PEANUT SCIENCE

The Journal of the American Peanut Research and Education Society

2023 Bailey Award Winner

ARTICLE

Discovery of a resistance gene cluster associated with smut resistance in peanut

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ARTICLE INFORMATION

Keywords:
Peanut, Smut, Resistance, Quantitative Trait Loci

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DOI: 10.3146/0095-3679-51-PS23-6

ABSTRACT

Peanut smut, caused by *Thecaphora frezzii* Carranza & J.C. Lindq., is an emerging threat for the global peanut industry. The disease's destructive potential can be exemplified by pod incidence values as high as 70% and yield losses reaching 30%. Because fungicides have shown moderate but highly variable levels of control, development and deployment of smut resistant cultivars are the best strategies for disease management. Screening for smut-resistant germplasm requires years of field trials and is currently the only option for breeders because genetic markers for resistance have not yet been developed. The objectives of this study were to perform whole genome sequencing (WGS) on populations developed for smut resistance mapping and subsequently fine map discovered QTL associated with smut resistance. An expedited strategy was employed by phenotyping in the F_3 generation. We phenotyped 200 families with 3 plants per family for smut resistance in infested fields during the 2019/2020 season in General Deheza (32°45′23″S 63°47′20″W), Argentina. Each individual was sequenced using iGenomXRipTide library preparation and Illumina NovaSeq sequencing to yield approximately 1 times genome coverage. Analysis of the phenotype and genotype data using Khufu resulted in the identification of a single major smut resistance QTL on chromosome 12 (B02). Chromosome level genome sequences were assembled for the resistant parent (Ascasubi) and susceptible parent (Granoleico) using PacBio HIFI sequencing. A validation population was sequenced using Khufu to validate the QTL region and analysis confirmed the major locus on chromosome 12. The identified variation will be used to develop smut resistant varieties quickly using molecular assisted breeding strategies.

INTRODUCTION

Peanut smut, caused by *Thecaphora frezzii* Carranza & J.C. Lindq. (*T. frezzii*), is an emerging threat for the global peanut industry (BICON, 2017; Rago *et al*., 2017). First described in 1962, *T. frezzii* was observed on a collected sample of a wild peanut from Mato Grosso do Sul, Brazil, that was sent to INTA's Manfredi Exp. Stn. in Córdoba Province, Argentina (Carranza & Lindquist 1962). In 1995, peanut smut was first detected on cultivated peanuts on three farms in north-central Córdoba (Marinelli *et al*., 2008). Since then, it has spread to all peanut-producing provinces in Argentina (Cazón *et al*., 2018). The disease's destructive potential can be exemplified by pod incidence values as high as 70% (Bonessi *et al*. 2011, Chamberlin et al. 2022) and yield losses reaching 30% (Oddino *et al*. 2010). Disease management strategies using pesticides or biocontrol agents (Figueredo *et al*. 2017), liming (Bonessi *et al*. 2011), tillage (Cignetti *et al*. 2010; Cazón *et al*. 2014), and rotation (Marraro Acuña and Haro, 2011) have been attempted with modest results. Fungicides have shown moderate but highly variable control levels (Rago *et al*. 2017; Paredes *et al*. 2021). Smut resistant cultivars seem the best tool for disease management as they suffer less crop loss and limit the pathogen's multiplication (Rago *et al*. 2017). Sources of smut resistance have been reported in wild peanuts (de Blas *et al*. 2019), synthetic amphidiploids (de Blas *et al*. 2021) and in *A. hypogaea* core collections (Chamberlin *et al*. 2022; Wann *et al*. 2020). Smut genetic resistance has shown high broad sense heritability (Bressano *et al*. 2019; de Blas *et al*. 2021). Recently, major and a minor quantitative trait loci (QTL) associated with moderate resistance have been identified in a recombinant inbred line (RIL) population developed from a cross between a susceptible *A. hypogaea* genotype and a resistant synthetic amphidiploid [*A. correntina* × *A. cardenasii]* × *A. batizocoi* (de Blas *et al*. 2021). Peanut reaction to *T. frezzii* is assessed by opening each pod by hand and inspecting it visually for incidence (i.e., the presence or absence of *T. frezzii* in pods), which is time and labor intensive. Screening for smut resistant genetic sources requires years of in-field trials along with pod phenotyping and is currently the only option for breeders because markers for resistance have not yet been developed.

Marker assisted selection (MAS) is now widely used in plant breeding to increase the efficiency of developing cultivars with highly desired traits. Early marker work identified several for disease resistance in peanut. Random amplified polymorphic DNA (RAPD) was used to identify root-knot nematode (RKN) resistance (Burow *et al*., 1996), restriction fragment length polymorphisms (RFLPs) were identified for resistance to *Meloidogyne arenaria* (Church *et al*., 2000), amplified fragment length polymorphisms (AFLPs) were found linked to resistance to the vector of groundnut rosette virus (Herselman *et al*., 2004), and single sequence repeats (SSRs) associated with resistance to *Sclerotinia minor* (Jagger) (Chenault, *et al*., 2009) along with those for tomato spotted wilt virus (TSWV) and leaf spot (LS) (Wang *et al*., 2013; Tseng *et al*., 2016; Zhao *et al*, 2018), rust (Khedikar *et al*., 2010; Mondal and Badigannavar, 2018), and bud necrosis disease (Jadhav *et al*., 2019) were identified.

Sequencing of the diploid progenitors (Bertioli *et al*, 2016), *A duranensis* and *A. ipaensis*, provided the reference genomes necessary to construct the high-density single nucleotide polymorphism (SNP) genotyping array Axiom_Arachis (Pandey *et al*., 2017) which led to the generation of SNP-based linkage maps enabling the identification SNPs associated with traits. SNPs are preferred over other marker types due to their wide distribution throughout the peanut genome (Liao and Lee, 2010). Whole genome resequencing (WGRS) of mapping populations has also been used to generate high-density SNP-based maps and identify QTL along with candidate genes for resistance to peanut diseases such as early LS (ELS), late LS (LLS), and spotted wilt (Agarwal *et al*., 2018). Such linkage maps have also been used to identify QTL for resistance in peanut to RKN (Leal-Bertioli *et al*., 2016), *Aspergillus flavus* (Khan *et al*., 2020), bacterial wilt (Luo *et al*, 2020a), stem rot (Luo *et al*. 2020b), Sclerotinia blight (Liang *et al*., 2021), and bud necrosis disease (Jasani *et al*., 2021). The recent release of the tetraploid (cultivated) peanut genome sequence (Bertioli *et al*., 2019) has further clarified questions regarding *A. hypogaea* evolution and the generation of a high-density SNP array for tetraploid peanut (Axiom_Arachis2) (Clevenger *et al*., 2017, Clevenger *et al*., 2018) provide further foundation for rapid marker-trait association. Using the Axiom_Arachis SNP array, potential SNPs and QTL associated with peanut smut resistance were identified by association mapping using a limited number of genotypes (Massa *et al*., 2021), but no confirmed QTL or candidate genes have been reported to date. Therefore, the objectives of this study were to perform whole genome sequencing (WGS) on a population developed for smut resistance mapping and subsequently fine map discovered QTL associated with smut resistance.

MATERIALS AND METHODS

Plant Materials and Population Development.

The mapping population consisting of 200 lines was developed by crossing Mf10_2870, smut susceptible (J. Baldessari, unpublished data), and Ascasubi Hispano, smut resistant (Ibañez *et al*., 2018). An additional population used for validation purposes was also developed by crossing Granoleico, smut susceptible (Soave, 2002; Oddino *et al*., 2013) and Ascasubi Hispano. To rapidly identify QTL associated with smut resistance, a non-traditional approach was used where families within the population were phenotyped and genotyped at familial stages prior to the F_7 generation rather than developing a uniform recombinant inbred line (RIL) population. Briefly, three individuals from each $F_{2:3}$ family (for the mapping population) and from each $F_{3:4}$ family (for the validation population) were phenotyped under heavily smut infested field conditions. This was done during the 2019-2020 season for the mapping population and during the 2020-2021 season for the validation population, in General Deheza, Córdoba Province, Argentina under rainfed conditions.

Experimental Design and Field Testing.

Smut resistance tests were conducted as described in Chamberlin *et al*., 2022, in side-by-side fields of the same farm (-32.759, -63.770) in the town of General Deheza (Córdoba Province, Argentina). Soil at the site was a General Deheza coarse-silty sandy loam type (0.3% slope, coarse-silty, mixed, thermic, Entic Haplustoll). The tests were planted one km

downwind from a peanut processing plant where peanut smut is prevalent. The production field site was on a rotation schedule of corn-corn-soybean-peanut, where the preceding crop of the test site was always soybean.

Two control entries, one resistant (Ascasubi Hispano) and one susceptible (Colorado Irradiado INTA) were planted in both seasons. They were replicated twelve times each. Each family was represented by three individuals, each one constituting a plot. Plots were arranged in a square grid and randomized within it. Controls were placed, alternately, along a diagonal of the grid. Individual plots were spaced one m apart among rows and between plants.

The tests were planted on 11/7/19 and 12/23/20, using an augmented grid design with three replications, using single plants as experimental units. Plants were respectively dug at 147 and 140 days after planting (DAP), and all pods from each plant were harvested by hand and placed in mesh bags to air dry for two months. Yearly soil spore count was done as described by Chamberlin *et al.* (2022) to determine disease pressure. The test was conducted following the extension guidelines of the Instituto Nacional de Tecnología Agropecuaria (INTA).

Harvest and Evaluation of Smut Resistance.

Each pod was opened by hand and declared as Infected if *T. frezzii* sori was present or spore masses on the kernels. Disease incidence (DI) was calculated as DI = Infected pods / Total pods.

QTL mapping and validation.

DNA was taken from 572 individuals (approximately 3 individuals from each of the 200 lines) of the $F_{2:3}$ family (Mf10_2870 and Ascasubi Hispano. DNA was also taken from 96 individual plants of $F_{2:3}$ family of Ascasubi x Granoleico selected for phenotype. DNA extraction was performed from 20 mg of dried young leaves in silica gel. The samples were ground for two 10-sec cycles in bead mill at 15.000 rpm (Super FastPrep-2 Bead Beating System, MP BiomedicalsLLC, Irvine, CA, USA). A modified CTAB method was used with a sorbitol cleaning wash before the lysis step (Inglis *et al.* 2018). DNA quality was determined using 0.8% agarose gel and the DNA quantity was estimated with DS-11+ Nano UV-Vis Spectrophotometer (DeNovix Inc., DE, USA).

Parental genomic DNA extraction.

For whole-genome sequencing, seeds of Ascasubi Hispano Cv, Granoleico Cv and Mf10_2870 were germinated in an incubation oven, under total darkness at constant temperature of 28 °C for 15 days. Fresh young etiolated leaves were collected for each genotype, immediately frozen with liquid nitrogen, and preserved at -80 C (Revco, Model ULT1386-5V41). The nucleii were extracted following the Nucleii Isolation – LN2 Plant Tissue Protocol (Ciculomics Inc./ PacBio) optimized from Workman *et al.* (2018). In the step 5 of protocol, filtration of the lysate with steriflip 20 µm pore was replaced with two lysate filtrations with a cell strainer of 100 µm pore and 40 µm pore. High-molecular weight (HMW) DNA was extracted from nucleii following the specifications of Nanobind Plant Nuclei Big DNA Kit (Circulomics Inc./ PacBio). DNA quality was determined using 0.8% agarose gel and the DNA quantity was estimated by Qubit V1 (Invitrogen ThermoFisher Scientific Inc.) using Qubit® dsDNA BR Assay Kits.

Genotyping: Affymetrix Axiom SNP Array.

Seeds from the peanut mini core accessions were sowed in 4.7-m pots with a mix of 50% Promix (Premier Tech Horticulture, Quaker, PA) and 50% steam-sterilized sandy soil from the Coastal Plain Experiment Station in Tifton, GA. Young leaf tissue was collected from 1-month-old plants for DNA extraction using DNeasy Plant mini kit (www.qiagen.com). Quantification of DNA was performed with Quant-iT dsDNA assay kit (www.thermofisher.com). DNA were submitted for genotyping with the *Arachis* version SNP array consisting of 47K SNPs features (www.thermofisher.com). SNP data were curated from the Axiom analysis suite (www.thermofisher.com). SNP markers were classified into six categories, i.e., PolyHighResolution, NoMinorHom, MonoHighResolution, CallRateBelowThreshold, OfftargetVariant, and Other according to the SNP QC matrix of the software (Clevenger *et al.*, 2017). All data analysis in this study was performed with markers in the PolyHighResolution category due to its high quality in signal separation.

Genotyping: PacBio Sequencing

High molecular weight DNA was received from *A. hypogea* Granoleico and *A. hypogea* Ascasubi. DNA was sheared using the Diagenode Megaruptor 3 targeting 20kb fragments. Sheared DNA was prepared for PacBio sequencing using the SMRTbell Express Template Prep Kit 2.0. The library was size selected with AMpure PB beads to remove fragments less than 3kb. Sequencing was performed on a Sequel IIe System (Pacific Biosciences, Menlo Park, CA) using Binding Kit 2.2, Sequel II Sequencing Kit 2.0, and SMRTCell 8M. To target HiFi reads, the library was sequenced using a 30-hour movie time using Instrument Control Software Version 10. Raw subreads were converted to HiFi data by processing with CCS to call a single high quality consensus sequence for each molecule, using a 99.5% consensus accuracy cutoff. *A. hypogea* Granoleico was sequenced on 2 SMRTcells yielding 73.11Gb of HiFi data, and *A. hypogea* Ascasubi was sequenced on 3 SMRTcells yielding 61.37Gb of HiFi data.

Genotyping: Khufu whole-genome sequencing and analysis

DNA from populations were prepped into sequencing libraries using iGenomX Riptide library prep. For the F2:3 population, Six Riptide libraries, representing 576 samples were sequenced on 2 lanes of NovaSeq S4 chemistry (800 Gb raw base pairs per lane) to yield an average of 0.99X genome coverage per individual. The raw reads were demultiplexed and processed using Khufu (hudsonalpha.org/khufudata). To identify QTL for resistance, bulks were identified using one year of field data. Analysis of the bulks were done *in silico*, by bulking individual sequenced samples and calculating allele frequency differences between the putative resistant and susceptible bulks. The F3:4 samples were processed and analyzed the same way, except were sequenced to an average of 1.14X genome coverage. Haplotype analysis was done using HawkHAP, which is a component of Khufu.

RESULTS AND DISCUSSION

Generation of reference genomes

Long read sequencing and assembly led to the generation of highly contiguous genome assemblies for smut resistant parent Ascasubi, and smut susceptible parent Granoleico (Table 1).

Mapping of smut resistance

A total of 576 individuals were sequenced to construct bulks for mapping. They represented 200 F2:3 sub-families where each family was represented by 3 individuals. The smut pressure was low due to drought stress during pod development, but there were enough 'susceptible' individuals to map construct bulks (Figure 1). The frequency of infection was heavily skewed to the low end of the distribution, indicating that there was a considerable amount of false negative disease scores. To construct bulks, we employed a conservative approach whereby we selected susceptible individuals when all three sub-family members had infection and the average infection was greater than 5%. It was more difficult to select resistant bulks, because more than 400 individuals had 0% infection. For the 'resistant' bulk we selected every family member if they each had 0% infection and at least one individual was located in the field close to a susceptible check that exhibited infection. These criteria were chosen to reduce false negatives in the bulk as much as possible.

We selected 45 individuals for the 'resistant' bulk and 49 individuals for the 'susceptible' bulk. The QTL-seq analysis was conducted by bulking bam alignment files *in silico* and assaying allele frequency differences genome-wide between the bulks (Figure 2A). Analysis of the bulks indicated one major QTL located on the proximal end of chromosome 12. There were no other peaks identified, indicating that a single, major locus accounted for smut resistance from Ascasubi. In the following year (2019-2020), we phenotyped a related population of F3:4 progeny with Ascasubi as a parent and Granoleico as the susceptible parent. We selected a set of 48 resistant individuals and the 48 most susceptible individuals. We sequenced those 96 individuals and again analyzed them with QTL-seq analysis. The major locus on chromosome 12 was again indicated as a single, major resistance locus controlling resistance (Figure 2B).

We have identified and validated a major locus controlling smut resistance in peanut. The qualitative inheritance of this trait, its high level of heritability, low environmental variance, and strong phenotyping methodologies, we were able to map and validate this resistance within early generations (F2:3 and F3:4), by phenotyping single plants rather than replicated plots. Phenotyping and genotyping early generation families within a developing population saves time and allows the potential for selection for backcross breeding. This method greatly decreased the time needed for discovery. The resistance is indicated on an approximal 2 Mb region that contains the largest cluster of R genes in the peanut genome (Bertioli *et al.*, 2016). We have generated highly contiguous genome assemblies of the resistant parent, Ascasubi, and of the most widely grown cultivar in Argentina, Granoleico. Granoleico is very susceptible to smut, and the differences between those two genomes within the 2 Mb region will contain the functional variation that confers strong resistance to smut. This resistance and the markers associated with it will be transformational in breeding smut resistant cultivars for Argentina and the world.

Table 1. Assembly stats for the Ascasubi and Granoleico genome assemblies. Contigs were scaffolded into 20 pseudomolecules comprising the 10 A chromosomes and 10 B chromosomes. N/L50 is the length of the contig after half of the assembly has been accounted for when contigs are sorted from largest to smallest. Briefly, for Ascasubi half of the genome is assembled into 16 contigs that are longer than 53 Mb. N/L90 is calculated the same, but for 90% of the assembly. Briefly, for Ascasubi, 90% of the assembly is in 39 contigs which are longer than 19 Mb.

	Ascasubi (R)	Granoleico (S)
Scaffold total	20	20
Contig total	92	97
Contig N/L50	16/53.095 Mb	18/53.926 Mb
Contig N/L90	39/19.502 Mb	39/27.963 Mb
Max contig length	136.375 Mb	159.756 Mb

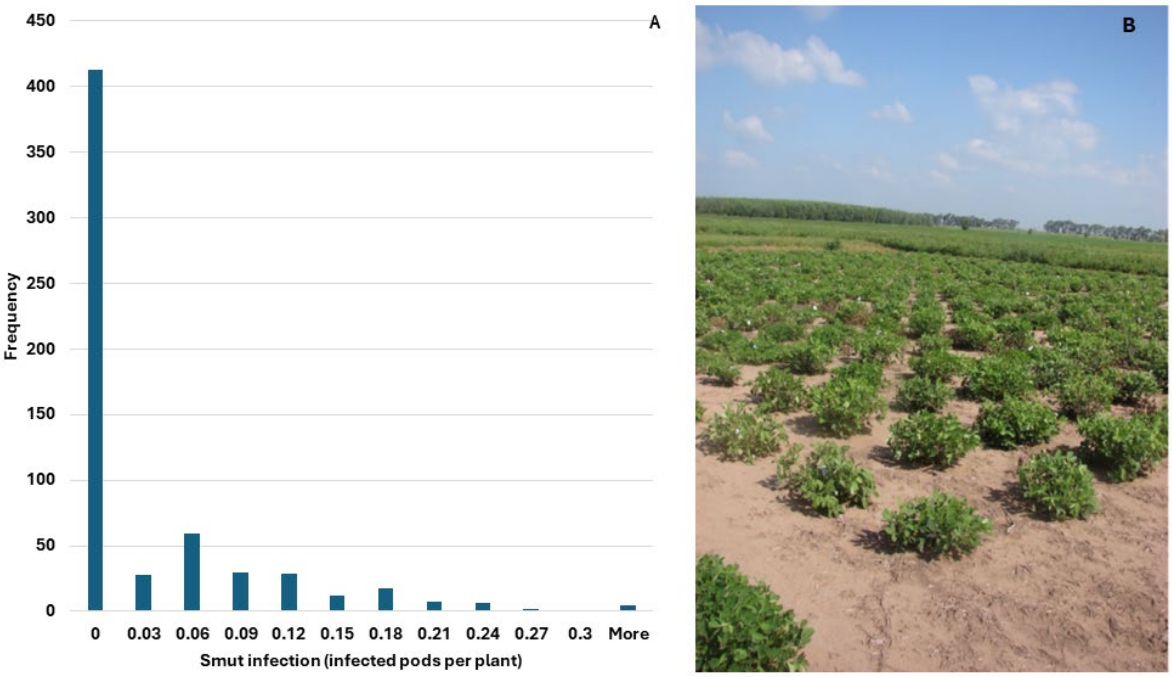

Figure 1: Smut infection as measured by infected pods/total pods in individual F2:3 progeny plants (A) tested at Manfredi in 2018-2019 field season (B).

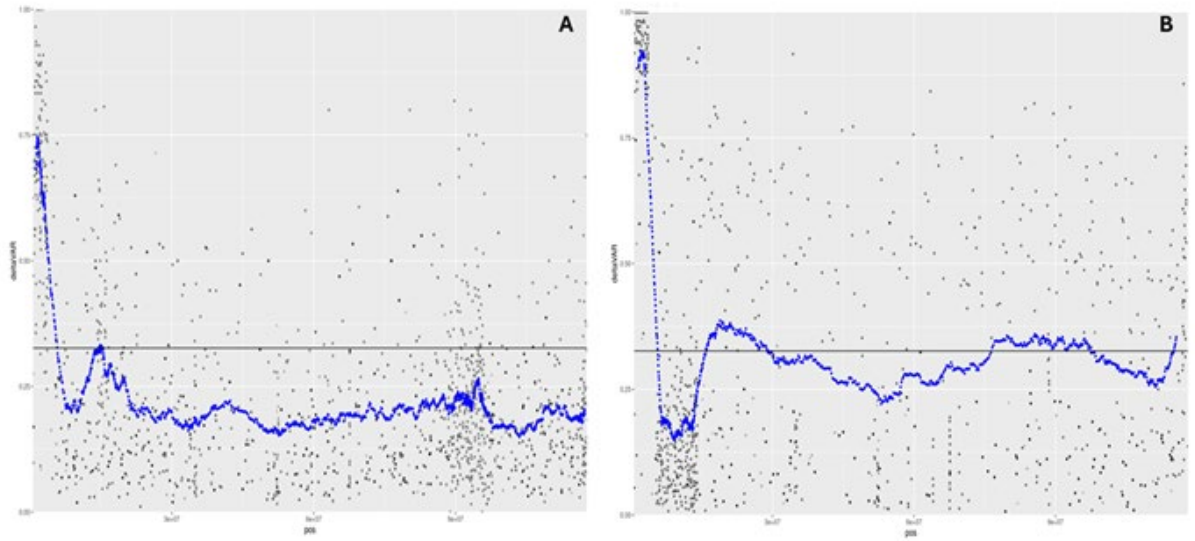

Figure 2: Mapping and validation of smut resistance. (A) A major QTL was identified on chromosome 12. Bulk analysis of 45 individuals with low infection and 49 individuals with infection >3% identifies a 2 Mb region conferring resistance. Y axis is the difference in allele frequency between the two bulks. A peak indicates a region where the allele frequencies significantly segregate between the bulks. The X axis is the physical location on the chromosome. (B) Validation of the QTL on chromosome 12 in a validation population of 48 resistant individuals and 48 susceptible individuals.

ACKNOWLEDGEMENTS

Funding for this project was provided by USDA ARS CRIS Projects 3072-21220-008-00D, 6048-21000-029-000D, 6066-21310-005-000D, and 6046-21000-012-000D, the USDA ARS National Plant Disease Recovery System (NPDRS,) and MARS WRIGLEY, Inc. The authors declare no conflict of interest. The authors would like to thank Lisa Myers for technical assistance. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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