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The Identification of the Peanut Wild Relative *Arachis stenosperma* as a Source of Resistance to Stem Rot and Analyses of Genomic Regions Conferring Disease Resistance through QTL Mapping

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Abstract: Peanut stem rot, also known as white mold, poses a significant threat to peanut production. It is typically managed using fungicides and moderately resistant cultivars. Cultivars with higher resistance can reduce fungicide dependency and increase sustainability. This study explores the potential of wild peanut species in stem rot resistance breeding programs by enhancing genetic diversity in cultivated peanut. Through greenhouse and field evaluations, 13 allotetraploid hybrids with *Arachis stenosperma* as one of the parents showed superior resistance compared to other wild genotypes. The genomic regions that confer the stem rot resistance were further identified by genotyping and phenotyping an F_2 population derived from the allotetraploid ValSten1 (*A. valida* × *A. stenosperma*)$^{4\times}$ and *A. hypogaea* cv. TifGP-2. A linkage map was constructed from 1926 SNP markers. QTL analysis revealed both beneficial and deleterious loci, with two resistance-associated QTLs derived from *A. stenosperma* and four susceptibility loci, two from *A. stenosperma* and two from *A. valida*. This is the first study that evaluated peanut-compatible wild-derived allotetraploids for stem rot resistance and that identified wild-derived QTLs for resistance to this pathogen. The allotetraploid hybrid ValSten1, that has *A. stenosperma* as one of the parents, offers a resource for resistance breeding. Markers associated with resistance QTLs can facilitate introgression from ValSten1 into cultivated peanut varieties in future breeding efforts, potentially reducing reliance on chemical control measures.

Keywords: stem rot; white mold; peanut; wild *Arachis*; QTL mapping; crop wild relatives

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1. Introduction

Peanut, or groundnut (*Arachis hypogaea* L.), is an essential oilseed crop and food legume cultivated in more than 100 countries, mainly in tropical and subtropical regions. In 2023, the world's total production of peanut accounted for 50.5 million tonnes, whereas the United States accounted for 5% of the world's total production (U.S. Department of Agriculture, 2023; https://ipad.fas.usda.gov/cropexplorer/cropview/Default.aspx, accessed on 28 February 2024). According to the United States Department of Agriculture database, the total harvested area of peanuts in 2023 was 637 thousand hectares producing 2.7 million tonnes. The warm and humid weather in the southeastern U.S. benefits peanut cultivation but also favors pests and diseases. Numerous fungi, nematodes, and virus pathogens cause significant economic losses, incurred through yield reduction and millions

of dollars in chemicals for plant protection. Pests and diseases also reduce the market value by decreasing the quality of pods and seeds. Hence, breeding cultivars with high yields along with disease resistance is the main objective of multiple peanut breeding programs.

Peanut stem rot caused by the fungus *Athelia rolfsii* (Curzi) C. C. Tu & Kimbr (syn. *Sclerotium rolfsii* Sacc.), also known as peanut white mold, is one of the most damaging soilborne diseases [1]. In Georgia, the largest producing state, the estimated losses caused by stem rot from 2017 to 2021 averaged $88.3 million per year [2–6]. Extensive screening programs for stem rot-resistant germplasm and breeding resistant cultivars were initiated in the late 1980s in the United States. Complete resistance to *A. rolfsii* has not been identified, but a few commercial cultivars present moderate resistance, including Georgia-12Y, Georgia-07W, Georgia-10T, Bailey, Florida-07, York, C-99R, TUFRunner '511', and TUFRunner '297' [7–14]. Among these resistant cultivars, Georgia-12Y is the most grown Runner-type peanut. The germplasm NC 3033 also shows partial stem rot resistance [15,16].

Peanut belongs to the genus *Arachis*, which has over 80 wild species in nine botanical sections [17–22]. These wild species, especially in the botanical section *Arachis*, are sources of genetic variation potentially conferring resistance to pathogens, tolerance to abiotic stress, and other desirable traits. Multiple resistances to diseases and pests have been identified in accessions of the wild species, *A. batizocoi*, *A. cardenasii*, *A. diogoi*, and *A. stenosperma* [23–29]. Though wild species show resistance to several diseases, their resistances are not readily transferable into cultivated peanuts by hybridization due to the ploidy barrier: while cultivated peanut is tetraploid, wild *Arachis* are diploid (except for *A. monticola* [17]). The development of interspecific hybridization techniques provided peanut breeders access to wild resistance sources and introgression of wild species into cultivated peanut is described in detail in the literature [30–33], allowing the production of lines and cultivars with superior performance and resistance to several peanut diseases [34–36]. Several commercial cultivars have already harnessed resistance from the wild species *A. cardenasii* [16,37–47]. Besides *A. cardenasii*, *A. stenosperma* serves as a new source of RKN resistance [48,49]. Resistance to stem rot, which is the focus of this study, has been found in the taxonomic sections *Arachis* (*A. duranensis*, *A. microsperma*, and *A. monticola*), *Heteranthae* (*A. pusilla*), and *Procumbentes* (*A. appressipila*) [50–52]. However, to date, no peanut-compatible wild-derived induced allotetraploid has been identified as having resistance to stem rot.

In plant breeding, selecting individuals based on phenotype is time-consuming and labor-intensive. When there are molecular markers strongly linked to the phenotype of interest, marker-assisted selection (MAS) aids in selecting desirable individuals based on genotype, significantly reducing the time needed and precision of selection by, for example, allowing selection at the seedling stage. Quantitative trait locus (QTL) analysis is an approach to finding genomic regions that confer the targeted trait, such as resistance. Generally, the QTL analysis method requires a population derived from two parents with different alleles that affect the variation in a trait [53,54]. After genotyping and phenotyping the targeted trait in the population, QTL analysis involves constructing the linkage maps for the population, mapping and characterizing the QTLs that affect the trait [55]. In peanut, QTL analysis has been applied in studying resistances to pathogens, such as leaf spots, rust, stem rot, and RKN; agronomic traits, such as seed size and seed weight; and plant morphology, such as main stem height and branch length [56–63]. Few QTL studies reported stem rot resistance loci. Earlier research reported single simple sequence repeat (SSR) markers weakly associated with stem rot disease in F_2 populations derived from crossing one susceptible and one resistant cultivated peanut [64,65]. A large-scale SNP array was developed specifically for peanut ('Axiom_*Arachis*') [66]. This SNP array has been used in QTL mapping studies for resistances against pests and diseases, such as root-knot nematode, late leaf spot, and peanut rosette, and for agronomic traits associated with yield and pod and seed characteristics [59,66–69]. Regarding stem rot, QTLs linked to 'Axiom_*Arachis*' markers were identified in recombinant inbred line (RIL) populations [57,70,71]. Notably, none of the above-mentioned studies utilized wild species as resistance donors.

This study has two main focuses: (1) the evaluation of stem rot resistance in wild-derived induced allotetraploids was conducted in greenhouse assays and field trials to understand their potential in resistance breeding; and (2) genetic analyses of the wild-derived segments that confer resistance and susceptibility. This is part of an introgression program that can aid the development of peanut cultivars with stem rot resistance. This is the first study evaluating and dissecting the genetic contribution of wild-derived allotetraploids for stem rot resistance.

2. Materials and Methods

2.1. Screening of Induced Allotetraploids for Peanut Stem Rot Resistance

2.1.1. Genotypes Tested in Greenhouse Assay and Field Evaluations

Thirteen induced allotetraploids derived from 12 wild species were screened for resistance to peanut stem rot (Figure 1; [33–35,72–75]). Resistances were assessed by greenhouse assay [76] and/or field evaluation [70] (Tables 1 and 2). Controls were the pure pedigree *A. hypogaea* cultivars Georgia-09B (susceptible) and Georgia-12Y (resistant) [8,77].

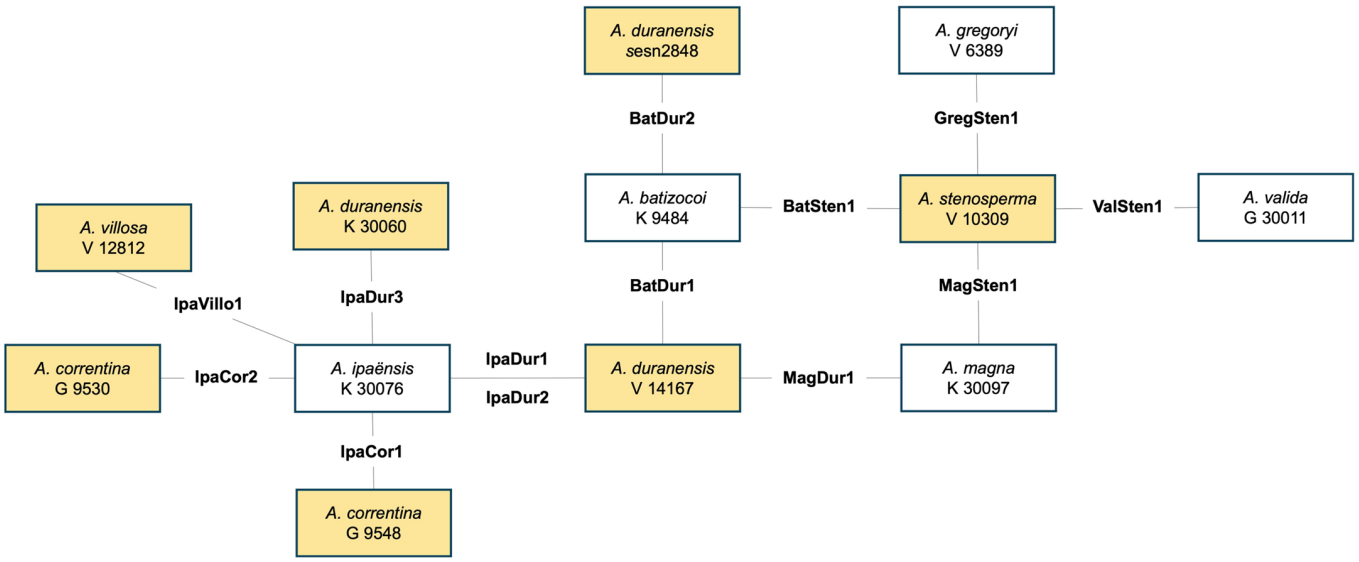


Figure 1. Thirteen induced allotetraploids (bold text) and their 12 diploid *Arachis* parents (shown in box). The boxes highlighted in orange contain the A genome parents of the induced allotetraploids, the others contain B or K (*A. batizocoi*) genome parents.

Table 1. Average lesion lengths and AUDPC value on stem cutting of 13 induced allotetraploids inoculated with *A. rolfsii* mycelial plugs in greenhouse assays.

Genotype Abbreviation	Genotype	Reference	5 DPI [a,b] Avg. [c]		7 DPI Avg.		9 DPI Avg.		AUDPC Avg.	
GregSten1	[*A. gregoryi* V 6389 × *A. stenosperma* V 10309]$^{4\times}$	[72]	0.67 [d]	a [e]	0.91	a	2.04	a	5.78	a
ValSten1	[*A. valida* G 30011 × *A. stenosperma* V 10309]$^{4\times}$	[34,74]	0.87	a	1.23	a	1.72	ab	6.57	a
MagSten1	[*A. magna* K 30097 × *A. stenosperma* V 10309]$^{4\times}$	[35]	0.89	a	1.24	ab	1.95	ab	6.97	ab
IpaDur3	[*A. ipaënsis* K 30076 × *A. duranensis* K 30060]$^{4\times}$	[34]	0.94	a	1.23	a	1.93	ab	6.96	a
IpaVillo1	[*A. ipaënsis* K 30076 × *A. villosa* V 12812]$^{4\times}$	[72]	0.98	a	1.53	ab	2.92	ab	8.63	ab
IpaDur2	[*A. ipaënsis* K 30076 × *A. duranensis* V 14167]$^{4\times}$	[75]	1.00	a	1.64	ab	3.86	ab	9.95	ab

Table 1. *Cont.*

Genotype Abbreviation	Genotype	Reference	5 DPI [a,b] Avg. [c]		7 DPI Avg.		9 DPI Avg.		AUDPC Avg.	
IpaCor1	[*A. ipaënsis* K 30076 × *A. correntina* G 9548]$^{4\times}$	[72]	1.09	a	1.54	ab	2.91	ab	9.06	ab
BatSten1	[*A. batizocoi* K 9484 × *A. stenosperma* V 10309]$^{4\times}$	[33]	1.10	a	1.26	a	1.74	a	7.41	a
IpaDur1	[*A. ipaënsis* K 30076 × *A. duranensis* V 14167]$^{4\times}$	[73]	1.11	a	1.64	ab	3.85	ab	10.02	ab
IpaCor2	[*A. ipaënsis* K 30076 × *A. correntina* G 9530]$^{4\times}$	[34]	1.13	ab	2.08	ab	3.48	ab	10.80	ab
BatDur2	[*A. batizocoi* K 9484 × *A. duranensis* sesn 2848]$^{4\times}$	[33]	1.15	ab	1.89	ab	3.32	ab	10.32	ab
BatDur1	[*A. batizocoi* K 9484 × *A. duranensis* V 14167]$^{4\times}$	[33]	1.20	ab	1.72	ab	3.52	ab	10.32	ab
GA-12Y [f]	*A. hypogaea* Georgia-12Y	[8]	1.32	ab	1.51	ab	1.99	ab	8.53	ab
MagDur1	[*A. magna* K 30097 × *A. duranensis* V 14167]$^{4\times}$	NP	1.51	ab	2.13	ab	4.63	ab	13.10	ab
GA-09B [f]	*A. hypogaea* Georgia-09B	[77]	2.27	b	2.85	b	3.84	b	15.40	b
	p-value of Kruskal-Wallis test		<0.001 *		<0.01 *		0.023 *		<0.01 *	

[a] days post inoculation; [b] 3 DPI not shown in the table, no significant difference was found among genotypes; [c] avg. represents the abbreviation of average; [d] unit of lesion length: mm; [e] means within columns for individual evaluations that are not followed by a common letter are significantly different according to Conover–Iman post hoc test; [f] Georgia-12Y (GA-12Y) and Georgia-09B (GA-09B) are the commercial cultivars used as resistant and susceptible control, respectively; NP = not published; * *p*-value of Kruskal-Wallis test < 0.05 = significant differences between means.

Table 2. Field evaluation of 11 induced allotetraploids for stem rot resistance on field evaluations.

Genotype Abbreviation	Genotype	2019 End-of-Season Evaluation Average		2020 Midseason Evaluation [a] Average		2020 End-of-Season Evaluation Average	
ValSten1	[*A. valida* G 30011 × *A. stenosperma* V 10309]$^{4\times}$	2.28	ab	0.76	a	2.31	a
GA-12Y [b]	*A. hypogaea* Georgia-12Y	1.44	b [c]	1.23	a	2.84	a
GA-09B [b]	*A. hypogaea* Georgia-09B	3.28	cd	1.45	ab	2.97	ab
GregSten1	[*A. gregoryi* V 6389 × *A. stenosperma* V 10309]$^{4\times}$	3.15	c	2.26	bc	3.79	bc
MagSten1 [d]	[*A. magna* K 30097 × *A. stenosperma* V 10309]$^{4\times}$			2.33	bc	3.64	bc
IpaVillo1	[*A. ipaënsis* K 30076 × *A. villosa* V 12812]$^{4\times}$	3.88	cd	2.48	cd	4.42	cd
IpaCor2 [d]	[*A. ipaënsis* K 30076 × *A. correntina* G 9530]$^{4\times}$			2.44	cd	3.75	cd
IpaCor1	[*A. ipaënsis* K 3076 × *A. correntina* G 9548]$^{4\times}$	3.42	acd	2.77	cde	4.00	cde
MagDur1 [d]	[*A. magna* K 30097 × *A. duranensis* V 14167]$^{4\times}$			2.92	cde	4.68	cde
BatSten1	[*A. batizocoi* K 9484 × *A. stenosperma* V 10309]$^{4\times}$	3.76	cd	2.92	cde	4.50	cde
BatDur2	[*A. batizocoi* K 9484 × *A. duranensis* sesn 2848]$^{4\times}$	4.32	d	3.08	cde	4.94	cde
IpaDur3 [d]	[*A. ipaënsis* K 30076 × *A. duranensis* K 30060]$^{4\times}$			3.26	de	4.86	de
IpaDur1	[*A. ipaënsis* K 30076 × *A. duranensis* V 14167]$^{4\times}$	4.05	cd	3.45	e	4.84	e
		<0.001 *		<0.001 *		<0.001 *	

[a] midseason evaluation data only collected in 2020; [b] Georgia-12Y (GA-12Y) and Georgia-09B (GA-09B) are the commercial cultivars used as resistant and susceptible control, respectively; [c] means within columns for individual evaluations that are not followed by a common letter are significantly different according to Conover–Iman post hoc test; [d] MagSten1, IpaCor2, MagDur1, IpaDur3 only tested in 2020; * *p*-value of Kruskal–Wallis test < 0.05 = significant differences between means within columns.

2.1.2. Greenhouse Assay

Seeds were germinated on Whatman No.1 filter paper (ThermoFisher Scientific, Waltham, MA, USA)) saturated with approximately 5 mL growth regulator (5% Florel, Monterey, Fresno, CA, USA) in a Petri dish at 28 °C for 24 h for breaking dormancy. Germinated seeds were transferred to six-cell seedling trays filled with potting mix (Pro-Mix Bx, Grow-Generation, Greenwood Village, CO, USA) and placed in a greenhouse. The 14-day-old seedlings were transplanted to a 25 cm round pot filled with soil with 4:2:2:1 sand/steamed

field soil/Pro-Mix (GrowGeneration)/perlite (Industries, Inc., Orwell, OH, USA). The controls were germinated two weeks later than the allotetraploids due to the differences in growth rate. The greenhouse assay was conducted using the method described by Tsai et al. [76]. In brief, *A. rolfsii* SR-18 strain [78] was grown on a 15 mL potato dextrose agar (PDA) in a Petri dish at room temperature with 8:16 h light/dark for four days as inoculum preparation. Stem cuttings were collected from primary lateral branches of sixty-day-old plants and were placed in a 355 mL cup filled with wet potting mix (Pro-Mix Bx, GrowGeneration). Cuttings were inoculated with a 0.7 cm diameter plug grown with active *A. rolfsii* on the next day of stem-cutting-in-cup preparation. Inoculated cuttings were placed in a mist chamber with a randomized complete block design (RCBD). Free water in the base of the mist chamber ensured the high humidity for disease development. About 5 mL of sterilized distilled water was carefully dripped on the potting mix using a wash bottle every two days. Lesion lengths on the stem were measured at 3, 5, 7, and 9 days post inoculation (DPI) using a digital caliper (Ted Pella, Inc., Redding, CA, USA). These four evaluations were used to calculate the area under the disease progress curve (AUDPC) to assess disease development over time [79]. The formula of AUPDC $A(t_k)$, at $t = t_k$, is the total accumulated disease until $t = t_k$, given by $A_k = \sum_{i=1}^{N_i-1} \frac{(y_i+y_{i+1})}{2} \times (t_{i+1} - t_i)$. Hence, the calculation in this study was AUDPC = ((DPI3 + DPI5)/2) × 2 + ((DPI5 + DPI7)/2) × 2 + ((DPI7 + DPI9)/2) × 2. Normally, eight replications per genotype were evaluated. Experiments were conducted three times in May, July, and October 2020. Average temperatures in the greenhouse were 24.2 °C, 26.4 °C, and 23.1 °C, respectively.

2.1.3. Field Evaluation

Induced allotetraploids (Figure 1) were evaluated during the summer from May to October 2019 and 2020 at the University of Georgia Blackshank Farm, Tifton, Georgia using the method described in Cui et al. with modifications [70]. The field was fumigated by injecting TRI-PIC 100 (336.26 kg/ha, TriEst Ag Group, Inc., Greenville, NC, USA) into the soil, and a plastic sheet covered the field for seven days to remove residual inoculum of *A. rolfsii* from the soil. Beds were 457.2 cm × 182.88 cm in size, and a strip-till rig with shanks spaced 91.4 cm was then run on each bed to break any compaction zones from equipment used to remove the plastic. Standard production practices were followed, including pre-plant incorporation of herbicide with a rototiller. Allotetraploids have a very distinct architecture from that of cultivated peanut. Therefore, Georgia-12Y seeds were planted to provide a canopy for adequate fungal development (referred to as background-GA-12Y hereafter). Seeds of background-GA-12Y were planted as 6 seeds per 30.48 cm in each of two rows per bed with a 91.5 cm row spacing. Planting dates for GA-12Y were 6 May 2019 and 18 May 2020.

Seed dormancy of the tested genotypes (allotetraploids and controls) was broken as described above. Seedlings were transferred to an 8 cm peat pot (Jiffy pots S8-15, Jiffy, Zwijndrecht, Netherland) filled with potting mix (Pro-Mix Bx, GrowGeneration) and kept in the greenhouse for 28 days prior to transplanting in the field. Georgia-12Y and Georgia-09B, the resistant and susceptible controls, respectively, were germinated two weeks after the allotetraploids. Four-week-old seedlings (grown in a greenhouse) were labeled with a plastic band at the base of the stem. All 40 seedlings per genotype were transplanted directly into the field, arranged in rows interspersed among two rows per bed of background-GA-12Y. Transplanting dates were 4 June 2019 and 2 June 2020. Eight rows of five individuals were planted for each genotype, with row distribution following an RCBD (Figure 2a). The objective was to achieve a more homogeneous microclimate among the tested plants (allotetraploids), as they exhibit a sparse canopy (Figure 2b,c).

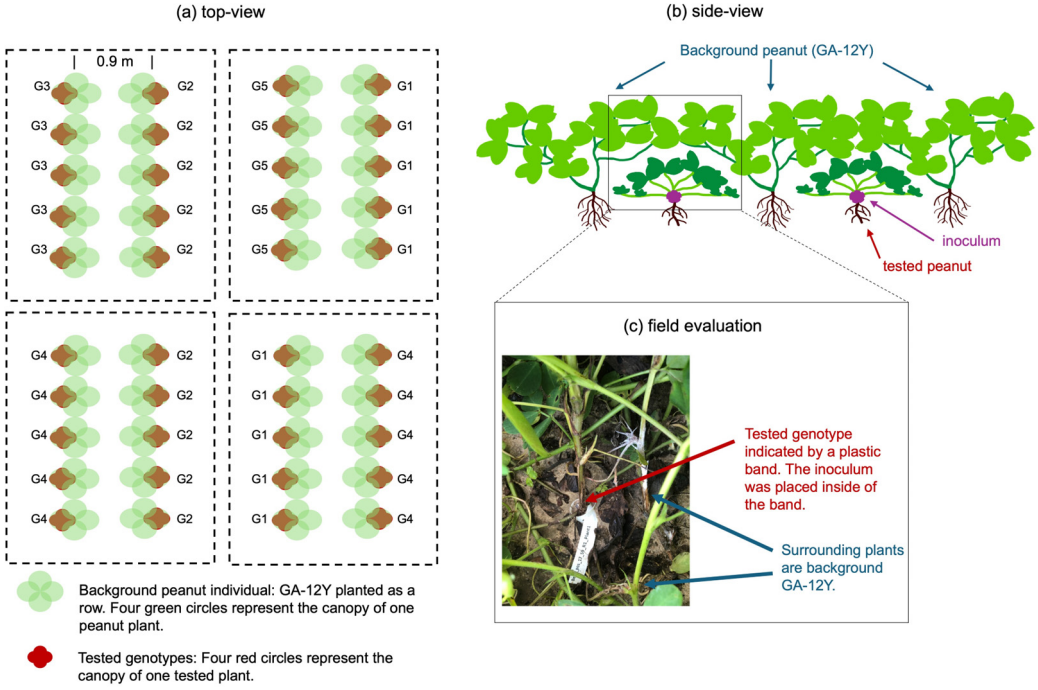


Figure 2. Schematic representation of the evaluation method of induced allotetraploids for stem rot resistance in the field. (**a**) Top-view of the field plan. Each set (e.g., G1–G5) was assigned to the field using a randomized complete block design. Each set has five reps of the same genotypes. (**b**) Side-view of the field evaluation; the tested genotype was nested under the canopy of background-GA-12Y peanut. (**c**) A close-up of the experiment: an individual being tested is surrounded by the background-GA-12Y and marked by a plastic band. The inoculum was placed inside of the band to keep it in place.

For inoculation, *A. rolfsii* SR-18 strain [78] was grown on PDA for three days. Individual tested plants were inoculated with a 1 cm diameter active mycelial plug cut from a PDA plate with the aid of a cork borer. Inoculation was performed 60 days after the seedlings were transplanting into the field (7 August 2019 and 11 August 2020). The mycelial plug was positioned between the plastic band and the plant to secure it in place, with the mycelial side directly in contact with the peanut stem. Acephate (Orthene 0.84 kg/ha) was applied once prior to the inoculation to control fire ants that can consume the agar plug. After the inoculation, three days of overhead irrigation were applied to the field to maintain high humidity long enough to achieve successful infection of the plants. Additional irrigation was applied as needed during the season to maintain plant growth. Standard management practices were employed, including applications of chlorothalonil (1.26 kg active ingredient per ha) every 14 days to control foliar diseases. Stem rot severity was evaluated before harvest (9 September of 2019 (32 DPI) and 6 October of 2020 (56 DPI)) by examining lesion development from the plant crown where the inoculum was placed. An additional midseason evaluation was undertaken in the 2020 field trial (27 August of 2020 (16 DPI)). A 0 to 5 rating scale modified from Cui et al. [70] was developed for disease evaluation, taking into account the wild phenotype of the plants (Table 3, Figure 3).

Table 3. The rating scale used for stem rot disease assessment of individual plants in the field.

Score	Description of Disease Development
0	No disease shown
1	A small lesion on the mainstem (<1 cm)
2	Larger lesions on the mainstem (1–4 cm), clean or small lesions on the laterals stem (<1 cm)
3	Larger lesions on the mainstem (>4 cm), more lesions on the lateral stem (1–4 cm)
4	Main stems dying, laterals largely affected (>4 cm)
5	Dead plant

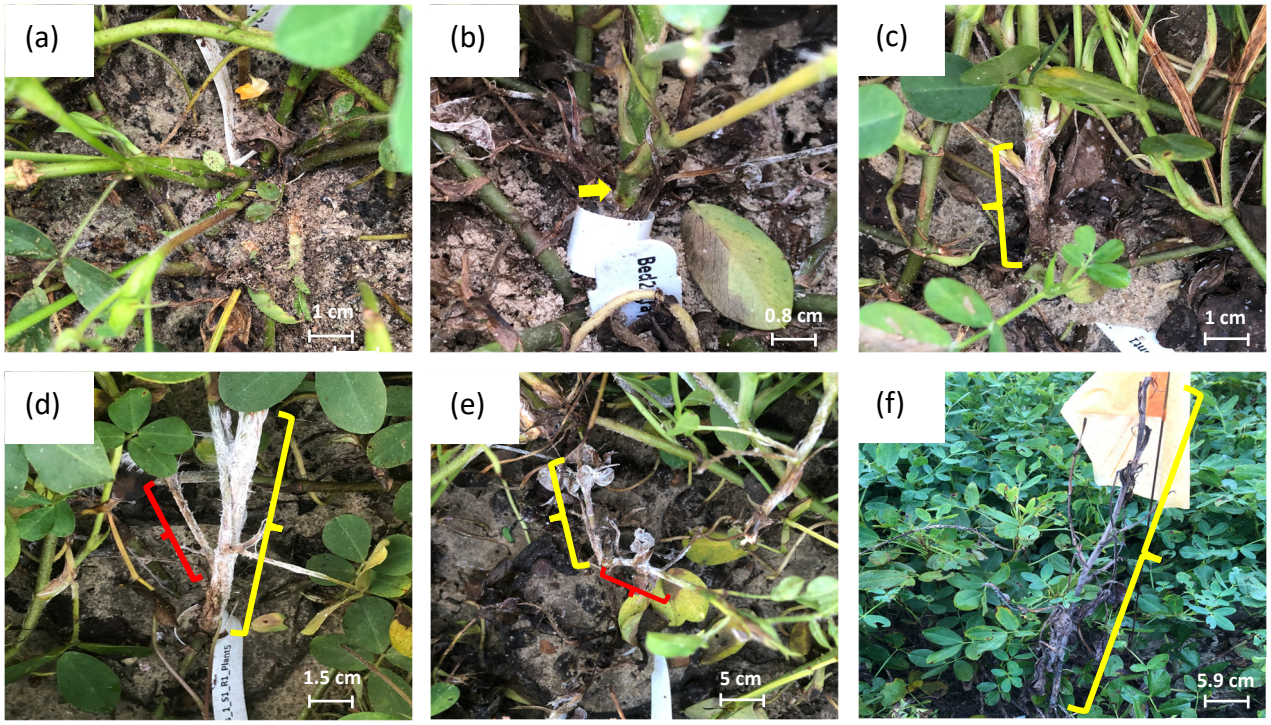


Figure 3. Rating scale used in field evaluation. The tested plants were labeled with a plastic band (**a**–**e**) and a field flag (**f**). Ratings are as follows: (**a**) score 0: no disease observed; (**b**) score 1: a small lesion on the mainstem (<1 cm) (yellow arrow); (**c**) score 2: a larger lesion (1–4 cm) on the mainstem and no or small lesions (<1 cm) on the lateral stem (yellow bracket); (**d**) score 3: a larger lesion (>4 cm) on the mainstem (yellow bracket) and secondary lesions on the lateral stem (1–4 cm) (red bracket); (**e**) score 4: main stem with large lesion and already dying (yellow bracket) and lateral stems largely affected (lesions > 4 cm) (red bracket); (**f**) score 5: dead plant (yellow bracket).

2.1.4. Statistical Analysis

The statistical analyses of data collected in the greenhouse assay and field evaluation were performed using the software RStudio (RStudio version 1.2.1335, Boston, MA, USA). First, the normality of datasets was verified with the Shapiro–Wilk test using the function *shapiro.test* [80]. As datasets did not follow normal distribution, means were compared by using the non-parametric Kruskal–Wallis rank sum test using the function *kruskal.test*, with significance levels set at 5% [81]. When significance levels were lower than 5%, the Dunn test was used post hoc for pairwise comparisons of means [82].

2.2. QTLs Mapping of Stem Rot Resistance

2.2.1. Population Development

The allotetraploid ValSten1 had the highest level of resistance in both greenhouse and field evaluations (Tables 1 and 2, Figures 4 and 5). ValSten1 is an allotetraploid derived from a cross of *A. valida* G 30011 and *A. stenosperma* V 10309 [34,74]. It was further crossed with the advanced line *Arachis hypogaea* cv. TifGP-2 [83], and the resulting F_1 hybrids were selfed to produce an F_2 population with 325 individuals. TifGP-2 is the sister line of Tifguard, which is moderately susceptible to stem rot with an Rx index value of 15 (https://site.extension.uga.edu/tiftcoag/files/2019/06/2019-Peanut-RX-Syngenta.pdf, accessed on 25 February 2021) [42,83]. Since ValSten1 showed resistance to stem rot among tested allotetraploids, we judged it very likely that the TifGP-2 × ValSten1 F_2 population would segregate for stem rot resistance and would be suitable for QTL mapping.

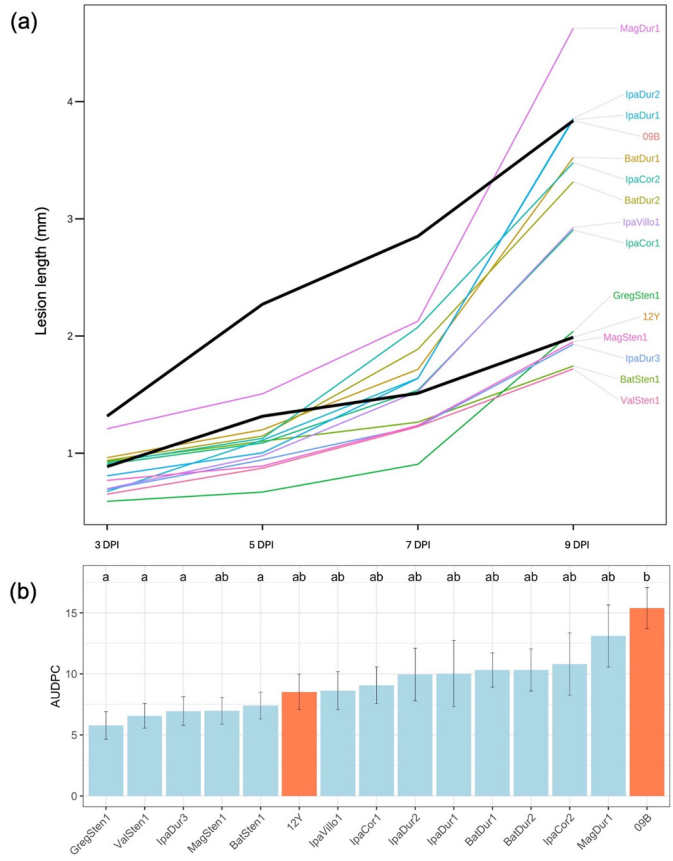


Figure 4. Greenhouse evaluation of induced allotetraploids for stem rot resistance. (**a**) Disease progress curves of stem rot based on the lesion length (mm). Black bold lines represent susceptible (Georgia-09B) and resistant (Georgia-12Y) controls. The *x*-axis shows the time after inoculation (3, 5, 7, and 9 days) and *y*-axis, the average length of stem rot lesion. (**b**) AUDPC value (*y*-axis) and standard error (bar on the top of each bar) of tested genotypes and controls (*x*-axis). Genotypes with different letters on the top of the bar are significantly different from each other at a p-value < 0.05. Blue boxes represent allotetraploids. Coral boxes represent resistant (Georgia-12Y) and susceptible (Georgia-09B) controls.

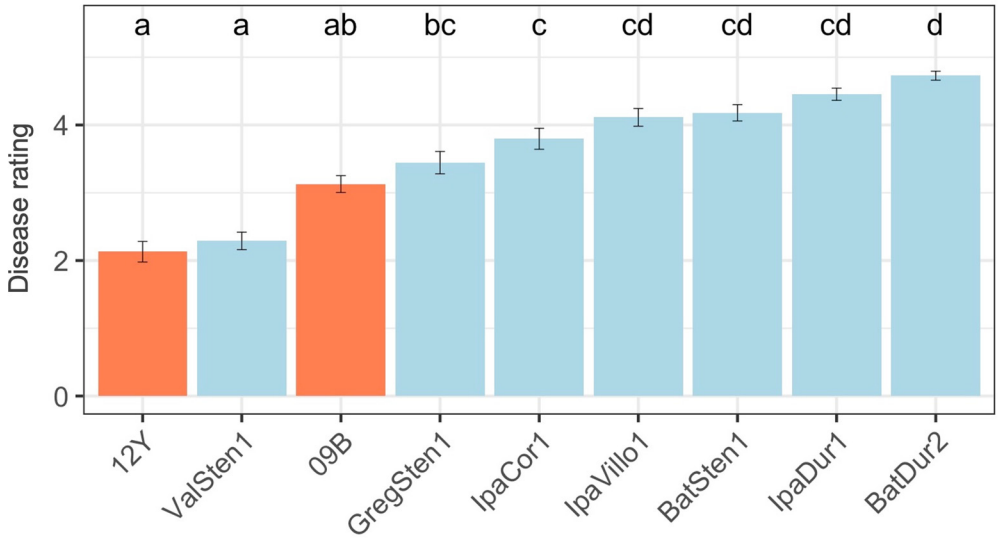


Figure 5. Two-year combined data of stem rot disease on induced allotetraploids evaluated in 2019 and 2020 in the field in Tifton GA. The *x*-axis shows the genotypes tested and the *y*-axis, the disease rating. Genotypes with different letters are significantly different from each other at a *p*-value < 0.05. Blue boxes represent allotetraploids. Coral boxes represent resistant (Georgia-12Y) and susceptible (Georgia-09B) controls. The two-year evaluations are significantly correlated (r = 0.87, *p*-value of r < 0.01).

2.2.2. Plant Germination and Leaf Tissue Collection

Seeds of the F_2 individuals and their parents were placed in a Petri dish on a Whatman No.1 filter paper (ThermoFisher Scientific) saturated with 5 mL of growth regulator to break the dormancy (5% Florel, Monterey). After incubating at 28 °C for 24 h, germinated seeds were transplanted to an 8 cm square peat pot (Huvai) filled with wet potting mix (Pro-Mix Bx, GrowGeneration) and cultivated in a greenhouse. The seeds of allotetraploid ValSten1 were germinated at the same time as the F_2s. As *A. hypogaea* genotypes grew faster and more vigorously than the wild-derived genotypes, they were germinated two weeks later using the same procedure. Each germinated individual was assigned a unique identification. Before transplanting plants into the field, 2–3 young leaves (around 100 mg) were collected and put into a 15 mL Falcon tube. The tubes were sealed with surgical tape (Micropore, 3M, St. Paul, MN, USA)) and lyophilized for 24 h. The dried leaves were stored on the laboratory bench at room temperature for further DNA extraction.

2.2.3. Stem Rot Resistance Evaluation

The individuals of the F_2 population were field evaluated along with the parents, ValSten1 and TifGP-2, and the susceptible and resistant controls, Georgia-09B and Georgia-12Y. There were 325 F_2 individuals, and as each F_2 individual was a unique genotype, there were no replications. The parents (ValSten1 and TifGP-2) and controls (Georgia-09B and Georgia-12Y), had 35, 35, 40, and 35 replications, respectively. Evaluations were conducted in the summer from May to October as described above at the same location, the University of Georgia Blackshank Farm in Tifton, Georgia. All plants in the same genotype (ValSten1, TifGP-2, Georgia-09B, and Georgia-12Y) or belonging to F_2 were randomly grouped into sets of five individuals each. There were 94 sets; each set was assigned to a row using an RCBD. The background-GA-12Y was planted in a fumigated field on 18 May. The use of background peanut is to provide a homogenous microclimate; the transplanting and inoculation were carried out as described above. Overhead irrigation was applied for three days to initiate the disease progress. Stem rot was evaluated twice: at midseason (7 August) and just before harvesting (4–5 October). The disease was rated individually based on the scale shown in Table 3 and Figure 3. The differences in stem rot resistance among ValSten1, TifGP-2, Georgia-09B, and Georgia-12Y were tested by the non-parametric

Kruskal–Wallis rank sum test using the function *kruskal.test* in R (RStudio version 1.2.1335, Boston, MA, USA) [81], with significance levels set at 5% as the dataset failed the normality test using the function *shapiro.test* [80].

2.2.4. SNP Genotyping, Analysis, and Data Filtering

DNAs were extracted from lyophilized dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. DNA was quantified with a Qubit 4 Fluorometer (Invitrogen, Fisher Scientific, Pittsburgh, PA, USA), and samples were submitted for genotyping with the 'Axiom_*Arachis* v02' 58K SNP array [84] (ThermoFisher Scientific, Affymetrix, Inc., Santa Clara, CA, USA). Two sets of markers were filtered, processed, and analyzed using the Axiom Analysis Suite software v5.1 (http://www.thermofisher.com, Santa Barbara, CA, USA, accessed on 7 June 2023) to construct an informative linkage map using the genotyping data (set 1) markers that specifically detect wild SNPs (referred to here as wild-species-specific markers) and (set 2) markers that differentiate the allotetraploid and cultivated parents (referred to here as parent-differentiation markers).

Wild-species-specific markers were classified as *A. stenosperma*-specific or *A. valida*-specific using the following procedures. First, raw genotyping data from *A. valida*, *A. stenosperma*, and TifGP-2 was extracted by the Axiom Analysis Suite software v5.1 using the diploid function. Then a Unix script (adapted from Ballén-Taborda et al. [59]; Supplemental File S1) was used to identify *A. stenosperma*-specific markers where the genotype of *A. stenosperma* was different from both *A. valida* and TifGP-2 (*A. stenosperma* $\neq$ (*A. valida* = TifGP-2)), and *A. valida*-specific markers where the genotype of *A. valida* was different from both *A. stenosperma* and TifGP-2 (*A. valida* $\neq$ (*A. stenosperma* = TifGP-2)). An initial set of 1753 *A. stenosperma*-specific and 973 *A. valida*-specific markers (a total of 2726) were identified. Then, genotypes of these markers were extracted from the tetraploids (325 F_2s and tetraploid controls; diploids were not included) using the Axiom Analysis Suite software v5.1 in polyploid mode. Each locus could then be assigned to one of four types: (1) homozygous wild, (2) heterozygous, (3) homozygous cultivated, and (4) missing data. Then, observing segregation in the F_2s, 1392 markers that did not fit the Mendelian segregation ratio 1:2:1 were filtered (using goodness-of-fit chi-square test for the F_2 population, p-value of the test < 0.05). The final set of wild-specific markers comprised 1334 markers (1011 *A. stenosperma*-specific, 323 *A. valida*-specific). These were combined with parent-differentiation markers (detailed in the following paragraph) and used for linkage map construction.

We identified an initial set of 16,423 parent-differentiation markers using the fitPoly package [85] in RStudio as markers that differentiated *A. hypogaea* TifGP-2 and the allotetraploid ValSten1. This initial set of 16,423 markers was evaluated in the two parents, *A. hypogaea* TifGP-2 and allotetraploid ValSten1, and 325 F_2 individuals. Markers were then filtered for missing data to reduce the marker set to 3978. The fitPoly package scores markers on a scale of 0 to 4, representing the five allele dosage possibilities in any particular allotetraploid genotype. Of the 3978 markers, 1758 demonstrated the expected single-dosage marker parental scores of 2 vs. 0 and 2 vs. 4 and were retained. The marker set was further filtered by retaining only markers whose modal scores in the F_2 population were the expected heterozygous score (1 for 2 vs. 0 and 3 for 2 vs. 4) and whose segregation in the F_2s fit the Mendelian ratio 1:2:1 (using the goodness-of-fit chi-square test for the F_2 population; p-value of the test > 0.05). This resulted in a filtered set of 1691 parent-differentiation markers. Dosages, as called in the fitPoly package, were then converted to diploid-like markers, where 2 and 0 represent opposite parents' homozygous states, and 1 represents the heterozygous state; or where 2 and 4 represent opposite parents' homozygous states, and 3 represents the heterozygous state. Subsequently, the alleles at each locus within the F_2 population were categorized as either cultivated-peanut-derived (TifGP-2) or allotetraploid-derived (ValSten1).

2.2.5. Genetic Mapping and QTL Discovery

Before constructing a genetic map using the two sets of markers (wild-species-specific and parent-differentiation markers), two further criteria were employed to filter markers, those with minor allele frequency in the F_2 population lower than 0.05 or with a proportion of missing data higher than 0.1 were excluded. In total, 2007 markers within 321 individuals were kept after filtering: 161 markers belonged to both *A. stenosperma*-characteristic markers and parent-differentiation markers; 26 markers were classified as *A. valida*-characteristic markers and also parent-differentiation markers; 499 were kept as only *A. stenosperma*-characteristic markers; 175 as only *A. valida*-characteristic markers; and 1146 as only parent-differentiation markers.

Subsequently, the 2007 markers were used to construct a genetic map using JoinMap software v4.1 with Kosambi's genetic map function and the maximum likelihood algorithm [86–88]. The resulting map consisted of 21 linkage groups (LGs), with 1926 markers clustered into groups and 81 markers not clustered. To further define the chromosomes of the LGs in the mapping results, each marker was then assigned to a chromosome of either the A or B subgenome. The LGs with *A. stenosperma*-specific markers are classified into the A subgenome, and those with *A. valida*-specific markers are classified into the B subgenome, as ValSten1 derived its A subgenome from *A. stenosperma* and its B subgenome from *A. valida*. Of these groups, 11 of 21 belonged to the A subgenome, and 10 belonged to the B subgenome. The corresponding physical position of each marker is based on the annotation by Bertioli et al. [75,89].

The genetic map, the F_2 genotyping data ('W' denotes the locus of an F_2 derived from a wild species, either *A. stenosperma* or *A. valida* on the A or B subgenomes, respectively), and phenotyping data from two stem rot evaluations (midseason and end-of-season) were used for QTL identification using R/QTL software v1.5. The analysis followed the procedure described by Broman et al. [90]. Composite interval mapping was employed chromosome by chromosome using the cim function with the arguments, map.function = "kosambi", imp.method = "imp", and method = "hk". The identified QTL with the logarithm of the odds (LOD) score was returned, representing the strength of evidence for the presence of a QTL at the particular location. The LOD score calculates the $\log_{10}$ likelihood ratio, comparing the hypothesis that there is a QTL to the hypothesis that there is no QTL. The 1000 permutations were used to identify genome-wise LOD significance thresholds for identified QTL at 1% and 5% significance levels. The nearest marker of the identified QTL was identified using find.marker function. The LOD support interval was calculated with lodint function. The percentage of phenotypic variance explained by each QTL was calculated with the fitqtl function [90]. The average stem rot rating for each identified QTL's nearest marker was extracted using the effectplot function in R/QTL. Subsequently, the percentage of decreased or increased disease levels between individuals with homozygous wild alleles and homozygous TifGP-2 alleles was calculated manually. The barplot function in RStudio was then utilized to visualize the stem rot ratings among F_2s with three genotypes (homozygous wild, heterozygous, and homozygous cultivated).

3. Results

3.1. Identification of Peanut Stem Rot Resistance in Induced Allotetraploids

3.1.1. Greenhouse Assay

Greenhouse assay enabled the successful development of stem rot lesions. Differentiation between genotypes was not very large; this bioassay distinguished susceptible controls from the resistant controls numerically, but the results were not statistically significant. All thirteen induced allotetraploids showed numerically lower AUDPC than the susceptible control Georgia-09B (Figure 4a,b). Five of them (GregSten1, ValSten1, IpaDur3, MagSten1, and BatSten1) had numerically lower AUDPC than the resistant control Georgia-12Y. The lesion lengths were statistically different among genotypes at 5, 7, and 9 days post inoculation (DPI), and the *p*-values of the AUDPC values are lower than 0.05 in the Kruskal–Wallis test (Table 1). Nine allotetraploids (GregSten1, ValSten1, MagSten1, IpaDur3, IpaVillo1,

IpaDur2, IpaCor1, BatSten1, and IpaDur1) had similar disease levels as the resistant control, Georgia-12Y, and statistically lower disease than the susceptible control Georgia-09B at 5 DPI. Four allotetraploids (GregSten1, ValSten1, IpaDur3, and BatSten1) were statistically more resistant than the susceptible control, Georgia-09B, at 7 and 9 DPI and AUDPC.

3.1.2. Field Evaluation

Both field evaluations showed disease development in every tested genotype. The resistant control Georgia-12Y and susceptible control Georgia-09B exhibited statistical differences in 2019 and numerical differences for the midseason and end-of-season evaluations in 2020. Disease scores in almost all wild-derived allotetraploids were significantly higher than those in the resistant control Georgia-12Y in both 2019 and 2020. ValSten1 was the only allotetraploid with a significantly lower disease score than the susceptible control Georgia-09B, and it exhibited a resistance level similar to Georgia-12Y in the 2019 end-of-season evaluation. In the 2020 midseason evaluation, 2020 end-of-season evaluation, and two-year combined data of the end-of-season evaluation, ValSten1 showed numerically lower disease ratings than the resistant control Georgia-12Y and susceptible control Georgia-09B (Table 2 and Figure 5). Therefore, we used ValSten1 for molecular mapping of stem rot resistance.

3.2. Mapping QTLs Associated with Allotetraploid ValSten1 Conferring Stem Rot Resistance

3.2.1. Stem Rot Resistance Evaluation of F_{2s}, Parents, and Controls

F_2 individuals were evaluated in one field season as the experiment was destructive. Inoculation was successful as most plants developed stem rot symptoms. In this experiment, one extra peanut genotype was included—TifGP-2—as it was a parent of the F_2 population being tested. The differences between controls and parents are shown in Table 4. Symptom ratings of the resistant control Georgia-12Y were only numerically lower than those of the susceptible control Georgia-09B. During the midseason, three cultivated peanuts (Georgia-12Y, Georgia-09B, and TifGP-2) showed similar stem rot ratings. Two parents of the F_2 (TifGP-2 and ValSten1) had similar disease severity, grouping with the susceptible control Georgia-09B. Surprisingly, ValSten1, which was more resistant both in the greenhouse and field evaluations in the first study (Tables 1 and 2, Figures 4 and 5), was numerically the most susceptible genotype here, and it was statistically more susceptible than Georgia-12Y during the midseason. In the end-of-season evaluation, the scores were not statistically different from each other (Table 4).

Table 4. Stem rot disease of F_2's parents, the controls, and the F_2 evaluated in the field.

Genotype	Evaluation		
	Midseason [a]		End of Season [a]
	Average [b]		Average
GA-12Y [c]	1.68 ± 1.07	a	2.34 ± 0.76
TifGP-2 [d]	1.81 ± 0.82	ab	2.48 ± 0.83
GA-09B [c]	2.00 ± 1.26	ab	2.57 ± 1.09
ValSten1 [d]	2.46 ± 0.82	b	2.83 ± 0.92
F_2	2.29 (data range 0–5)		2.82 (data range 0–5)
p-value of Kruskal–Wallis	0.013 [e]		0.328 [e]

[a] midseason evaluation was undertaken on 7 August 2021, the end-of-season evaluation was undertaken on 4–5 October 2021; [b] means within columns for individual evaluations that are not followed by a common letter are significantly different according to Conover–Iman post hoc test; [c] cultivated peanut Georgia-12Y (GA-12Y) and Georgia-09B (GA-09B) were resistant and susceptible control; [d] parents of the F_2 population; [e] *p*-value of Kruskal–Wallis test. When < 0.05 = significant differences between means; the normality assumption of the data is not satisfied.

The stem rot disease scores of segregating F_2 individuals averaged 2.29 and 2.82 during the midseason and end-of-season evaluation, respectively, ranging from the lowest (score

0 = no symptoms) to the highest (score 5 = dead plant) for both (Table 4). Transgressive segregation was observed: many F_2 individuals exhibited scores lower than those of the more resistant parent (ValSten1) or higher than those of the more susceptible parent (TifGP-2) in both evaluations (Figure 6).

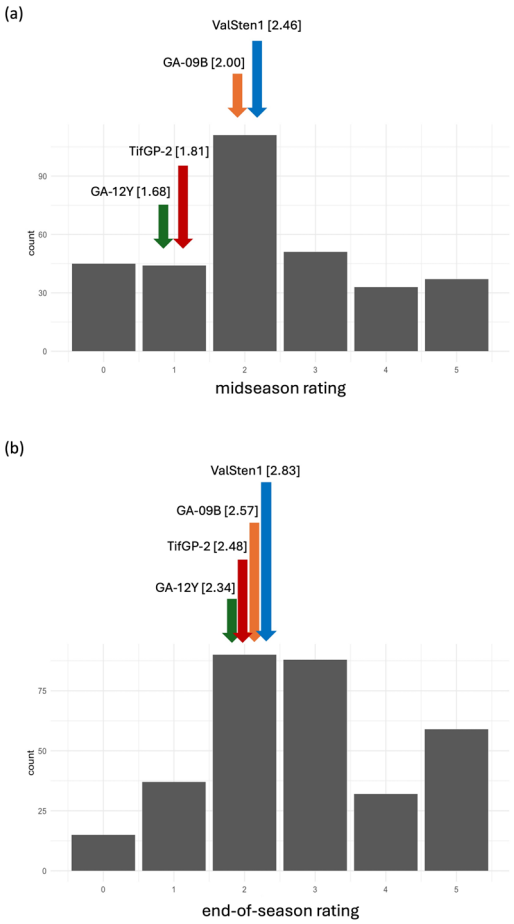


Figure 6. Frequency distribution of stem rot rating of 321 individuals of an (TifGP-2 × ValSten1) F_2 population at (**a**) midseason and (**b**) end of season. The ratings of parents for the F_2 population and controls (resistant GA-12Y, susceptible GA-09B) are shown by the different colors of arrows, and the averaged disease ratings are indicated in brackets.

3.2.2. Genetic Mapping and the Genotyping Result of F_2s

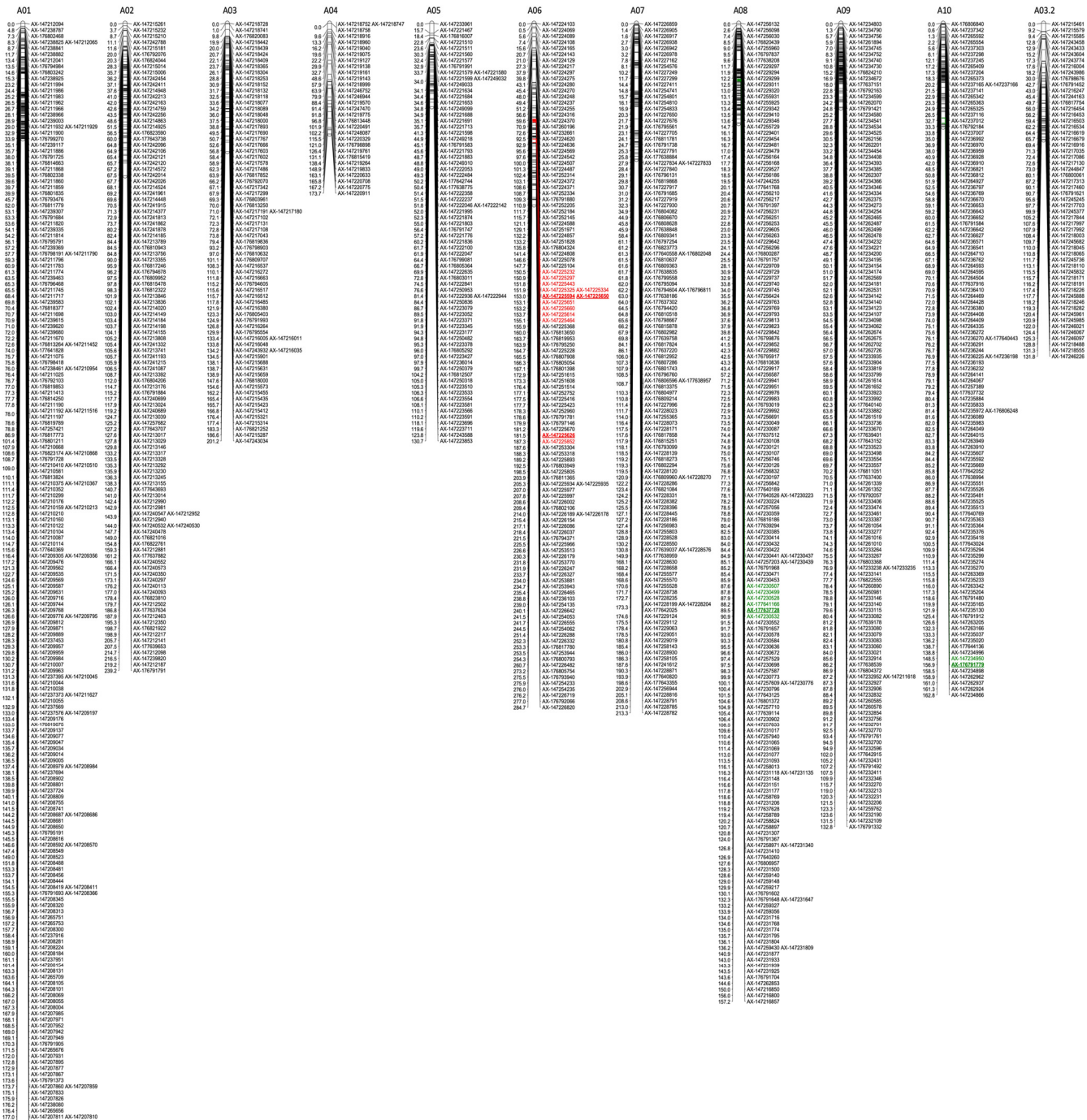
The genotyping of 321 individual F_2s with 2007 informative markers was used for genetic mapping. In total, 1926 loci were clustered into 21 linkage groups (LGs), with details shown in Table 5 and Figure 7. Based on the information of chromosome and physical position annotated by Bertioli et al. [75,89], 11 LGs with 1220 markers were assigned to the A01 to A10 peanut subgenome, and the other 10 LGs with 706 markers were arranged into the B01 to B10 subgenome. One linkage group, LG A03.2 was inferred as partial chromosomes of A03. The mapped SNP markers spanned a total genetic distance of 3856.27 cM (Table 5). The LG results with markers are detailed in Figure 7 and Supplemental File S2. Sizes of the 21 LGs vary from 97.42 cM (LG B07) to 353.45 cM (LG A03.2), the average distance of LGs ranged from 0.85 cM (LG A01) to 6.31 cM (LG A03.2); the maximum distance between adjacent markers of LGs ranged from 6.58 cM (LG B07) to 30.4 cM (LG B03). The Marey plots in Supplemental File S3, generated using the scatter plot function in Excel, display the markers identified in each LG, illustrating the correlation between the genetic map of the 21 linkage groups and the physical map annotated by Bertioli et al. [75,89]. These 21 Marey plots correspond to markers in the 21 LGs (A01–A10, A03.2, and B01–B10), with an additional plot for the original A03 linkage group. In these plots, the *x*-axis represents

each marker's genetic position within the respective LG, while the *y*-axis indicates the physical position in the diploid ancestors of cultivated peanut: *A. duranensis* for LG A01–A10, and A03.2; *A. ipaënsis* for LG B01–B10. The genotyping results of 321 F_2 individuals with 1926 markers of 21 LGs are shown in detail in Supplemental File S4.

Table 5. Statistical data of the genetic map obtained by the analysis of the 321 TifGP-2 × ValSten1 F_2 population.

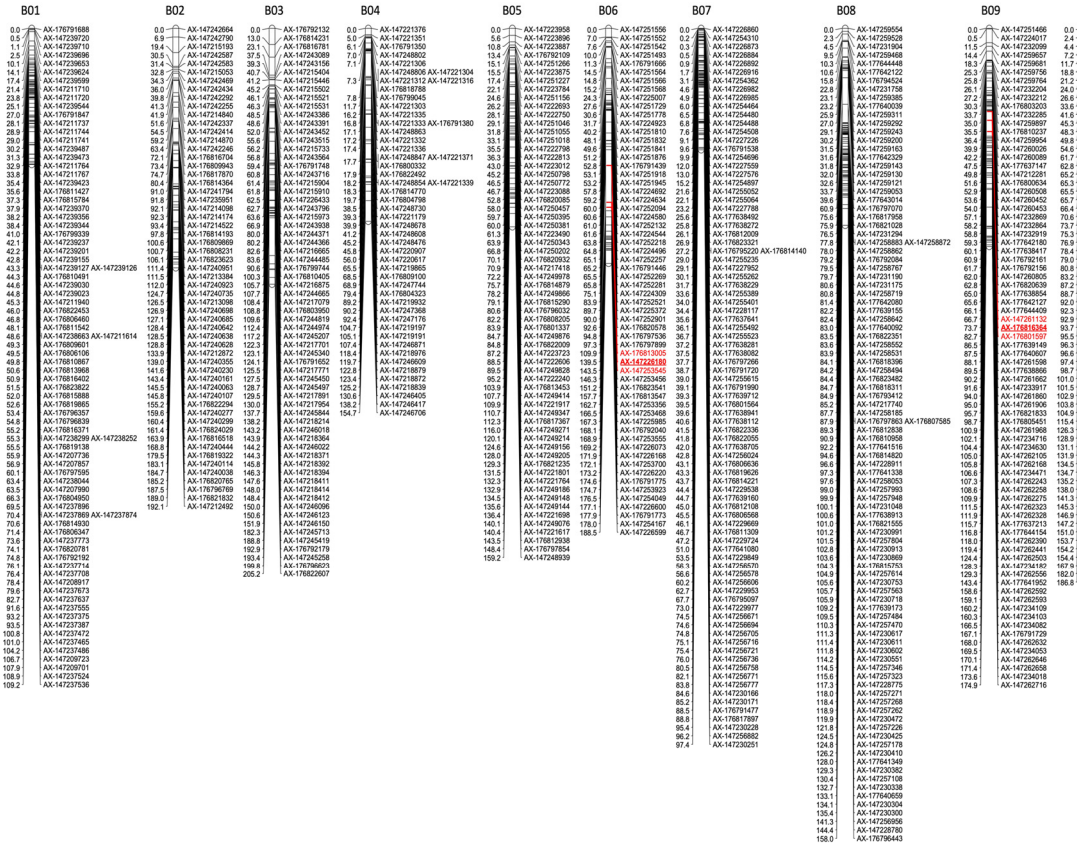
LG [a]	No. of Mapped SNPs [b]	Map Length [c]	Average Distance [c]	Maximum Distance [c]
A01	214	181.25	0.85	14.46
A02	110	239.23	2.17	20.02
A03	73	201.25	2.76	14.69
A04	30	173.73	5.79	15.97
A05	74	130.70	1.77	15.74
A06	118	284.70	2.41	19.83
A07	122	213.28	1.75	19.97
A08	171	157.17	0.92	14.03
A09	136	132.75	0.98	7.90
A10	116	162.76	1.40	9.69
B01	82	109.20	1.33	8.91
B02	57	192.06	3.37	12.70
B03	65	205.21	3.16	30.40
B04	51	154.71	3.03	18.83
B05	63	159.16	2.53	10.76
B06	60	188.52	3.14	29.58
B07	86	97.42	1.13	6.58
B08	98	158.05	1.61	21.33
B09	78	174.89	2.24	15.16
B10	66	186.78	2.83	14.10
Subtotal	1870	3502.82		
A03.2	56	353.45	6.31	20.40
Total	1926	3856.27		

[a] LG = linkage group; [b] the SNP markers per linkage group (LG) are expressed in absolute numbers; [c] map length, average distance between markers, and maximum distance between markers are expressed in Kosambi cM.



(**a**)

Figure 7. *Cont.*



(**b**)

Figure 7. The genetic linkage map constructed from a population with 321 F_2s obtained from a cross of *A. hypogaea* TifGP-2 and the induced allotetraploid ValSten1 [*A. valida* G 30011 × *A. stenosperma* V 10309]$^{4\times}$. The map has 11 LGs of the A subgenome (**a**) and 10 LGs of the B subgenome (**b**). The name of each linkage group (LG) is shown at the top of each group. The 1926 SNP markers are indicated on the right side of the 21 LGs. The position of a marker on the LG is shown on the left side. Markers are termed by the probe set name in the 48K 'Axiom_*Arachis* v02' SNP array [84]. The co-localized markers are shown parallelly on the right. Identified quantitative trait loci (QTLs) are highlighted in green (resistance) or red (susceptibility to stem rot). The underlined colored marker is the nearest marker of the identified QTL. The visualization of the LGs with QTLs was graphed using MapChart [91].

3.2.3. QTL Discovery

Two sets of QTLs were detected using the phenotypic and genotypic data: one set is associated with lower stem rot disease, and the other is related to higher disease. In the first set, two QTLs were identified on LG A10 and LG A08 (Figure 8a,b), therefore originating from *A. stenosperma*. In the other set, four QTLs correlated with higher stem rot ratings on LGs of A06, B06, and B09 (Figure 8c–f), originating from *A. valida*. Detailed QTL information and the correlation between the QTL and phenotypes are shown in Table 6 and Figure 8.

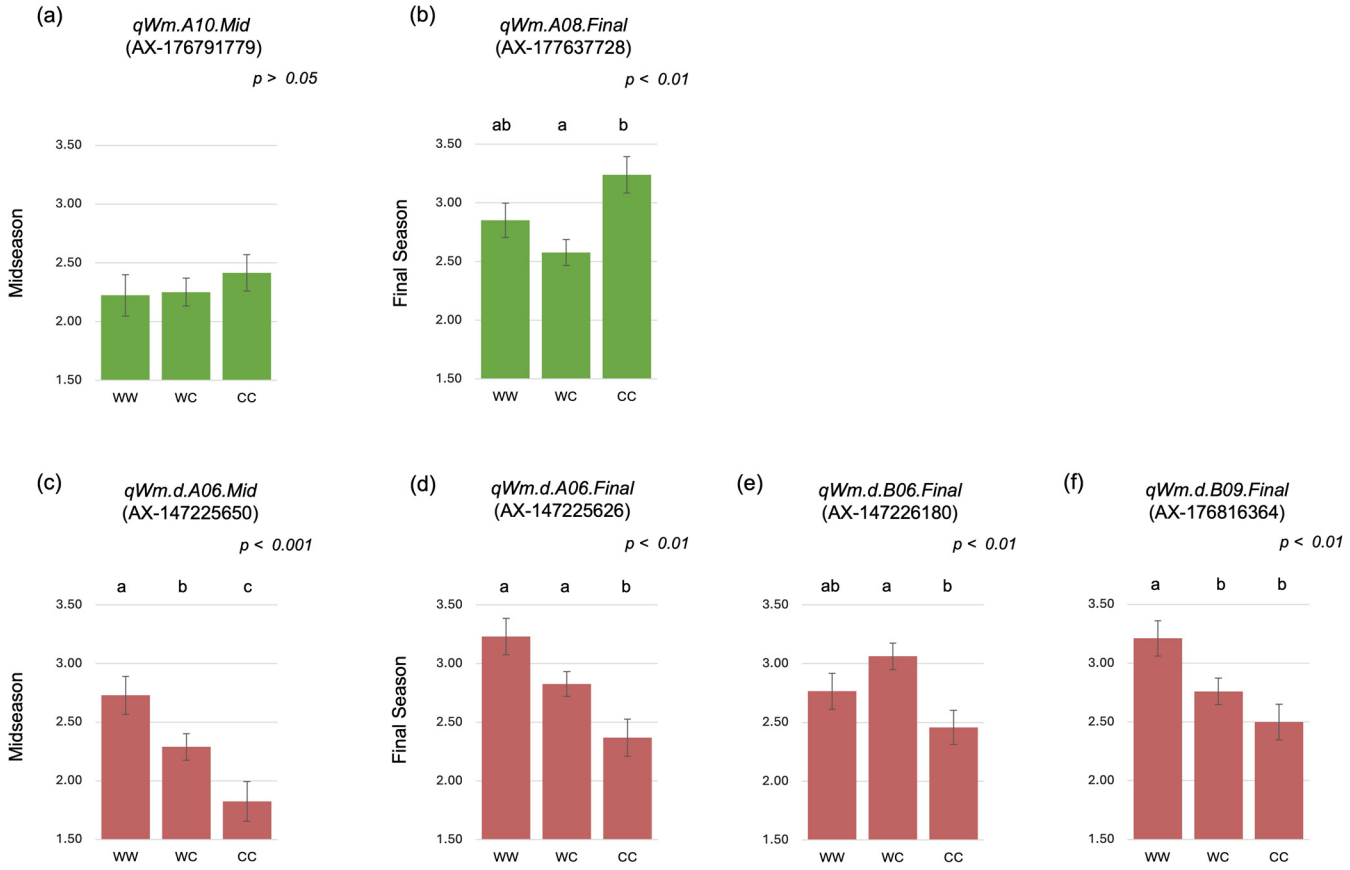


Figure 8. Bar graph of the contribution of wild and cultivated-derived alleles to stem rot ratings: in (**a**,**b**) the wild alleles decrease, and in (**c**–**f**) wild alleles increase stem rot ratings. Each graph displays the name of the identified quantitative trait locus (QTL), with the linkage group where it is located (e.g., A10) and the evaluation time (Mid = midseason, Final = end of season). Below the QTL name is the nearest single nucleotide polymorphism marker from the Axiom_*Arachis* v02. The *y*-axis represents the rating score of stem rot, while the *x*-axis depicts three groups of individuals: those with homozygous alleles derived from the wild (WW), heterozygous (WC), and homozygous TifGP-2 alleles (CC). The *p*-value in the top right corner of each plot indicates significant differences among the three genotypes, as determined by the Kruskal–Wallis test in RStudio (when $p < 0.05$). Columns marked by different letters denote significant differences among groups according to the post hoc Dunn test in R Studio (RStudio version 1.2.1335, Boston, MA, USA).

Set 1: QTLs associated with lower stem rot rating

Two QTLs were identified to be associated with either midseason or end-of-season lower stem rot rating. The QTL *qWm.A10.Mid*, associated with lower disease at midseason, was detected at LG A10 with an LOD score of 2.88, above the genome-wise empirical threshold at *p*-value = 0.05. The nearest SNP marker of *qWm.A10.Mid* is AX-176791779 (Table 6). The QTL *qWm.A08.Final*, associated with a lower rating at the end of season, was detected at LG A08 with an LOD score of 2.60, and the nearest SNP marker is AX-177637728. The QTLs *qWm.A10.Mid* and *qWm.A08.Final* have PVE values of 0.02% and 3.13%, respectively (Table 6). F_2 individuals with homozygous wild alleles (WW) at markers AX-176791779 and AX-177637728 exhibited stem rot ratings that were 8.60% and 13.64% lower, respectively, compared to individuals with homozygous TifGP-2 alleles (CC) (Table 6 and Figure 8a,b).

Table 6. QTLs associated with lower or higher stem rot disease identified from 321 TifGP-2 × ValSten1 F_2 population.

QTL Name	LG [a]	Genetic Position [b]	Nearest Marker [c] (Position)	Physical Position of Nearest Markers [d]	LOD Interval [e]	LOD [f]	LOD Threshold [g]	PVE (%) [h]	% [i]	Effect on Disease Evaluation
qWm.A10.Mid *	A10	154	AX-176791779 (156.87)	Aradu.A10 694,007	148.52–156.87	2.88	3.78 (1%), 2.86 (5%)	0.02	−8.60	Lower disease @ midseason
qWm.A08.Final	A08	89.5	AX-177637728 (89.49)	ambiguous	87.60–90.92	2.60	4.2 (1%), 2.79 (5%)	3.13	−13.64	Lower disease @ end of season
qWm.d.A06.Mid *	A06	153	AX-147225650 (153.03)	Aradu.A06 82,779,126	150.51–155.08	3.16	3.55 (1%), 2.96 (5%)	4.38	33.14	Higher disease @ midseason
qWm.d.A06.Final *	A06	182	AX-147225626 (181.53)	Aradu.A06 81,148,227	181.53–187.31	3.25	3.48 (1%), 2.88 (5%)	4.70	26.69	Higher disease @ end of season
qWm.d.B06.Final	B06	140	AX-147226180 (139.53)	ambiguous	109.95–143.48	2.37	3.46 (1%), 2.70 (5%)	2.96	11.15	
qWm.d.B09.Final	B09	73.7	AX-176816364 (73.72)	ambiguous	66.71–82.71	2.49	3.36 (1%), 2.7 (5%)	3.50	22.16	

[a] linkage group of identified QTL; [b] mapped position in cM; [c] probe set name of 'Axiom_*Arachis* v02' SNP marker which was mostly near the identified QTL; the position on LG for each marker was represented in parentheses; [d] physical positions of nearest marker for identified QTL are based on the diploid genomes annotated by Bertioli et al. 2016 [75,89]. Aradu. means the marker is on the diploid ancestor of cultivated peanut *Arachis duranensis*; [e] logarithm of the odds (LOD) support interval; [f] LOD score at QTL peak; [g] LOD threshold based on 1000 permutations at 1% and 5% significance levels; [h] proportion of the phenotypic variance explained by the QTL; [i] percentage of change in peanut stem rot disease score (calculated the disease between individuals with homozygous wild-derived genotype and homozygous TifGP-2-derived genotype).; * The LOD of QTL is higher than the 5% LOD threshold.

Set 2: QTLs associated with higher stem rot rating

Four QTLs correlated with higher stem rot ratings, with two on subgenome A and two on subgenome B. Both QTLs on subgenome A were located on LG A06. The *qWm.d.A06.Mid*, associated with higher disease during the midseason, was detected with LOD scores of 3.16, surpassing the genome-wise empirical threshold at *p*-value = 0.05. The nearest marker of this segment is AX-147225650, with a 4.38 PVE value for this QTL (Table 6). Comparing the disease in individuals with homozygous wild alleles (WW) and homozygous TifGP-2 alleles (CC) of marker AX-147225650, a 33.14% higher disease rating was observed in individuals with wild alleles (Table 6 and Figure 8c). Individuals with two different homozygous derivations showed significant differences from each other (Figure 8c). Another QTL, *qWm.d.A06.Final*, had an LOD score of 3.25, surpassing the genome-wise empirical threshold at *p*-value = 0.05. The nearest marker is AX-147225626, with a 4.7 PVE value for this QTL. A 26.69% higher disease rating was observed in F_2 individuals with homozygous wild alleles (WW) compared to individuals with homozygous TifGP-2 alleles (CC), showing statistically higher disease (Table 6 and Figure 8d).

Two QTLs were associated with higher disease on the B subgenome. The *qWm.d.B06.Final*, located on LG B06, had an LOD score of 2.37, with the nearest marker being AX-147226180. The *qWm.d.B09.Final*, situated on LG B09, had an LOD score of 2.49, with the nearest marker being AX-176816364. The QTLs *qWm.d.B06.Final* and QTL *qWm.d.B09.Final* have PVE values of 2.96% and 3.50%, respectively. F_2 individuals with homozygous wild alleles (WW) in markers AX-147226180 and AX-176816364 showed 11.15% and 22.16% higher stem rot ratings, respectively, compared to individuals with homozygous cultivated alleles (CC), indicating significantly higher disease levels of marker AX-176816364 (Table 6, Figure 8e,f).

4. Discussion

Peanut stem rot is challenging to control because of its broad host range and persistent presence in the field. The cost of controlling it is high. There is no strong resistance in the

primary gene pool of peanut. For this reason, tapping into the secondary gene pool of peanuts with resistance, its wild relatives, is promising [52]. However, the process of introgressing wild alleles can be time-consuming, typically beginning with the production of wild species-derived allotetraploids that are compatible with peanut, followed by multiple rounds of backcrossing [92]. Therefore, we screened several newly induced allotetraploids before initiating introgression.

Screening for stem rot resistance is not straightforward on cultivated peanuts. Screening wild-derived genotypes has the extra complication that there are significant differences in plant architecture between wild-derived allotetraploids and cultivated peanut [74]. To try to circumvent these difficulties, we screened allotetraploids in both field and greenhouse assays that were designed to make the comparisons as meaningful as possible. We recently developed the greenhouse method and showed that its results correlate with those of field assays [76]. However, the resistant and susceptible controls did not exhibit significant differentiation from each other in the greenhouse assays, nor did they show substantial distinction in the field evaluations. This lack of differentiation can be attributed to the highly aggressive nature of this necrotrophic pathogen. In traditional field assays, the measurement is typically not on the effect of the fungus on individual plants but rather on the spread of infections within a row of plants after inoculation [93,94]. More resistant genotypes are expected to allow for less spread of the pathogen. However, for such evaluations to be effective, genotypes must be genetically homogeneous, and those being compared should have a similar architecture. Additionally, enough seeds (in the range of hundreds to thousands) should be available for all genotypes being tested. Most of these requirements are not met in the case of a peanut pre-breeding program using wild species: induced allotetraploids exhibit a very different architecture from cultivated peanut and produce few seeds. Consequently, the only feasible method to evaluate them is by inoculating and assessing the pathogen development on individual plants. However, the highly aggressive nature of the pathogen complicates screening. Thus, while we acknowledge that our methods may not capture the full differential response to the pathogen, pragmatically, the general trends need to be used to guide research and the formation of hypotheses to be tested. During the development of the greenhouse evaluation method, it became apparent that even small numerical differences underscored field resistance in cultivated peanuts [76].

Results from the field and greenhouse tended to follow the same general trends. However, in the field, induced allotetraploids were less resistant. This can be explained by the lack of adaptation of the wild-derived plants to field conditions, which influences their growth, vigor, and overall health, subsequently affecting their resistance response to *A. rolfsii*. Moreover, in the field evaluation, background peanut plants were planted next to the tested allotetraploids to make the environment more homogeneous. However, as the background peanut plants had a well-developed canopy, and the allotetraploids were shaded beneath it, they received insufficient sunlight for vegetative development and, therefore, were disadvantaged in the field evaluation. Considering these factors, the potential of the wild genetics as resistance donors was likely underestimated by field assays. In the greenhouse assay, a clearer trend was observed, with all allotetraploids being more resistant than the susceptible controls. Four induced allotetraploids, GregSten1, ValSten1, IpaDur3, and BatSten1, among the tested genotypes, exhibited significantly shorter lesion lengths from 5 DPI to 9 DPI and lower AUDPC values compared to the susceptible control (Georgia-09B). The evaluations of these four allotetraploids closely resemble those of the resistant control (Georgia-12Y) in the greenhouse assay (Table 1 and Figure 4). The wild parent, donor of the A-subgenome of three of the most resistant allotetraploids, GregSten1, ValSten1, and BatSten1, is *A. stenosperma* accession V 10309. This accession is also resistant to several other pests and diseases, such as root-knot nematode, rust, late leaf spot, and tomato spotted wilt virus [34,59,74,95–97]; therefore, the use of these allotetraploids to breed for stem rot resistance also has the potential to improve the resistance to these other diseases. Three allotetraploids that have as parents the progenitor species of peanut (*Arachis*

ipaënsis and *A. duranensis*) were also assessed, IpaDur1, IpaDur2, and IpaDur3. Among them, only IpaDur3 displayed resistance to stem rot (Table 1 and Figure 4). The discrepancy in resistance levels between allotetraploids with parents from the same species can be attributed to the fact that IpaDur1 and IpaDur2 are the result of the cross using the same accessions (*A. ipaënsis* K 30076 and *A. duranensis* V 14167; in different labs and at different times) [73,75]. IpaDur3, however, is derived from a different accession of *A. duranensis* K 30060 [34], which is likely the source of resistance. IpaDur3 has also been found to be resistant to fall armyworm [34,98].

One of the most resistant allotetraploids in the greenhouse test, ValSten1, was also confirmed to exhibit partial resistance in field evaluation. It is noteworthy that, although there is variability in the placement of ValSten1's resistance score over the years, the 4-year field evaluation (2019 to 2022) confirms that Georgia-12Y is more resistant than Georgia-09B and suggests that ValSten1 is more resistant than Georgia-09B and comparable to Georgia-12Y (Table 4, Supplemental File S5). Results obtained in the greenhouse were much clearer, showing the superior resistance of ValSten1 compared to the susceptible control (Figure 4). ValSten1 was chosen as the material for subsequent resistance breeding due to its partial resistance to stem rot and also for its vigorous growth, prolific seed production, and excellent compatibility with peanut. ValSten1 was crossed with the *A. hypogaea* advanced line, TifGP-2, and the highly prolific F_1 hybrid obtained yielded a large F_2 population consisting of 325 individuals for further study. To identify the genomic regions that encode for stem rot resistance, all 325 individuals were field evaluated and subsequently genotyped. During field evaluations, numerical differences were observed between the resistant control Georgia-12Y and the susceptible control Georgia-09B; however, this difference was not statistically significant. The symptoms that developed during midseason on Georgia-12Y and Georgia-09B are shown in Figure 9. Most plants of Georgia-12Y and Georgia-09B were scored as 2 or 3 both in the midseason and end-of-season evaluations. Differentiating the disease at this stage using the current evaluation system and rating scale proved challenging, and the rating scale will probably need to be further refined. Surprisingly, ValSten1 exhibited an unexpectedly similar rating to Georgia-09B and higher than Georgia-12Y (Table 4).

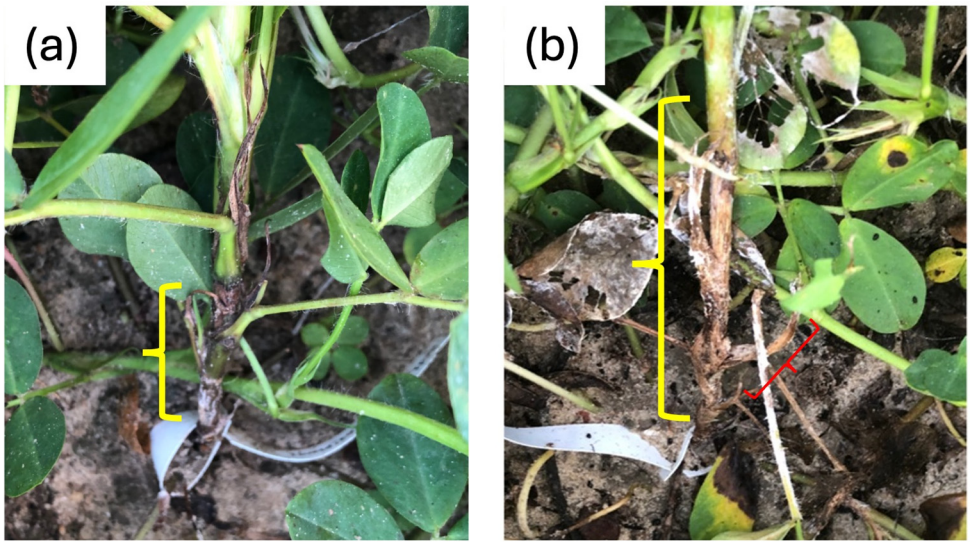


Figure 9. The disease developed on (**a**) resistant control Georgia-12Y with lesions mainly developed on the main stem (yellow bracket) and (**b**) susceptible control Georgia-09B with longer lesions on the mainstem (yellow bracket) and lesions on the lateral branch (red bracket). The evaluation was undertaken during midseason.

In our mapping analysis, we report 21 linkage groups (LGs). Although the JoinMap software v4.1 initially returned 19 LGs, these effectively represent the A01 to A10 of the

peanut A subgenome, B01–06, and B08–B10 of the peanut B subgenome. After examining the sizes of LGs in centimorgans (cM) and comparing the genetic position of each group with the coordinated physical position of the peanut chromosome in the Marey plot, we manually split original LG 07 and LG 03 into two groups due to insufficient separation from clusters in the JoinMap. The original LG 07 clustered chromosomes A07 and B07 of the peanut genome together, with a large gap of about 10,000 cM in between. Therefore, LG 07 was manually split as A07 and B07. Furthermore, a manual separation was also applied to LG 03 due to an obvious distorted pattern observed in the Marey plot (Supplemental File S3). The LG 03 was split into LG A03 and LG A03.2, with the LG A03 mainly constructed from *stenosperma*-specific markers and A03.2 primarily grouping parent-differentiation markers. The sizes of the 21 LGs ranged from 97.42 cM (LG B07) to 353.45 cM (LG A03.2), spanning a total map distance of 3856.27 cM (Table 5). This mapping result is comparable to other similar studies conducted recently. For example, Ballén-Taborda et al. (2019) reported peanut root-knot nematode resistance QTL mapping in 2019 with 20 LGs ranging in size from 100.7 cM to 359.5 cM, spanning a total map distance of 3984.9 cM, mapped from an interspecific population developed from crosses between an allotetraploid (also derived from *A. stenosperma* V 10309) and cultivated peanut lines [59]. Similarly, de Blas et al. reported peanut smut resistance QTL mapping in 2021 with 21 LGs ranging in size from 17.29 cM to 236.22 cM, spanning a total map distance of 2531.81 cM, mapped from another interspecific population developed from crosses between an allotetraploid and elite lines [99].

The QTL analysis identified two sets of QTLs: one set correlated with lower disease ratings and the other with higher disease ratings (Table 6 and Figure 8). The QTLs associated with lower disease ratings are both derived from *A. stenosperma* V 10309; one is linked to midseason, and the other is correlated with the end-of-season's lower rating, located at LG A10 and A08, respectively. Two QTLs associated with higher disease ratings are also from *A. stenosperma* V 10309, both located at A06. One is related to the midseason, while the other is associated with the end-of-season's higher stem rot rating. The coexistence of beneficial and deleterious QTLs in *A. stenosperma* V 10309 complicates its utilization for resistance breeding, and QTL-linked markers may facilitate the transfer of resistance into the target population while avoiding the transfer of the regions associated with susceptibility. Additionally, the B subgenome donor *A. valida* G 30011 contributed two deleterious QTLs at LG B06 and B09.

The identified QTLs in this study are all estimated to be minor QTLs (PVE < 10%, Table 6). This is consistent with published studies that mostly report stem rot QTLs as being minor [57,70,71,100]. Among these published studies, one QTL associated with lower stem rot disease colocalized with the QTL related to the branching habit. The weak-effect QTLs identified in this study may not solely arise from their genetic characteristics but could also be because analyses were conducted in a single season/location, lacking confirmation in other locations. Moreover, phenotyping wild-derived materials presented inherent difficulties, with the differences in architecture between wild-derived and pure cultivated peanuts and the genetic uniqueness of each F_2 plant constraining experimental design. In the context of both evaluation methods used here, a small single lesion that encircles the main stem can result in a severe disease rating by causing the demise of the entire stem. This contrasts with standard field evaluations where the spread from plant to plant in a row is the most crucial factor. The difficulties in evaluation effectively add noise to the phenotyping, very probably contributing to the weak-effect QTLs observed in this study. Despite the small effect estimated by the software and the low LOD scores, when we calculated the effect of identified QTL on the susceptibility of genotypes by comparing individuals with or without the QTLs, the results showed that the effects were larger than estimated (Table 6 and Figure 8). Notably, the QTL *qWm.A08.Final*, which had an estimated PVE of only 3.13%, showed an observed reduction in disease of approximately 13%, highlighting its significance as the primary QTL for subsequent focus in resistance breeding.

The polyploid origin and selection during domestication and breeding have resulted in peanut having very limited genetic diversity. This constrains the advances that can be made in breeding programs using only pure pedigree cultivated peanut. Crop wild relatives, which are subjected to natural selection in their native environments, represent crucial reservoirs of genetic diversity for crop breeding programs. In the case of peanut breeding, the narrow genetic diversity poses a significant challenge. However, although wild relatives of cultivated peanut have been identified as potential sources of resistance to stem rot [50–52], they have yet to be extensively utilized in peanut breeding programs targeting stem rot resistance. Therefore, the identification of stem rot resistance in allotetraploid species in this study underscores their potential as valuable resources in peanut breeding efforts, particularly due to their readily crossable ploidy level. Resistance breeding involves stacking different sources of resistance into one cultivar to achieve stronger and more durable resistance to a particular disease. The practice of stacking resistance genes has been increasingly adopted worldwide for nearly a century. A prime example is the control of wheat stem rust through the stacking of resistance genes [101]. Other examples are resistance against bean rust, wheat powdery mildew, and potato late blight [102–104]. Currently, cultivated peanuts exhibit only moderate to low stem rot resistance. Combining multiple resistances into a single genotype should enhance durability and provide more effective resistance than cultivars with a single source of resistance. Therefore, while ValSten1 exhibits similar resistance to the most resistant cultivar, Georgia-12Y, it likely remains valuable to include this wild-derived allotetraploid in breeding programs to achieve even higher levels of resistance. Pyramiding resistance to various diseases together can be another objective for breeders. Given that the three allotetraploids derived from *A. stenosperma* V 10309 revealed superior stem rot resistance [34,59,74,95–97], breeding stem rot resistance with any allotetraploid derived from *A. stenosperma* V 10309 may additionally improve other disease resistances.

5. Conclusions

This is the first report on the evaluation of peanut-compatible wild-derived allotetraploids for peanut stem rot resistance. Allotetraploids derived from *Arachis stenosperma* exhibit the highest levels of resistance, with ValSten1 performing the best in both greenhouse assays and field evaluations. Additionally, this study identified wild chromosome segments associated with the disease using a ValSten1-derived population. While the validation of these QTLs in different populations is still needed, this represents a crucial step toward identifying wild-derived chromosome segments that contribute to increased resistance to stem rot. The breeding program can be more informed by choosing individuals with markers linked to lower stem rot-associated QTLs and excluding those with markers linked to higher stem rot-associated QTLs. This is the first study that reports wild-derived QTLs related to resistance to stem rot. ValSten1 is a promising candidate donor for stem rot resistance breeding.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14071442/s1; Supplemental File S1: Unix script used for distinguishing wild-species specific markers and identifying which loci in F_2 individuals derived from the wild species *A. valida* or *A. stenosperma*, Supplemental File S2: detailed genetic position and physical position for 1926 SNP markers, Supplemental File S3: Marey Plot of 1926 SNP markers with genetic position and physical position information, Supplemental File S4: genotyping color map of 321 F_2 individuals arranged in 21 linkage groups, Supplemental File S5: Four-year stem rot disease evaluation of allotetraploid ValSten1, resistant control (Georgia-12Y), and susceptible control (Georgia-09B).

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manuscript. S.L.—assistance in linkage mapping construction and manuscript writing. D.J.B.—supervision of the linkage map construction and QTL analysis, review and editing of the manuscript, and funding acquisition. S.C.M.L.-B.—conceptualization, methodology, supervision of the analyses, funding acquisition, project administration, review and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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