genetics ratios are fundamental to deduce the genetic basis of the dependence of one gene on another (Gusmini et al., 2004; Tetteh et al., 2013). Our study indicates that resistance to GSB in watermelon should not be attributed to a single recessive gene (i.e., *db* gene). The inconsistency between observed and expected ratios of several progenies in four different crosses suggests a more complex mode of inheritance for resistance to GSB, as well as a large environmental effect (greenhouse vs. field). The inconsistency of the observed ratios should be attributed, perhaps, to several loci interacting with each other, and also interacting with the environment (Kumar, 2009). In the greenhouse (a controlled environment), uniform conditions were created for plant and pathogen development. In the field (a variable environment), the evaluation of a high number of cultigens in each test required the use of large areas, increasing the environmental variation within the field per year and site. Across families, field goodness-of-fit-tests (F_2 and/or BC_1P_r) showed inconsistency to fail to reject the single recessive gene hypothesis, suggesting possible confounding genetic and environmental effects for resistance to GSB. However, greenhouse goodness-of-fit-tests (F_2 and/or BC_1P_r) consistently reject the single recessive gene hypothesis, suggesting a more complex mode of inheritance (Table 1). Indeed, if the resistance to GSB was solely due to the *db* gene, similar results should be expected in both greenhouse and field tests. Furthermore, in all four crosses, our F_2 data showed a continuous and mixture distribution, arguably, influenced by genetic and non-genetic factors (Fig. 1). In the F_2, there were few plants with disease ratings similar to the resistant parent. That was the case for the F_2 of all crosses whether they were tested in the field or greenhouse. The distribution pattern also supported the presence of a more complex mode of inheritance, with large environmental effects, and modifier genes (Hopkins and Levi, 2008).

The lack of progress in breeding effectively for resistance to GSB is partially explained by our results. Our study showed that the observed segregation ratios varied from the expected segregation ratios in the greenhouse and field, giving evidence of large environmental effects and multiple loci involved in controlling resistance to GSB

(Gusmini and Wehner, 2005). We suggest that GSB-resistant cultivars be developed using more intercrossing of resistant with elite lines before selection is started to help break linkages and increase the chance of getting nonparental types (high resistance combined with high quality). Because of high trait variability, selection should be based on progeny rows (F_3) rather than single plants (F_2). Finally, high environmental variability should be controlled by basing selection on data from multiple replications using both field and greenhouse tests.

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